Control of Metabolism in Brown Adipose Tissue[1]

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ABSTRACT

Cells and mitochondria were isolated from brown adipose tissue of the adult hamster. Isolated mitochondria did not show respiratory control. Reversed electron transport was demonstrated and the oxidation rates of various substrates were compared. α-Glycerophosphate gave the highest oxidative rate with isolated mitochondria. The low basal respiration of isolated brown fat cells could be stimulated by catecholamines, oleate, succinate, α-glycerol phosphate and uncoupling agents. Only norepinephrine or oleate induced respiration was sensitive to inhibition by oligomycin, but this inhibition could not be released by uncoupling agents. Neither atractyloside nor (+) decanoylcarnitine were found to affect respiration, suggesting that mitochondrial nucleotide exchange is slow and that fatty acid oxidation might be carnitine independent. In resting brown fat cells, ATP amounts to 75% of the total adenine nucleotides. NE or oleate caused a small decrease of ATP and a corresponding increase of ADP. Oligomycin caused a partial depletion of ATP content, but subsequent NE addition increased ATP back to control values. This effect was abolished by arsenite. Similarly, uncoupling agents diminished the ATP level which was increased only slightly by NE. Arsenite alone decreased ATP levels to a small extent but a rapid depletion occurred upon subsequent NE addition while respiration was inhibited. Thus, substrate level phosphorylation may be the major energy producing reaction for the generation of ATP and GTP for the activation of fatty acids. Norepinephrine addition to brown fat cells caused an oxidation of pyridine nucleotides, a reduction of flavoproteins and an oxidation of cytochrome b. In contrast, succinate produced a reduction of all the components of the respiratory chain. The bioenergetic basis of thermogenesis in brown fat is its high respiratory rate. The rapid respiration induced by norepinephrine or fatty acids appears to be characterized by a low yield of ATP from oxidative phosphorylation and may be controlled by fatty acid mediated release of energy coupling, possibly by an indirect mechanism.

INTRODUCTION

In a previous paper (1), in vivo measurements were made of the oxygen tension, temperature and the fluorescence attributable to reduced pyridine nucleotides, using the interscapular brown fat of the hamster. Brief periods of anoxia produced an increase of fluorescence and a decrease of the brown fat temperature, while infusion of norepinephrine increased the respiration and temperature of the tissue and caused a decrease of the tissue fluorescence. This fluorescence change was interpreted as indicating an oxidation of mitochondrial pyridine nucleotides in the tissue. Chance and Fain (unpublished observations) in experiments with isolated brown fat cells found that norepinephrine produced an oxidation of cytochrome b. Norepinephrine is known to promote the release of free fatty acids from triglycerides through the mediation of cyclic AMP (2). It is generally accepted that during conditions of stimulated thermogenesis, free fatty acids provide the substrate necessary to support the increased respiratory activity. If electron transport is uncoupled from phosphorylation, as suggested by Lindberg et al (3), then a change in the redox state of the respiratory chain components towards reduction would be expected with an increased supply of substrate. The fact that the opposite change occurs after stimulation of respiration with norepinephrine suggests that respiration in brown fat is subject to respiratory control, but may become uncoupled either directly by the released fatty acids, or by stimulation of an energy consuming ion pumping mechanism (1).

Further experiments with isolated brown fat cells are reported in this paper which attempt to answer the basic questions: What is the chemical mechanism for heat production in brown fat, and how is it controlled? As we shall see, only a partial answer to this question is available at the present time, but we hope that this presentation will clarify some guidelines for further experiments.

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[1]One of nine papers to be published from the Symposium "Brown Adipose Tissue," presented at the AOCS-AACC Joint Meeting, Washington, D. C., March 1968.

[2]This work was done during the tenure of an Established Investigatorship from the American Heart Association.

TABLE I

Heat Production During Substrate Oxidation

Reaction	ATP yield	Enthalpy change	
		Uncoupled	Coupled
		Kcal/mole	
ATP → ADP + P_i		4.7	
NADH + H^+ + O → NAD^+ + H_2O	3	61.6	47.5
Succinate → fumarate	2	36.2	26.8
Acetate → CO_2 + H_2O	12	209	153
Palmitate → CO_2 + H_2O	130	2400	1790

METHODS

Brown Fat Cells

Brown fat cells were prepared by collagenase treatment of minced brown fat from 48 hr fasted male hamsters (120-180 g) using a slight modification of the method described by Fain et al. (4). The layer of triglyceride which formed above the layer of unbroken cells during the centrifugation procedure was removed by suction. The free triglyceride content of the cells was greatly reduced by repeatedly washing the cells with Krebs Ringer phosphate, pH 7.4 (5) containing 4% bovine serum albumin (Pentex Inc.). The cells were suspended in 6 vol of Krebs Ringer phosphate, pH 7.4, containing 4% albumin, and kept at room temperature with gentle rotary shaking.

Respiration Studies

Brown fat cells were suspended in 4 to 13 vol of Krebs Bicarbonate Ringer containing 20 mM Tris, pH 7.2 (final albumin concentration, 3 to 10 mg/ml). Upon equilibration of the buffer with gas mixtures containing oxygen and 5% CO_2, the pH of the buffer stabilized at 7.2. The oxygen consumption of the cells was measured with a Clark electrode (Yellow Springs Instrument Co.) at 28 C in a chamber with a total volume of 0.6 to 4 ml. The oxygen electrode was fitted with a double membrane of cellophane and teflon to improve its stability (6). A polarization voltage of -0.6 v was applied to the combination electrode and the current was recorded on a 10 in. Beckman linear-log recorder. Additionally, the rate of oxygen consumption was recorded directly by differentiating the output of the oxygen electrode using a modification of the circuit described by Chappell and Crofts (7).

Analytical

Samples of diluted cells (0.2 to 0.5 ml) from the oxygen electrode chamber were rapidly pipetted into 5 vol of 12% perchloric acid, and an aliquot of the supernatant after centrifugation for 10 min at 25,000 *g* was neutralized with 3 N K_2CO_3 containing 0.5 M triethanolamine. Adenine nucleotides were measured by specific enzyme methods using fluorometric techniques as described by Williamson and Corkey (8). Enzyme activities were measured by following the techniques of Bucher et al. (9) after suitable modification for fluorometric assays. Lipid fractions were separated by thin layer chromatography (10) and fatty acids released by saponification were determined by gas chromatography as the methyl derivatives (11).

Mitochondria

Mitochondria were prepared from brown fat obtained from hamsters which had been kept at 4 to 10 C for at least one month. Preparative techniques were similar to those described by Hohorst and Rafael (12).

RESULTS AND DISCUSSION

General Properties of Brown Fat Cells and Mitochondria

Heat production is undoubtedly linked to the rate of respiratory activity. Unlike other organs, such as the heart or kidney which also have a high rate of oxygen utilization, brown fat appears to be poorly endowed with mechanisms for the transduction of metabolic energy into physiologically useful forms of work. Its main function is to serve as a specialized site of thermogenesis in animals during arousal from hibernation, adaptation to cold, and in the newborn of some species (13-19). For this purpose, the distribution of brown fat around vital organs and the special arrangement of the vascular network is of particular significance (15).

The question as to whether brown fat mitochondria in the intact tissue are coupled or uncoupled to respiratory chain phosphorylation is of fundamental importance in connection with the nature of the chemical processes responsible for the continuous supply of phosphate acceptor to the respiratory chain, but is of less importance in relation to the total heat production for a given respiratory rate. This point is illustrated in Table I, which shows the heat production for the oxidation of a number of compounds. Since the heat production (as distinct from the free energy change) for the

TABLE II

Fatty Acid Characterization of Hamster Brown Fat[a]

Lipid	Myristic 14:0	Palmitic 16:0	Palmitoleic 16:1	Stearic 18:0	Oleic 18:1	Linoleic 18:2	Linolenic 18:3
Triglyceride	2.5	33.0	8.3	9.9	61.7	54.5	3.1
Phospholipid	0.01	0.16	0.04	0.40	0.36	0.67	----
Cholesterol esters	0.007	0.06	0.01	0.02	0.08	0.08	----
Free fatty acid acid	0.007	0.08	0.03	0.03	0.02	0.17	----

[a]μmoles/100 mg fresh weight.

hydrolysis of ATP to ADP and inorganic phosphate is relatively low (20), there is only about a 25% increment of heat production in the uncoupled versus the coupled state. However, it is necessary to resolve the issue of energy conservation in the intact tissue in order to define the control properties of the system. Thus, if net oxidative phosphorylation does not occur, e.g., due to the presence of a highly active adenosine triphosphatase, respiration may be controlled primarily by substrate availability. However, when fatty acids provide the major fuel, a source of ATP is required to activate the fatty acids to the acyl CoA derivatives. If, on the other hand, electron transport through the respiratory chain is coupled to phosphorylation, respiration may be controlled by ADP. In this case, a mechanism for the regeneration of ADP is required, such as that proposed by Ball and Jungas (22) for white adipose tissue. Before an evaluation is made of these alternative possibilities some general biochemical properties of brown fat cells and mitochondria will be described.

An analysis of the lipid content of brown fat from fed hamsters (Table II) shows that triglyceride accounts for over 99% of the total lipid. The major fatty acids present in the triglyceride are palmitic, oleic and linoleic. The triglyceride content of brown fat decreases greatly, relative to the cytochrome c content, if the animals are fasted for several days or if they are kept in the cold for a number of weeks.

A preliminary investigation of the enzyme contents of brown fat shows that it contains all the glycolytic enzymes, and that the constant proportion group of enzymes is similar to that found in other tissues (23). Figure 1 compares the relative activities of a number of enzymes in brown fat with corresponding activities in rat cerebral cortex. It is noteworthy that the hexokinase content of brown fat is very low relative to the constant proportion enzymes, whereas that of α-glycerophosphate dehydrogenase, α-glycerophosphate oxidase and succinate dehydrogenase is very high. Other studies have shown that the contents of glutamate oxalacetate transaminase and glutamate dehydrogenase are very low in brown fat compared with NAD^+ and $NADP^+$-linked isocitrate dehydrogenase, succinate dehydrogenase, fumarase and malate dehydrogenase. These results are in accordance with the generally accepted proposal that brown fat has a low glycolytic capacity but a high citric acid cycle capacity. The significance of the high activities of α-glycerophosphate dehydrogenase and oxidase, which are key enzymes of the α-glycerophosphate shuttle (24) is not clear at present. One possibility is that extensive re-esterification of the fatty acids liberated by the hydrolysis of triglyceride is prevented by removal of α-glycerophosphate. This would ensure the availability of fatty acids for oxidation.

α-Glycerophosphate oxidase is a mitochon-

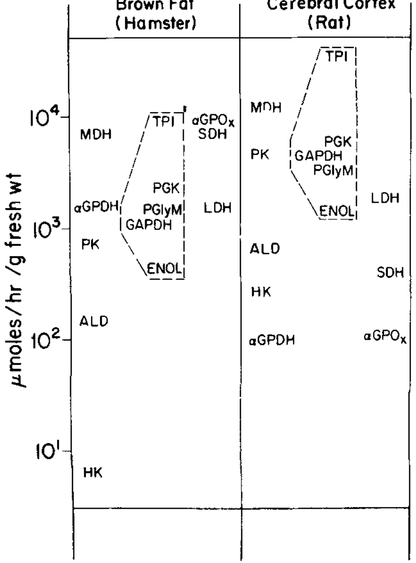

FIG. 1. Enzyme profile patterns of hamster brown fat and rat cerebral cortex (23).

TABLE III

Substrate Respiration Rates in Mitochondria of Brown Fat From Cold-adapted Hamsters[a,b]

Substrate	O_2 Uptake
α-Glycerophosphate	300
Succinate + rotenone	148
Palmityl carnitine + malate	109
Pyruvate + malate	88
α-Ketoglutarate + malonate	64
Pyruvate	28
Malate	11

[a]Cytochrome c = 0.81 μmoles/mg protein; cytochrome a = 0.34 μmoles/mg protein.

[b]μatoms/min per milligram of protein.

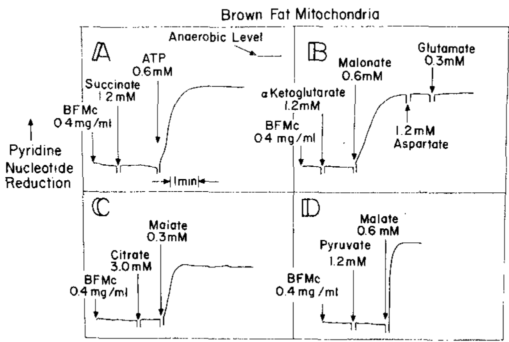

FIG. 2. Properties of brown fat mitochondria (BFMc). Pyridine nucleotide changes were measured in an Eppendorf fluorometer.

drial flavin-linked enzyme, and preliminary studies with isolated brown fat mitochondria (Table III) show that α-glycerophosphate is oxidized more rapidly than other substrates. The mitochondria showed no respiratory control. However, preparation of brown fat mitochondria with respiratory control has been achieved either by the use of high albumin concentrations (25-27) or by ATP and carnitine (28). In the present experiments with brown fat from adult hamsters, these techniques were not successful for producing mitochondria with coupled respiration. With α-glycerophosphate as substrate, the cytochrome c turnover is 12 sec^{-1}. This value is considerably smaller than the cytochrome c turnover of about 120 sec^{-1} observed with isolated cells stimulated by norepinephrine, suggesting that the respiration of the isolated mitochondria is grossly inhibited compared with respiration in their natural state. In the presence of malate, malonate and fluorocitrate (29), palmityl carnitine is oxidized stoichiometrically to citrate. Palmityl CoA plus carnitine is oxidized at comparable rates to palmityl carnitine, and fatty acids are also oxidized under suitable conditions if ATP is supplied to increase the intramitochondrial ATP level.

Brown fat mitochondria possess the ability to reduce NAD^+ by an energy-linked reversal of electron transport from succinate (Fig. 2A). Also shown is the malonate requirement for reduction of NAD^+ by α-ketoglutarate (Fig. 2B). However, aspartate, even in the presence of glutamate (30), was unable to provide enough oxalacetate by transamination to oxidize the NADH. Reduction of NAD^+ by citrate or pyruvate required the presence of malate (Fig. 2C,D). Malate alone at the concentration used was unable to reduce the intramitochondrial NAD^+. These studies indicate that brown fat mitochondria in common with liver or heart mitochondria, possess several of the specific anion transport systems which impose permeability restrictions on intermediates of the citric acid cycle. The apparent failure of aspartate to be metabolized by brown fat mitochondria is in accordance with the low activity of glutamate oxalacetate transaminase in the tissue.

Returning to the properties of isolated brown fat cells, we have found that the endogenous respiration is normally very low and is capable of being stimulated by very few substrates. Thus, contrary to reports of studies with brown fat fragments (3), α-ketoglutarate and pyruvate plus malate did not stimulate endogenous respiration appreciably. Presumably a permeability restriction prevents the oxidation of these intermediates in the intact cells. Epinephrine, norepinephrine and dibutyryl cyclic AMP, after a characteristic delay (31), stimulated respiration of isolated brown fat cells 30- to 40-fold (Fig. 3). In these studies, the rate of respiration started to decrease when the oxygen concentration fell below about 25 μM. Dose-response relationships with norepinephrine showed that a half maximum effect was obtained with 0.02 μg/ml. Oleate also induced a large increase of the respiratory rate (Fig. 3D), whereas α-glycerophosphate and succinate were much less effective. The respiratory response to oleate in this experiment was submaximal compared with the norepinephrine response, and the effects of α-glycerophosphate, succinate and oleate were additive. Other experiments showed that the concentration of oleate required for a maximum effect was raised by an increase of the albumin concentration. At low albumin concentrations (e.g., 3 mg/ml), supra-maximal concentrations of oleic acid were inhibitory.

A comparison of the respiratory stimulation induced by norepinephrine between Krebs Bicarbonate Ringer and Krebs Phosphate Ringer,

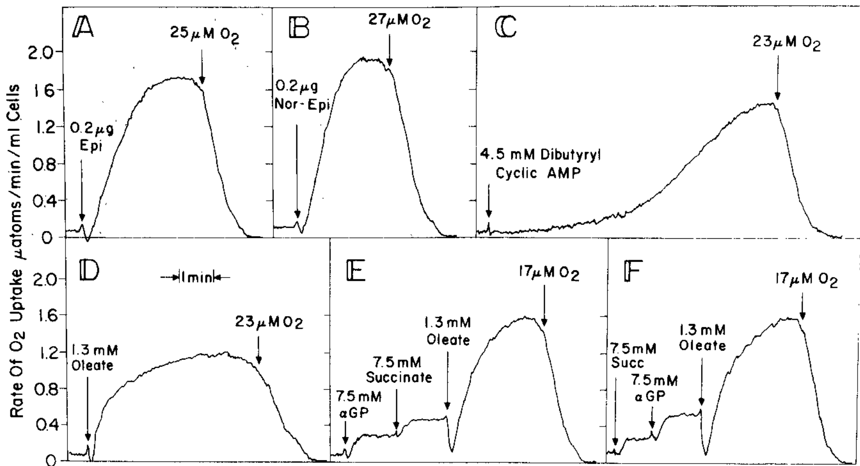

FIG. 3. Effect of different substrates and hormones on the rate of oxygen uptake by isolated brown fat cells.

at pH 7.2, showed that a higher rate of oxygen consumption was obtained with the bicarbonate buffer when a maximal norepinephrine concentration was used (Fig. 4). Succinate and α-glycerophosphate added together with the norepinephrine did not increase the maximum respiratory rate induced by norepinephrine alone with the bicarbonate buffer, but an extra response was obtained with the phosphate buffer. These results show that under suitable conditions, the norepinephrine induced respiration is able to saturate the respiratory chain.

Adenine Nucleotide Measurements in Brown Fat Cells

In order to elucidate the mechanism by which norepinephrine and fatty acids stimulate respiration, we attempted to answer the following questions: (a) What are the rate controlling steps for the oxidation of different substrates? (b) What is the evidence that the respiratory chain is coupled to phosphorylation in intact cells? (c) What are the major energy forming and utilizing steps when the respiration of the tissue is stimulated?

Two independent experimental approaches were used to answer these questions. The first was to measure the adenine nucleotide content of isolated cells under different metabolic conditions, and the second was to follow the redox changes of the respiratory carriers after addition of suitable substrates and inhibitors.

Figure 5 shows the changes of ATP and ADP in brown fat cells stimulated with norepinephrine. The rate of oxygen utilization by the cells is shown on the left hand side of the Figure along with the changes of the ATP/ADP ratio. Norepinephrine (0.1 μg) was added after 2 min of incubation and there was a distinct lag before oxygen consumption increased, whereas the ATP/ADP ratio fell immediately after norepinephrine addition. In the resting cells, ATP accounted for about 75% of the total adenine nucleotides and ADP for about 12%. Perhaps the most striking facet of this experiment is the relatively small change of the nucleotides in relation to the 20-fold increase of respiratory activity. If it is assumed that respiration is coupled to phosphorylation with an average P/O ratio of 3, the terminal phosphate turnover rate of ATP is calculated to be 4 per second. This figure may be compared with values of 0.13 and 0.09 for rat heart and liver, respectively, which are calculated from the respiratory rates

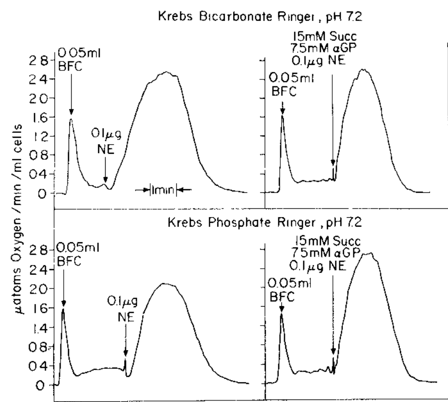

FIG. 4. Effect of different buffers on the stimulation of respiration induced by norepinephrine in isolated brown fat cells.

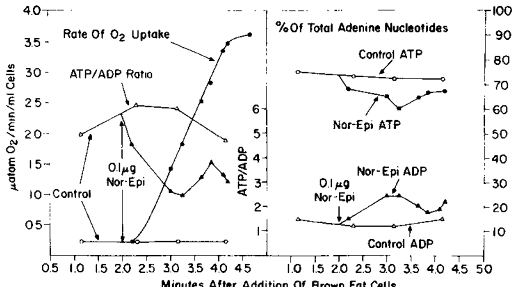

FIG. 5. Changes of the adenine nucleotide content of brown fat cells during stimulation by norepinephrine.

and ATP contents of these tissues (32-34). A high turnover rate of ATP in brown fat implies either a very active ATP utilization step in the tissue, or, on the other hand, that the basis for the calculation is invalid, and that the net phosphorylation yield per atom of oxygen consumed is very small. The latter explanation is probably correct.

Addition of oleate (Fig. 6) produced changes of the adenine nucleotides which were similar to those elicited by norepinephrine. Thus, initially ATP fell, while ADP increased as the rate of respiration increased, but subsequently the nucleotides tended to cycle back towards control levels. In this experiment, 1.6 mM oleate increased the rate of oxygen consumption by about the same amount as norepinephrine. Antimycin A rapidly inhibited respiration and decreased ATP levels to 10% of the total nucleotides, while the proportion of the total nucleotides, as AMP, increased from about 10% to 50%.

The basic function of brown fat as a heat generating organ is not inconsistent with a full oxidative phosphorylation capacity of the mitochondria provided that sufficiently active metabolic processes occur to regenerate phosphate and ADP. Indeed, the rise of ADP upon addition of norepinephrine to brown fat cells is not unlike that observed in the perfused rat heart with epinephrine (35), where the response was interpreted as a change in the metabolic state towards the more active, ADP-controlled, state 3 (36).

The fuel for the norepinephrine mediated increase of respiration in brown fat is undoubtedly fatty acids. Taking palmitate as an example, it may be calculated that one mole of palmitate consumes 14 g atoms of oxygen during its β-oxidation to 8 moles of acetyl CoA and produces a maximum of 35 moles of ATP. Each mole of acetyl CoA uses 4 g atoms oxygen for oxidation in the citric acid cycle and produces 12 moles of ATP. Thus, the total yield of ATP is 131 moles per mole of palmitate oxidized, with the consumption of 46 g atoms of oxygen. Of the total ATP yield, 1 to 2 moles are required to activate palmitate to palmityl CoA (depending on the enzyme system used), while 8 moles are formed from GTP produced by substrate level phosphorylation. It is clear, therefore, that the net high energy phosphate yield per mole of palmitate oxidized ranges from a minimum of 6 in the fully uncoupled state to a maximum of 130 with theoretical yields for oxidative phosphorylation.

In most tissues the major ATP-utilizing steps are extra-mitochondrial, and ATP produced in the mitochondria is exchanged for cytoplasmic ADP in a reaction which is inhibited by atractyloside (37,38). This process is shown schematically in Figure 7. The necessity of an adenine nucleotide exchange reaction arises from the fact that the inner mitochondrial membrane is not directly permeable to nucleotides (39). It follows, therefore, that if respiration in brown fat is accompanied by a high yield of phosphorylation, there should be a high rate of transport of nucleotides through the atractyloside sensitive barrier. However, addition of atractyloside up to concentration of 15 mM, which is

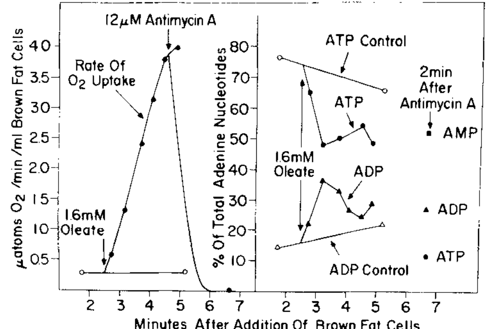

FIG. 6. Changes of the adenine nucleotide content of brown fat cells during stimulation by oleic acid.

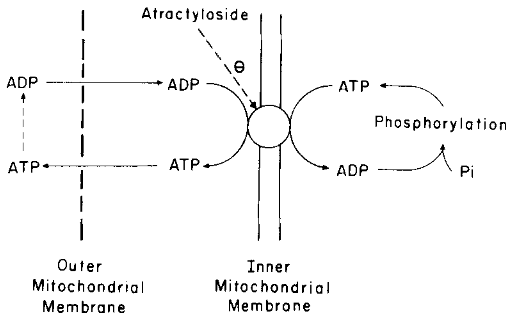

FIG. 7. Schemes showing the site of action of atractyloside.

two orders of magnitude greater than the concentration required to fully inhibit ADP-stimulated respiration in mitochondria, failed to affect the increase of respiration induced by norepinephrine in brown fat cells (Fig. 8). Unfortunately, this experiment does not provide a definitive answer to the question whether nucleotide translocation across the mitochondrial membrane is occurring since there is no independent assessment that the inhibitor is reaching its postulated site of action. It may be noted, however, that an inhibition of respiration with 1 to 2 mM atractyloside has been obtained in rat diaphragm (40) as well as in other tissues (41).

Classical studies of the relationship between the electron transport chain and the energy transfer pathway (42,43) have led to the postulation of energized intermediates whereby part of the free energy change at three of the oxidoreduction steps of the respiratory chain is transferred to a phosphorylated intermediate which donates its phosphate to ADP. This hypothetical scheme is shown in Figure 9, where A and B represent components of the electron transport chain. Studies with isolated mitochondria and sub-mitochondrial particles have shown that ATP formation by the energy transfer pathway is inhibited by oligomycin. In the coupled state, respiration is inhibited to an extent dependent on the rate of dischange of an energized intermediate which is depicted in Figure 9 as $X \sim I$. Uncoupling agents will normally release oligomycin inhibited respiration. In Figure 9, this effect is depicted as a stimulation of the reaction $X \sim I$ to $X + I$. Oligomycin and uncoupling agents may, therefore, be used as diagnostic tools to reveal whether high energy phosphate formation is occurring in respiring systems.

Figure 10 shows the respiratory response of

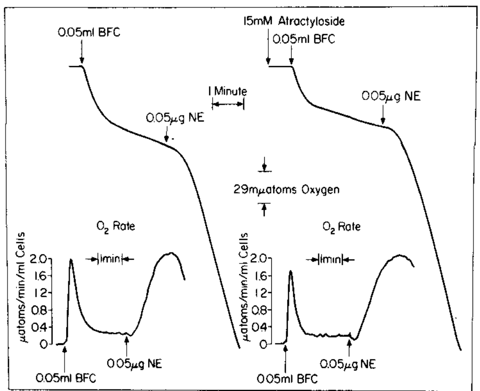

FIG. 8. Effect of atractyloside on the response of isolated brown fat cells to norepinephrine.

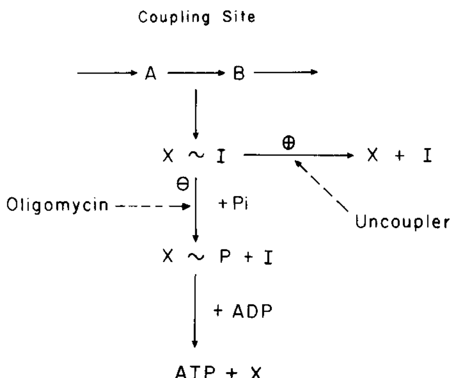

FIG. 9. Scheme showing the postulated site of action of oligomycin and uncouplers in the energy transfer pathway of oxidative phosphorylation.

brown fat cells to norepinephrine in the presence of the uncoupling agent dicumarol (Fig. 10B), and oligomycin (Fig. 10C). Dicumarol stimulated endogenous respiration, but subsequent addition of norepinephrine caused a much smaller increase of the respiratory rate than the control response shown in Figure 10A. Likewise, the response to norepinephrine was inhibited after prior addition of oligomycin, and further addition of dicumarol was found to have no effect on the respiratory rate. Rotenone completely inhibited the effect of both dicumarol and norepinephrine. The fact that endogenous respiration is stimulated by uncoupling agents indicates that the low rate of metabolism in the resting cells is not caused by substrate deficiency, but by respiratory inhibition by lack of phosphate acceptor. Similarly, inhibition by oligomycin of the norepinephrine effect may be interpreted as evidence in favor of coupled respiration. However, it should be pointed out that the oligomycin induced inhibition of respiration is atypical of that seen with isolated mitochondria in that it is not relieved by uncoupling agents. Since both oligomycin and uncouplers interfere with the formation of

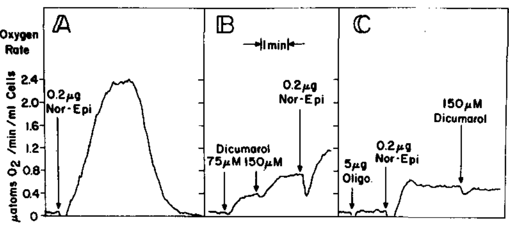

FIG. 10. Effects of dicumarol and oligomycin on the rate of oxygen uptake of isolated brown fat cells and their effects on the response of the cells to norepinephrine.

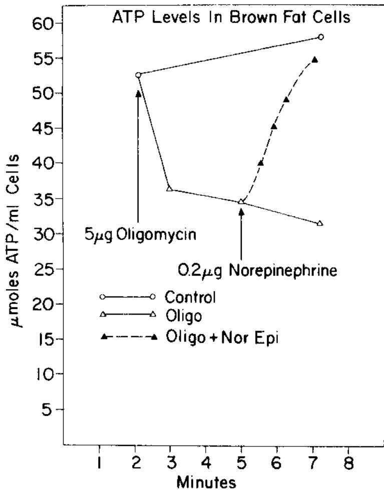

FIG. 11. Effects of oligomycin and norepinephrine on the ATP content of isolated brown fat cells.

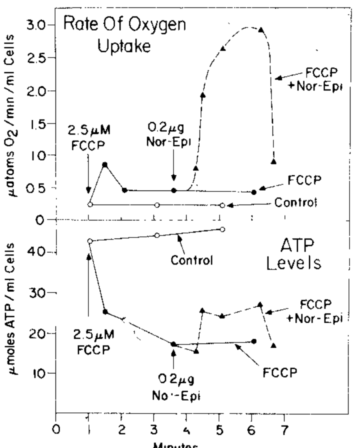

FIG. 12. Effects of FCCP and norepinephrine in the oxygen uptake and ATP content of isolated brown fat cells.

ATP by oxidative phosphorylation, it may be anticipated that their inhibitory effects on the norepinephrine response are partly mediated by a lack of ATP, which is required for the activation of fatty acids.

Measurements of the ATP content of brown fat cells after addition of oligomycin (Fig 11), showed only a moderate drop of ATP. However, subsequent addition of norepinephrine caused the ATP level to increase almost up to the control levels. Further experiments showed that this rise of ATP was abolished by arsenite, thereby proving that ATP was produced by substrate level phosphorylation during the conversion of succinyl CoA to succinate. Although GTP is the immediate product of this reaction, ATP is readily formed through the mediation of nucleotide diphosphate kinase. A similar increase of ATP after addition of oleate in the presence of oligomycin, which was arsenite sensitive, has been observed by Rossi et al. (44) with rat kidney mitochondria.

The role of substrate level phosphorylation in the maintenance of the cellular ATP level was investigated further by measuring ATP levels in the presence of the uncoupling agent FCCP ((*p*-trifluoromethoxyphenyl hydrazone of carbonyl cyanide)(Fig. 12). Unlike dicumarol, FCCP produced an initial burst of respiration before the respiration reached a constant rate which was about twofold higher than that of the control. The ATP content of the cells fell rapidly after addition of the uncoupler and leveled off at values about 60% lower than the controls. In this experiment, the maximum norepinephrine induced respiration was inhibited about 30% by FCCP. Under these conditions, norepinephrine induced a small but definite increase of the ATP content above the FCCP-inhibited level (Fig. 12). This relative increase of ATP during stimulation of respiration by norepinephrine is probably caused by a substrate level phosphorylation, and its small extent compared with the larger rise shown in Figure 11 may be a reflection of increased adenosine triphosphatase activity in the presence of uncoupler which will tend to deplete the ATP level even though it is supported by uncoupler insensitive substrate level phosphorylation.

Figure 13 shows that addition of arsenite to resting cells caused only a small depletion of the ATP content, but subsequent addition of norepinephrine produced a rapid fall of ATP, and greatly inhibited the respiratory response. Addition of succinate at this point had a very interesting effect (Fig. 13). The respiratory rate increased immediately by a small amount, which is the normal response to succinate (cf. Fig. 3), but subsequently a large increase of respiration occurred characteristic of a normal norepinephrine response. Furthermore, the ATP level increased significantly. This experiment confirms that Krebs cycle substrate level phosphorylation is the major energy producing reaction responsible for the maintenance of ATP levels in isolated brown fat cells. When this reaction is inhibited by arsenite, ATP levels are rapidly depleted by the energy requirements for fatty acid activation, and the respiration induced by norepinephrine becomes inhibited as shown in Figure 13. Other experiments have shown that the more complete the arsenite

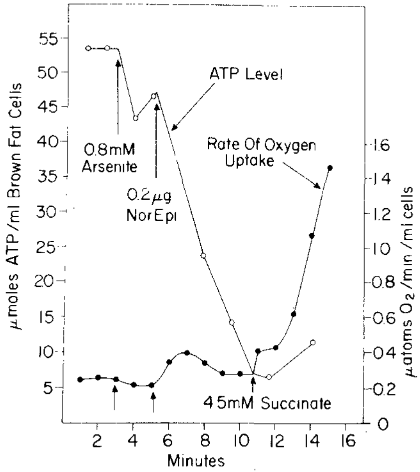

FIG. 13. Effects of arsenite and norepinephrine on the oxygen uptake and ATP content of isolated brown fat cells.

block, the faster is the onset of respiratory inhibition after norepinephrine addition. The small rise of ATP and the large increase of respiration which occurred some minutes after succinate addition (Fig. 13) suggests that a small amount of oxidative phosphorylation is occurring during succinate respiration, and that enough ATP is produced to allow activation and further respiration of fatty acids. However, an alternative explanation is that the arsenite-inhibited system is deficient in oxalacetate as well as ATP, and that succinate oxidation to malate is able to spark fatty acid oxidation by supplying the acceptor for acetyl CoA since brown fat, unlike liver, has a poor capability for ketone formation. This latter explanation may be the more probable one since the succinate-induced respiration in brown fat cells is oligomycin insensitive, and is unaffected by uncouplers in the presence of rotenone.

It is clear from the above results that interpretations based on respiration data alone may not be valid in the absence of knowledge concerning the cellular ATP levels. Specifically, it appears that ATP deficiency is not responsible for the norepinephrine-induced inhibition of respiration in the presence of oligomycin or uncouplers unless a small specific pool of ATP is utilized for fatty acid activation. Further experiments are required before a final assessment of the implications of these findings can be made in relation to the extent of coupled oxidative phosphorylation in intact brown fat tissue. The possibility should not be ignored that part of the effects observed with oligomycin and uncouplers might be mediated by secondary ef-

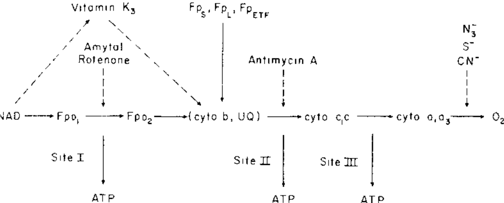

FIG. 14. Scheme of the electron transport chain.

fects resulting from an altered redistribution of cations in the cell. At present, there is little experimental evidence to substantiate this hypothesis.

Redox Changes in Brown Fat Cells

A schematic representation of the respiratory chain showing the location of the three coupling sites is shown in Figure 14. The sequence of the flavin intermediates and the terminology is based on the work of Chance et al. (45) and Garland et al. (46). These authors showed that in rat liver and beef heart mitochondria the flavin complex denoted by F_{pD1} had a higher fluorescence yield relative to its absorption than the other flavin pools. If the rate of electron transfer through the respiratory chain is limited by energy coupling at the conservation sites, removal of this limitation will result in an increase of flux, and according to the Crossover Theorem (47) the dominant control site may appear as a forward crossover; that is the respiratory component on the substrate side of the energy conservation site will become more oxidized while that on the oxygen side will become more reduced. In practice, however, as a consequence of the multi-site interactions in the electron transport chain, the changes of some of the components may be very small.

As previously noted (1), norepinephrine produced a decrease of the tissue fluorescence attributable to pyridine nucleotides in brown fat of the hamster in vivo. That this change was at least partly caused by an oxidation of pyridine nucleotides in the cytoplasmic compartment is seen from the results shown in Figure 15. Addition of norepinephrine to isolated brown fat cells produced a decrease in the ratio of lactate to pyruvate, which corresponds to a decrease of the $NADH/NAD^+$ ratio provided that the lactate dehydrogenase equilibrium does not change. The shift in the ratio was caused primarily by a rise of pyruvate, while lactate levels (not shown) did not change appreciably. The observed increase of pyruvate is probably a result of its inhibited entry into the citric acid

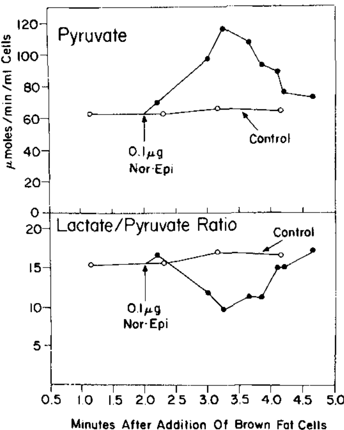

FIG. 15. Effect of norepinephrine on the level of pyruvate and on the lactate-pyruvate ratio in isolated brown fat cells.

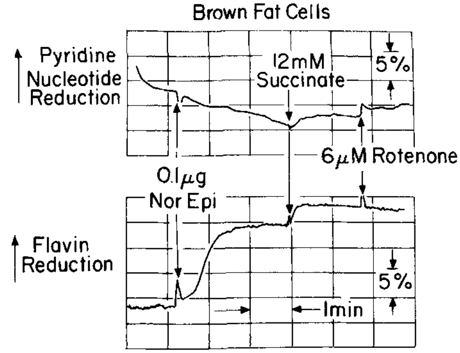

FIG. 16. Responses of the pyridine nucleotides and flavins of isolated brown fat cells to norepinephrine, succinate and rotenone. Reduction of pyridine nucleotides corresponded to an increase of fluorescence using an excitation wave length of 366 mμ and a measuring wave length of 450 mμ. Reduction of flavins corresponded to a decrease of fluorescence measured at 570 mμ with an excitation wave length of 436 mμ.

cycle when acetyl CoA formation from fatty acids is augmented.

Direct optical measurements of pyridine nucleotide changes in isolated brown fat cells by fluorescent techniques gave rather unsatisfactory responses, but changes of flavin fluorescence were considerably larger (Fig. 16). Norepinephrine produced a slow oxidation of pyridine nucleotides but a faster reduction of flavin pigments. Subsequent addition of succinate caused a small reduction of pyridine nucleotides and a further small flavin reduction. Rotenone increased the reduction state of the pyridine nucleotide pool, but did not affect the flavin pool. The lack of effect of rotenone on the flavin fluorescence suggests that most of the signal is derived from highly fluorescent flavins located prior to the first phosphorylation site (i.e., lipoyl dehydrogenase).

This point is illustrated further by an example of some results obtained with isolated brown fat mitochondria (Fig. 17). In this experiment both flavin fluorescence (upper trace) and flavin absorption (lower trace) were measured. Prior to the start of the recording, the mitochondria were uncoupled and incubated for 3 min with arsenite and antimycin A. Addition of 10 μM (-)palmityl carnitine caused a large reduction of flavin as measured both by fluorescence and absorption. Malate further enhanced the fluorescence response but had no effect on the absorption response. Since β-oxidation provides reducing equivalents both at the level of NADH and flavin (Fp_A and Fp_{ETF}), addition of palmityl carnitine serves to reduce most of the flavin pool in the mitochondria. Rotenone diminished the flavin fluorescence response slightly, but caused a large diminution of flavin absorbance. These changes may be interpreted with reference to Figure 14, where it is seen that as electrons leak through the antimycin A block, the highly fluorescent FpD1, which is before the rotenone site stays reduced, while the flavins of lower fluorescent yield, which are after the rotenone site, become oxidized. Subsequent addition of succinate and then α-glycerophosphate (Fig. 17) reduces the flavin components associated with succinate dehydrogenase and α-glycerophosphate oxidase, and it is seen that these flavins have a relatively low fluorescence yield.

Cytochrome b measurements were made in suspensions of brown fat cells to follow redox changes after the first phosphorylation site. Absorbance difference measurements were made in the Soret region in a double beam spectrophotometer using the wave length pair 410-430 mμ. An increase of absorbance at 430 mμ corresponds to a reduction of cytochrome b. The top trace of Figure 18 shows that norepinephrine caused an oxidation of cytochrome b. The onset of the response occurred almost immediately after addition of norepinephrine and prior to the onset of the increased oxygen uptake. It will be remembered that ADP also increased prior to the increase of respiration after norepinephrine addition (see Fig. 5). Apparently, norepinephrine induced complete oxidation of cytochrome b since subsequent addition of dicumarol did not cause a further oxidation. The bottom trace of Figure 18 shows that dicumarol oxidized cytochrome b fully, and that subsequent norepinephrine addition had no further effect. Antimycin A caused

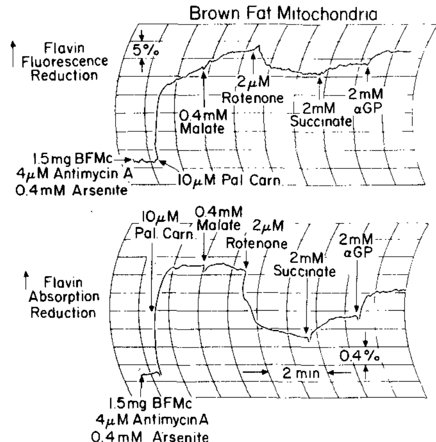

FIG. 17. Flavin responses of brown fat mitochondria. Flavin reduction corresponded to a decrease of fluorescence measured at 570 mμ with an excitation wave length of 436 mμ and to a decrease of absorption using the wave length pair 475 and 510 mμ.

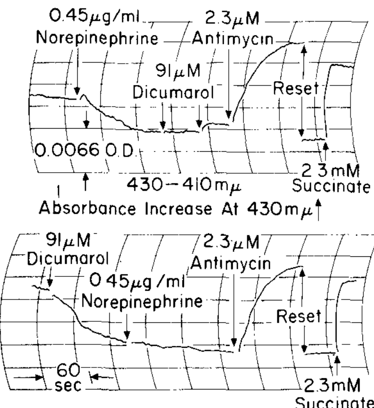

FIG. 18. Cytochrome b responses of isolated brown fat cells. Reduction of cytochrome b corresponded to an increase of absorption at 430 mμ relative to 410 mμ.

a partial reduction of cytochrome b, which was enhanced by the subsequent addition of succinate. Other experiments showed that succinate would also reduce cytochrome b in the absence of antimycin A. Further studies showed that norepinephrine produced a reduction of cytochrome c (absorbance difference using wave lengths of 540 and 550 mμ), thus locating a forward crossover at the second conservation site, namely between cytochrome b and c.

Although the data from the spectroscopic and fluorometric measurements cannot be considered conclusive, they suggest that endogenous respiration in the isolated cells is indeed maintained at a low level by phosphate acceptor control. The primary effect of norepinephrine on the respiratory chain appears to be the release of phosphate acceptor control which results in an oxidation of cytochrome b. This effect may even precede the delivery of fatty acids since the onset of respiratory stimulation is delayed after norepinephrine addition, and free fatty acids do not accumulate. Presumably the flavin reduction represents increased substrate delivery to the respiratory chain, and Figure 16 shows that the onset of rapid flavin reduction was somewhat delayed. The similarity of the cytochrome b response to uncouplers and norepinephrine suggests that fatty acids may be releasing phosphate acceptor control directly, or indirectly, by a change of the intramitochondrial ionic environment (e.g., of K^+ or Ca^{++} ions).

Site of Fatty Acid Activations

Brief mention should be made of the possible sites of fatty acid activation and the carnitine dependence for fatty acid oxidation. Studies, mainly with rat liver, have established that there are at least four separate fatty acyl CoA synthetase enzymes, which differ in their cellular location, nucleotide requirement and sensitivity to inhibitors (44,48-52). The first is the classical microsomal enzyme described by Kornberg and Pricer (53) which uses extramitochondrial CoA and ATP and forms fatty acyl CoA in the cytosol. The second is located on the outer surface of the inner mitochondrial membrane and also uses extramitochondrial CoA and ATP. The third is located in the mitochondria and uses intramitochondrial CoA and ATP, while the fourth enzyme, also intramitochondrial, uses internal CoA and GTP and is inhibited by phosphate, arsenite and arsenate. When fatty acyl CoA is synthesized outside the mitochondria it must be converted to the carnitine ester for acyl transport through the carnitine barrier of the inner mitochondrial membrane, this being impermeable to CoA and its derivatives.

A schematic representation of the carnitine carrier mechanism is shown in Figure 19. Extramitochondrial fatty acyl CoA is converted to the acyl carnitine ester by an outer palmityl carnitine transferase (E_1). The acyl carnitine reacts with a second palmityl carnitine transferase (E_2) in the inner membrane, which utilizes intramitochondrial CoA, and forms intramitochondrial fatty acyl CoA, which then undergoes β-oxidation to acetyl CoA. Both palmityl carnitine transferase enzymes E_1 and

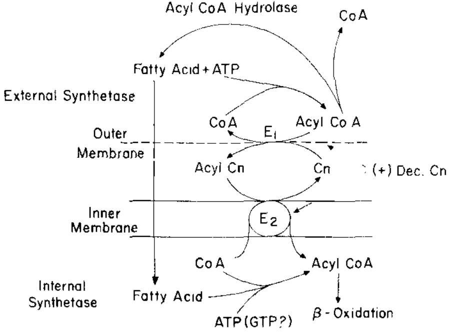

FIG. 19. Scheme showing the carnitine carrier mechanism for the transport of acyl groups into the mitochondria, and the site of action of (+)decanyl carnitine (Dec. Cn).

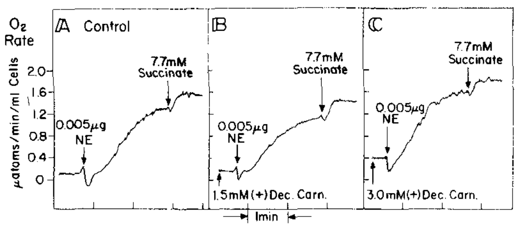

FIG. 20. Effect of (+)decanyl carnitine on the oxygen uptake of isolated brown fat cells upon addition of norepinephrine (NE) and succinate.

E_2 are inhibited by the unnatural analogue (+)decanyl carnitine (54,55). On this basis, if fatty acids are activated by either of the first two acyl CoA synthetase systems, their oxidation will be carnitine dependent, while if the third or fourth types of acyl CoA synthetase systems are used, fatty acid oxidation will be carnitine independent. However, it may be pointed out that there is some controversy as to whether the GTP-dependent synthetase is carnitine dependent (48,50) or independent (51,52) for fatty acid oxidation in rat liver mitochondria. To date, each of the four acyl CoA synthetizing systems have not been well characterized in brown fat. We have found that in brown fat, the microsomal synthetase is considerably less active than the soluble fatty acyl CoA hydrolase or the mitochondrial synthetases.
We have found that in brown fat, the microsomal synthetase is considerably less active than the soluble fatty acyl CoA hydrolase or the mitochondrial synthetases.

Figure 20 shows that the norepinephrine induced respiration in brown fat cells was not inhibited by up to 3 mM (+)decanyl carnitine. Succinate produced the same increment of respiration in the presence and absence of the inhibitor. Other experiments show that oleic acid induced respiration in brown fat cells was likewise unaffected by (+)decanyl carnitine. Although these experiments are not conclusive, they suggest that in brown fat, fatty acids are oxidized by carnitine independent pathways. This proposal is in accordance with the lack of atractyloside inhibition of respiration in the cells (see Fig. 8), since activation of fatty acids to the CoA esters by the carnitine independent pathways requires the use of intramitochondrial ATP or GTP. However, it is clear from the work of Kornacker and Ball (56) with homogenates of brown fat that the tissue possesses the capability of oxidizing fatty acids by carnitine dependent pathways. Further work is needed in this area before any firm conclusions can be drawn concerning the quantitative aspects of which pathway is used for activation of fatty acids. Contrary to the reports quoted by Kornacker and Ball (56), we have found a very low level of free carnitine in brown fat cells, indicating that it may be a limiting component for the carnitine dependent pathways of fatty acid oxidation.

In summary, it appears that the basis for the heat generating capacity of brown fat is its high respiratory rate. Fatty acids provide the major respiratory fuel in the intact tissue, and are liberated from triglyceride stores through the mediation of norepinephrine, which triggers the formation of cyclic AMP; a specific activator of an intracellular triglyceride lipase. The low endogenous respiration of isolated brown fat cells is stimulated additively by oleate, succinate or α-glycerophosphate, suggesting that the respiratory rate is largely controlled by the availability of oxidizable substrate. Norepinephrine or oleate induced respiration is sensitive to inhibition by oligomycin, but this inhibition cannot be released by uncoupling agents. Succinate and α-glycerophosphate induced respirations are insensitive to oligomycin. Uncoupling agents stimulate the endogenous respiration of brown fat cells and inhibit the norepinephrine response. Although there is some evidence for energy coupling at the first phosphorylation site of the electron transport chain, the high respiratory rate of brown fat induced by norepinephrine or fatty acids appears to be characterized by a very low yield of ATP by oxidative phosphorylation. Substrate level phosphorylation in the Krebs cycle is the major energy producing reaction for the generation of ATP and GTP required for fatty acid activation. Since mitochondria can be prepared from brown fat with normal respiratory control and

other properties, it is unlikely that they are deficient in any of the coupling factors required for oxidative phosphorylation. Fatty acids, either directly or indirectly, may be involved in releasing the respiratory chain from control by energy coupling and transfer reactions. Thus, control may be released at the same time as substrate is delivered to the respiratory chain. The oligomycin sensitivity of fatty acid respiration suggests that energy driven ion translocation cannot account for the necessary energy dissipation with a fully coupled electron transport system. It appears that a final answer to the question of what is the chemical mechanism for heat production in brown fat will be provided only after the nature of the energy transfer mechanisms of the respiratory chain have been elucidated.

ACKNOWLEDGMENTS

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The Comparative Oxidation of Palmitic, Oleic and Succinic Acids by Rat and Bat Brown Adipose Tissue Homogenates as a Function of Temperature[1]

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ABSTRACT

The oxidation of 1-^{14}C-palmitic, 1-^{14}C-oleic and 2-^{14}C-succinic acids to $^{14}CO_2$ by homogenates of brown adipose tissue (BAT) from rat and bat was studied as a function of temperature. In all cases bat BAT gave a greater conversion of added fatty acids to $^{14}CO_2$ than did rat BAT. The conversion of labeled succinate to $^{14}CO_2$ is greater in the bat than in the rat only at low temperatures. In all paired observations below 30 C the energy of activation (E_A) of the bat preparations are lower than the rat. This indicates a greater thermal efficiency for the oxidation of the indicated substrates. The bat BAT homogenates show a greater efficiency than rat homogenates in the amount of succinate oxidation associated with the oxidation of long chain fatty acids to CO_2. The significance of these findings to thermogenesis by BAT in hibernation and cold adaptation is discussed.

INTRODUCTION

A working definition of hibernation is "that slowed state of life, characterized by periods of naturally induced hypothermia, which occurs during winter in the colder regions of the temperate zone" (1-3). It has been stated that brown adipose tissue (BAT) is a characteristic of all mammalian hibernators thus far examined (4); in addition, BAT is found in many mammalian species which do not hibernate. Mammalian non-hibernators cannot withstand artificially-induced deep hypothermia, below 15 C for prolonged periods (5). In contrast, non-mammalian hibernators are not noted for BAT deposits but can withstand extended periods of hypothermia (1). Thus the relationship of BAT as an essential adjunct to hibernation is not altogether clear.

Considerable evidence has been gathered linking BAT to heat production and thermoregulation in both hibernating and non-hibernating mammals (6-11). Marquis and Fritz (12) have stated that BAT from rat has a high capacity to oxidize palmitic acid. It also has a high level of carnitine and carnitine acetyl transferase which is a characteristic of tissues having a high fatty acid oxidizing capacity. Electron micrographs of BAT cells from two species of bats clearly demonstrate the high percentage of the cell volume occupied by mitochondria (13,14). The cristae are tightly packed and highly developed, also indicative of great oxidative capacity.

The unique thermogenic behavior and great oxidative capacity of BAT makes it an obvious choice for comparative biochemical examination at the particulate and enzymatic level. The data from the experiments to follow demonstrate basic quantitative and qualitative differences in the enzymology of a hibernator (big brown bat, *Eptesicus fuscus*) and a non-hibernator (albino rat).

MATERIALS AND METHODS

Experimental Animals

Male albino rats (Holtzman, Madison, Wis.) were cold-adapted at 5 C for a minimum of six weeks prior to killing. Bats of either sex were collected and maintained in the cold, 5 C, as described elsewhere (15). Preliminary investigations showed no sex difference in the bat with regard to fatty acid and succinate oxidation.

Homogenate Preparation

Animals were killed by decapitation. Rats and bats were killed promptly after removal from the cold-room, and were handled so as to minimize agitation or trauma. The BAT was rapidly excised, freed from any adherent white adipose tissue, and placed immediately in ice-cold sucrose-EDTA (0.25 M and 10^{-3} M, respectively) solution adjusted to pH 7.4 with HCl. The BAT was then homogenized in fresh ice-cold sucrose-EDTA solution by means of a Potter-Elvehjem type homogenizer; the crude homogenate was centrifuged at 300 x *g* for 10 min. After centrifugation the floating fat was aspirated and the supernatant carefully

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TABLE I

Comparison of Rat and Bat Oxidative Capacities[a]

Temperature (C) (1/T x 10^{-3}) (1/°A)	5 (3.60)	10 (3.53)	15 (3.47)	20 (3.41)	25 (3.36)	30 (3.30)	37 (3.23)
1-^{14}C-palmitate							
+ Succinate	10.5	8.3	6.9	7.9	5.9	3.7	2.4
- Succinate	16.8	11.2	7.9	6.2	5.8	4.6	2.5
1-^{14}C-oleate							
+ Succinate	15.0	8.6	5.4	6.3	4.7	2.9	1.8
- Succinate	16.2	10.1	7.7	4.6	4.3	3.8	1.9
2-^{14}C-succinate							
+ Palmitate + Carnitine	1.8	1.3	1.0	0.78	0.60	0.46	0.34
+ Palmitate - Carnitine	3.6	2.2	1.4	1.0	0.66	0.34	
+ Oleate + Carnitine	1.3	1.0	0.74	0.58	0.47	0.37	0.17
+ Oleate - Carnitine	2.7	1.7	1.0	0.74	0.52	0.34	

[a]Tabular values = $\frac{\text{Bat } ^{14}CO_2/\text{mg-hr}}{\text{Rat } ^{14}CO_2/\text{mg-hr}}$

decanted so as not to disturb the pelleted debris. Protein concentration of the supernatant was normally 1.0 ± 0.3 mg/ml as determined by the method of Lowry et al. (16). This partially resolved homogenate was used throughout all of the experiments described.

Incubation Medium

The fatty acid oxidation medium contained, in a final volume of 2.5 ml; 1 mM MgCl, 1 mM adenosine triphosphate (ATP), 80 mM KCl, 8 mM Na_2HPO_4, 0.04 mM coenzyme A, 0.12 mM 1-^{14}C-palmitic or 1-^{14}C-oleic acid (as the potassium salt), 0.5 mM DL-carnitine-chloride, 100 mM sucrose, and 0.4 mM EDTA. Final pH of the medium was 7.4. In those experiments where succinate was added, a final concentration of 0.12 mM was employed.

The 2-^{14}C-succinic acid oxidation medium differs from the above in two ways. Unlabeled fatty acid was substituted for the labeled material, and in some experiments carnitine was deleted.

Fatty Acid-Albumin Complex

Unlabeled succinic acid was purchased from Sigma Chemical Co. (St. Louis, Mo.) and palmitic and oleic acids from Applied Science Laboratories (State College, Pa.). Radio-labeled materials, 1-^{14}C-palmitic, 1-^{14}C-oleic, and 2-^{14}C-succinic acids were used as obtained from Tracerlab, Inc. (Waltham, Mass.). Requisite amounts of the fatty acids were dissolved in a slight excess of KOH, added to crystallized bovine serum albumin (Pentex, Kankakee, Ill.) and adjusted to a final pH of 7.4 with HCl. The concentration of the stock solution of the fatty acid-albumin complex was 0.6 mM in acid and 0.075 mM in albumin. In the experiments involving the addition of succinic acid, it was added directly to the fatty acid-albumin complex at the same molarity as the fatty acid.

Incubation Procedure

Incubations were performed in Kontes reaction vessels fitted with polypropylene center wells (Kontes Glass Co., Vineland, N.J.). The center wells contained 0.2 ml of hyamine hydroxide (Packard Instr. Co., Downer's Grove, Ill.) pipetted onto a small folded piece of filter paper. Trapping efficiency for $^{14}CO_2$ was better than 99% as determined with standard solutions of ^{14}C-sodium bicarbonate. All measurements of $^{14}CO_2$ were made by clipping the plastic center well from its stem and adding the entire assembly to 15 ml of toluene-PPO-POPOP scintillation fluid. Radioactivity was measured in a Packard Tri-Carb scintillation spectrometer.

Aliquots of the fatty acid-albumin complex (0.5 ml) and incubation medium (1.0 ml) were pre-incubated to insure thermal equilibrium in each of a series of Eberbach metabolic shakers (Eberbach Corp., Ann Arbor, Mich.) thermostatted to ± 0.5 C at approximately 5 degree intervals from 5 to 40 C. The reaction was initiated by addition of homogenate (1.0 ml) in a sequential order with respect to the thermostatted baths. Triplicate flasks were used at each temperature, and the entire experimental protocol was repeated. Oxidation of 2-^{14}C-

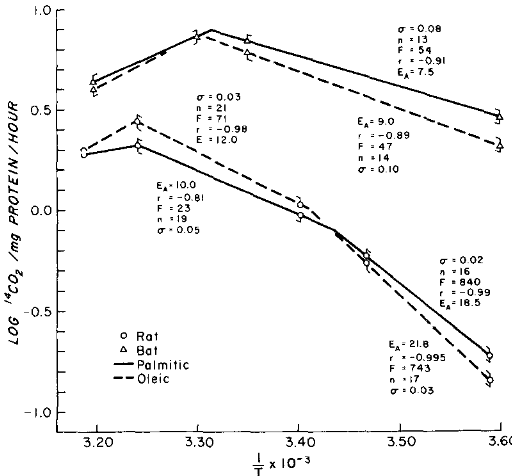

FIG. 1. Per cent conversion of 1-^{14}C-fatty acids to $^{14}CO_2$ in the absence of succinic acid. For clarity, only the end points of the line segments are shown. Abbreviations: E_A, activation energy in k cal/mole; r, the correlation coefficient; F, the variance ratio; n, number of observations; and σ, the standard deviation. See text for further details.

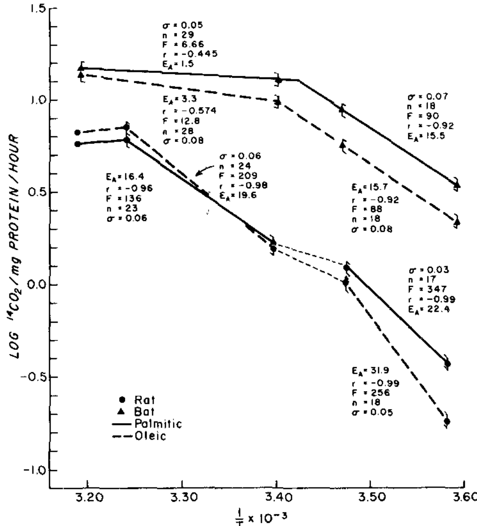

FIG. 2. Per cent conversion of 1-^{14}C-fatty acids to $^{14}CO_2$ in the presence of succinic acid. The symbols have the same meaning as in Figure 1. See text for further details.

succinate was always performed in the presence of either unlabeled palmitate or oleate in order that their effect on the conversion of labeled succinate to $^{14}CO_2$ could be observed.

Statistical Analysis

Statistical analysis of the data was performed by an IBM 7044/1401 computer supplied with a multiple regression analysis "can" program (University of Iowa Computer Center REGAN-1, Iowa City, Ia.).

RESULTS

The data for the oxidation of the indicated substrates by rat or bat BAT are reported in the form of Arrhenius plots in Figures 1-4. The ordinate represents the log of the per cent of total added label converted to $^{14}CO_2$ per milligram of protein per hour. The slopes of the lines were obtained directly from the computer analysis of the primary data. The indicated statistical parameters were similarly obtained, except for the values of E_A (energy of activation) which were obtained by multiplying the computer-derived slopes of the bracketed line segments by 2.303 R (R = 1.986 calories per mole). The data tables were derived from the computer-calculated values by taking the antilog of the differences in the log rates of the paired conditions indicated in the legends and at the same temperature. In this way, comparisons of rat and bat oxidative capacities, succinate stimulation of fatty acid oxidation (FAO), carnitine stimulation of succinic acid oxidation (SAO), and succinate-fatty acid utilization ratios could be more easily visualized. For convenience, only the end points of computer-derived line segments are shown in the figures.

Figure 1 shows the Arrhenius plot for the conversion of 1-^{14}C-labeled fatty acid to $^{14}CO_2$ in the absence of added succinate. It is quite evident that the bat exhibits a much greater oxidative capacity for palmitate and oleate than does the rat. Table I illustrates the magnitude of these differences. The degree to which the bat BAT FAO exceeds that of the rat is clearly temperature-dependent. The largest differences occur at the lower temperatures. The same is true for FAO in the presence of added succinate as is shown in Figure 2 and Table I.

Figures 3 and 4 are Arrhenius plots of the conversion of 2-^{14}C-succinate to $^{14}CO_2$ by rat and bat BAT homogenates. Obviously the results are quite different from those for FAO. The bat oxidation of succinate exceeds that of rat only at the lower temperatures; at the higher temperatures the rat has a higher capacity for SAO. The crossover point is influenced by the fatty acid present in the medium. Table I gives the values for these differences. Values greater than unity are indicative of a greater oxidative capacity by bat BAT than rat.

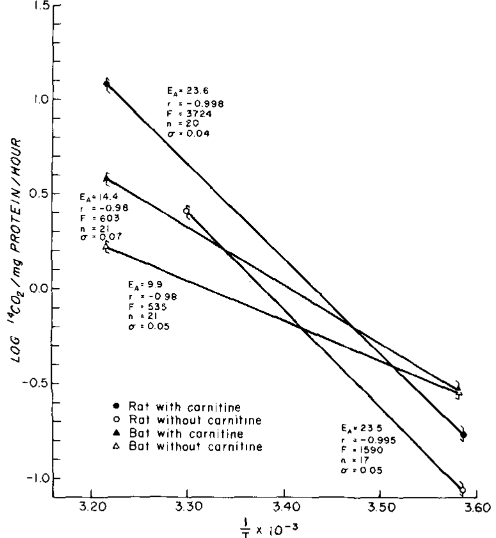

FIG. 3. Per cent conversion of 2-^{14}C-succinic acid to $^{14}CO_2$ in the presence of palmitic acid. The symbols have the same meaning as in Figure 1. See text for further details.

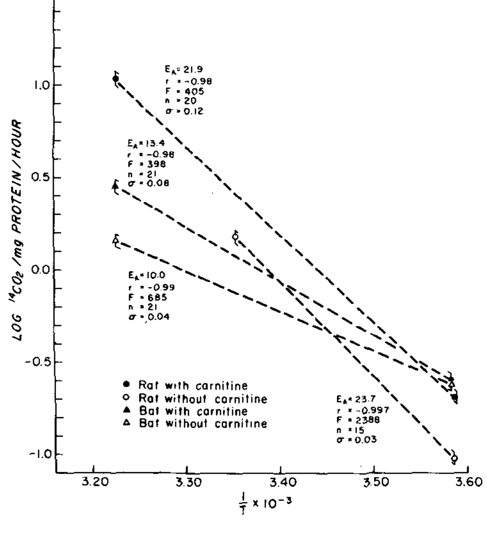

FIG. 4. Per cent conversion of 2-^{14}C-succinic acid to $^{14}CO_2$ in the presence of oleic acid. The symbols have the same meaning as in Figure 1. See text for further details.

A distinct feature of Figure 1 are the breaks in the Arrhenius plots for rat and bat FAO. Both rat and bat show clear thermal optima for FAO; the rat BAT at 35 C and bat near 28 C to 30 C. Furthermore, the rat FAO gives a change in slope at 18 C to 20 C. Figure 2 is qualitatively similar to Figure 1 in most respects. Notable differences are the effect of succinate on the bat FAO optimum and the unusual nature of the thermal break in the rat FAO between 15 and 20 C. Addition of succinate to bat preparations caused a nearly flat thermal response of FAO from near 20 C to 40 C. The unusual break in FAO by rat BAT between 15 and 20 C prompted a computer solution of the entire range of 5 C to 35 C. The resultant statistical data showed conclusively that the best treatment is that shown in Figure 2, i.e., two separate line segments. The same conclusion was reached upon re-examination of the rat data in Figure 1.

In contrast to the FAO data, SAO shows a linear response to temperature. Rat SAO without added carnitine does show an oxidative plateau between 30 C and 37 C (this data does not appear on the Arrhenius plots for the sake of clarity of data presentation).

In the previous discussion it was mentioned that breaks or changes in slope occur in the plots of both rat and bat FAO. In the rat these breaks occur at the threshold of the hypothermic range. Since the rat is unable to withstand hypothermia for extended periods of time, this thermal range is of great comparative interest. A consistent feature of Figures 1 through 4 is the higher E_A values for rat BAT oxidations of the indicated substrates. Below 15 C the average E_A for rat FAO is 20.2 k cal/mole in the absence of added succinate and 27.2 k cal/mole in the presence of added succinate. Corresponding values for the bat are 8.2 k cal/mole and 15.6 k cal/mole respectively. The E_A values from 20 C to 35 C are much higher for rat than bat, but the dramatic changes in bat FAO makes any comparison at these temperatures difficult to assess.

The E_A values for succinate oxidation (Fig. 3 and 4) are similar to those for FAO. E_A for rat BAT SAO averages 22.8 k cal/mole in the presence of added carnitine and 23.6 k cal/mole in the absence of added carnitine. Corresponding values for the bat are 13.9 k cal/mole and 10.0 k cal/mole, respectively.

Succinate stimulation of FAO in the presence of added carnitine (Table II) and carnitine stimulation of SAO in the presence of added fatty acid (Table III) give remarkably similar stimulation values. In the bat the values for both parameters are at a minimum at the lowest temperatures and maximal at the highest. Carnitine stimulation of SAO in BAT from rat is a rather constant twofold increase, regardless of which fatty acid is present. Succinate stimulation of FAO in rat BAT prepa-

TABLE II

Succinate Stimulation of Fatty Acid Oxidation (FAO)[a]

Temperature (C) (1/T x 10^{-3}) (1/°A)	5 (3.60)	10 (3.53)	15 (3.47)	20 (3.41)	25 (3.36)	30 (3.30)	35 (3.25)	40 (3.20)
Bat								
Palmitate	1.2	1.5	2.0	2.3	2.0	1.9	2.5	3.3
Oleate	1.0	1.3	1.6	2.0	1.8	1.6	2.3	3.3
Rat								
Palmitate	1.9	2.1	2.3	1.8	2.0	2.4	2.8	3.0
Oleate	1.2	1.6	2.0	1.4	1.6	2.0	2.5	3.2

[a]Ratio = $\frac{\text{FAO with succinate}}{\text{FAO without succinate}}$

rations gives slightly lower values with oleate as the substrate than with palmitate. Succinate provides up to a threefold increase in FAO in both rat and bat BAT homogenates.

The experiments in Figures 2, 3 and 4 were performed under identical conditions except that the fatty acids were labeled in the experiments shown in Figure 2 and succinate was labeled in the experiments shown in Figures 3 and 4. This allows one to compare the number of fatty acid carbons oxidized for each carbon of succinate. Implicit in this calculation are two assumptions. One, $^{14}CO_2$ released from the fatty acid, represents total combustion of that acid. That is, as the fatty acid passes through the β-oxidation scheme, the labeled acetate formed is representative of the entire, fatty acid-derived acetate pool, which is then oxidized to CO_2 within the Krebs cycle. The second assumption is that the $^{14}CO_2$ derived from succinate represents oxidation of the entire succinate molecule. The ratio of label to total carbon is 1:4 in succinate, 1:16 in palmitate and 1:18 in oleate. On this basis the values obtained for the succinate-fatty acid oxidation ratio must be corrected for the ratio of label frequency (4.0 for palmitate and 4.5 for oleate). Table IV is the result of these comparisons. From this table it is obvious that the bat can oxidize far more fatty acid per molecule of succinate than the rat. The values are temperature-dependent. The most efficient utilization of succinate occurs below 25 C for both BAT preparations. The potential importance of this observation will be discussed in the section to follow.

In an earlier communication (15) we reported that homogenate preparations of bat BAT oxidize oleate less well than palmitate. At incubation temperatures less than 20 C the same is true of BAT for rat. At higher temperatures oleate is more rapidly oxidized by rat BAT than palmitate. An additional factor evident in Figures 1 and 2 is the higher E_A values for oleate oxidation than for palmitate. This latter observation is of particular interest in view of a recent communication by Pande and Mead (17) suggesting the presence of two different activating enzymes for fatty acyl CoA formation; one for saturated acids and another for unsaturated fatty acids. The difference in E_A for the oxidation of palmitate and oleate seen in the rat BAT system may be the result of a difference in the E_A of the two activating enzymes. This possibility is currently under investigation in our laboratory.

DISCUSSION

The thermogenic capability of BAT has been the subject of considerable investigation in recent years, and several biochemical mechanisms have been proposed to explain this phenomenon (18-20). No general agreement exists as to which mechanism, if any, is correct. One of the problems could stem from the use of BAT from different species of mammals. The similarity of BAT as a thermogenic tissue notwithstanding, the fundamental mechanism(s) may not be the same in all cases.

The two examples of BAT used in these experiments clearly show differences in the oxidation of the substrates tested. The differences are not only those of quantitative capacities but of qualitative thermal reactivities as well. If the latter is due to a species-specific change in the E_A of a certain step, a difference in excess of 7 k cal/mole would be necessary in some cases. We do not suggest that this is impossible, since work recently concluded in our laboratory demonstrated that differences of this magnitude do occur (Paulsrud et al., in preparation); however, an alternative interpretation is possible. A rate-limiting step in one BAT may be replaced by another in the BAT from a different species.

Comparison of bat FAO in Figures 1 and 2 and SAO in Figures 3 and 4 shows that the E_A values and rates of oxidation change when

TABLE III

Carnitine Stimulation of Succinate Oxidation (SAO)[a]

Temperature (C) ($1/T \times 10^{-3}$) ($1/^{\circ}A$)	5 (3.60)	10 (3.53)	15 (3.47)	20 (3.41)	25 (3.36)	30 (3.30)	37 (3.23)
Bat							
Palmitate	1.0	1.2	1.3	1.5	1.7	2.0	2.2
Oleate	1.0	1.1	1.3	1.4	1.6	1.7	2.0
Rat							
Palmitate	2.0	2.0	1.9	1.9	1.8	1.8	3.7
Oleate	2.1	2.0	1.9	1.8	1.7	1.7	3.4

[a]Ratio = $\frac{\text{SAO with carnitine}}{\text{SAO without carnitine}}$

either succinate or carnitine was removed from the system. This strongly suggests the establishment of a new rate-limiting enzymatic step with a lower E_A. Deletion of the same components from an identical system using rat BAT results in a lower level of oxidation. The low temperature E_A values are virtually unchanged for rat SAO in contrast to the 4 k cal/mole shift seen in bat SAO. The E_A for rat FAO shifted to lower values in the deletion experiments, especially those values for the oxidation of oleate. Despite the shift, indicative of the establishment of a new rate-limiting step, even the lower rat E_A values are in excess of the highest comparable bat E_A. Regardless of the cause for the gross differences in E_A values, the main feature of these data is that the bat exhibits a far greater capacity and efficiency for FAO and SAO at low temperatures than does the rat.

Johnson et al. (21), summarizing earlier theories of Crozier and Blackman and Putter, stated the following regarding the thermal reactivity of biological systems: "(a) a catenary series of reactions, each with a definite μ value . . . underlies every physiological process; (b) the overall process is limited, within definite ranges of temperature, by a slowest step or master reaction; (c) at a certain critical temperature a different master reaction may assume control, and a sharp "break" will be apparent in the slopes of lines in an Arrhenius plot of the data; and (d) it might be possible to identify corresponding master reactions in different . . . processes . . . by correspondence in their measured micro values." The μ referred to is identical to E_A used above. The notion of sharp breaks occurring in plots of complex systems has a well-established counterpart in Arrhenius plots of purified enzymes (22). Thus it is not without precedent that we report sharp breaks in Figures 1 and 2.

Whether or not the E_A of a complex biological system reflects the E_A of a single rate-limiting enzymatic step is not yet firmly established. It is tempting to speculate that such is the case. Such speculation, based on Part 4 of the Crozier hypothesis and on literature values for the E_A of some of the enzymes involved in FAO and SAO, would lead one to the suspicion that the rat E_A values are determined by succinic dehydrogenase (23) or DPNH cytochrome c reductase (24) whose E_A values bracket those for rat FAO and SAO. Extending such speculation to the bat, malic dehydrogenase (Paulsrud et al., in preparation) matches the higher values; fumarase (22), the lower. Until the rate-limiting enzyme(s) for these processes are determined, and their E_A values measured for each animal, the entire question remains speculative. Studies involving BAT, such as were performed by Williamson (25) and Lardy's group (26) for other tissues and pathways, will likely result in the discovery of which enzymes are rate-controlling.

The high E_A values and lower oxidative capacity exhibited by rat preparations might account for the rat's inability to withstand hypothermic body temperatures. The sharp decline in oxidative capacity may well result in the operation of some systems below a level necessary to the maintenance of life. Perhaps the key physiological observation explaining the inability of a non-hibernator to survive hypothermia is that severe cardiac malfunctions occur at body temperatures below 20 C (27). It is interesting that the abrupt breaks we report in Arrhenius plots of rat FAO occur near this temperature. The slopes of the FAO plots below 20 C indicate a much lower thermal efficiency than above 20 C. Comparison of our rat BAT data with that of Fritz (28) for rat heart homogenates shows that the heart preparations have an even greater FAO capacity. If FAO in the rat heart is essential and thermally similar to rat BAT, it may be that the observed decrease in thermal efficiency of FAO is responsible for the cardiac crisis in the hypothermic non-hibernator. In contrast, a characteristic of the hibernator heart is the ability to

TABLE IV

Succinate: Fatty Acid Utilization Ratio[a]

Temperature (C) (1/T x 10^{-3}) (1/°A)	5 (3.60)	10 (3.53)	15 (3.47)	20 (3.41)	25 (3.36)	30 (3.30)	37 (3.23)
Bat							
Palmitate	51	55	57	54	39	26	17
Oleate	42	45	49	53	42	28	22
Rat							
Palmitate	8.4	8.3	3.0	4.9	3.9	3.2	2.4
Oleate	3.5	4.9	6.6	4.7	4.1	3.8	3.1

[a]Ratio = $\frac{FAO}{SAO}$

beat at temperatures near 0 C (29). Thus the data we report for FAO and SAO would seem consistent with certain observations at the physiological level.

Succinate oxidation in the rat and bat BAT preparations showed the same thermal relationships observed for FAO below 15 C; however, SAO maintained that relationship throughout the entire range of temperatures examined. The fact that E_A values for FAO and SAO are very similar under equivalent incubation conditions suggests that both processes are controlled by the same step at the low thermal range. It therefore seems possible that both are controlled by a step within the Krebs cycle or the electron transport chain associated with it.

The release of $^{14}CO_2$ from 2-^{14}C-succinate by bat preparations exceeds that of the rat only at the lower temperatures (Table I), which is in direct contrast to the case for FAO. This does not mean that oxidation of succinate in the Krebs cycle occurs at a lower rat in bat BAT. On the contrary, since CO2 from fatty acid-derived acetyl groups arises almost entirely within the Krebs cycle, there must be a greater net oxidation of succinate by bat BAT. What was measured was the amount of radio-labeled CO_2 released from succinate, not the activity level of succinic dehydrogenase. Theoretically the cycle can operate with only catalytic amounts of dicarboxylic acids as "sparking" agents for the oxidation of acetyl groups. However, the processes of diffusion and or transport, transamination and decarboxylation of oxaloacetate and malate to form phosphoenolpyruvate (PEP) and pyruvate may result in the loss of intermediates necessary to the continued operation of the cycle. A loss by any of these means would require replacement from the added extramitochondrial pool of 2-^{14}C-succinate. The result of such replacement in the face of active FAO would be to increase the specific activity of the mitochondrial succinate pool, thereby increasing the yield of succinate-derived $^{14}CO_2$.

Regardless of the mechanism, the bat uses less succinate to oxidize fatty acid than the rat. Such a situation would be extremely beneficial since the bat, as a hibernator, is faced with the problems of surviving for six months with virtually no food intake. During this time, the bat occasionally arouses to an active state to seek better shelter or obtain water. Data obtained by Stones and Wiebers (31) indicate that the bat undergoes daily bursts of metabolic activity during hibernation resulting in an increased body temperature. To be sure, not all of these bursts result in complete thermal arousal but nonetheless create additional metabolic demands. Therefore, any mechanism which would conserve metabolites derived from carbohydrate or amino acids and not from the stores of body fat would help to bridge the gap between the times of food availability. On the other hand, the rat apparently lacks such a conservative mechanism and has to have a constant food supply in order to survive.

Since the bat BAT has a great low-temperature efficiency and capacity, one might well ask what controls exist to prevent the bat from using up its energy reserves long before the end of hibernation. Reports in the literature and observations from our laboratories point to a very straightforward mechanism for the control and release of this metabolic capability. Joel (31) and others (32,33) have shown that BAT is very responsive to stimulation by lipolytic hormones. Microscopic examination of BAT revealed a high degree of innervation largely adrenergic in nature (34). In an earlier publication we reported that the ATP levels in bat BAT are maximal during the torpid state, but as arousal from hibernation progressed (as measured by body temperature) the levels fell rapidly (35). Observations in our artificial cave and in the natural caves where the animals were captured reveal that the bat responds almost instantly to the slightest disturbance with an increase in respiration and heart rate. Such a response is obviously the result of a nervous

system which is highly irritable even at body temperatures less than 5 C. Often a bat disturbed in this manner will arouse completely. In summary, BAT is a tissue well supplied with nerve endings capable of releasing norepinephrine which stimulates lipolysis, high levels of ATP for the activation of fatty acids, and high metabolic capacity to oxidize fatty acid. The BAT oxidative capacity at low temperatures could be controlled by the release of fatty acid. Since the bat has a high degree of irritability at low body temperatures, any stimulus resulting in the release of catacholamines into BAT would reverse the control and assist in the process of arousal. Unfortunately it is still not known how the bat is able to reduce its metabolism and become hypothermic.

The theories on the mechanism of thermogenesis are all concerned in part with the means by which large amounts of reduced pyridine and flavin nucleotides are reoxidized in order to be able to continue FAO. The inability to obtain high P/O ratios in BAT under conditions which give P/O ratios of nearly 3 for liver mitochondria suggests a poorly coupled electron transport system (36). If this is a characteristic of the BAT mitochondria, the reduction of molecular oxygen to water without concomitant phosphorylation would yield significant amounts of heat. There is ample evidence in the literature showing the electron transport chain is uncoupled by a high level of fatty acid (37). Such a level might arise during the lipolysis associated with arousal from hibernation.

Now that fundamental differences between the rat and bat BAT metabolism have been established it should be possible to further define the thermogenic mechanisms, with full realization that the mechanisms may not be the same in different species.

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Bill Snider and Mrs. Clara Augustine gave assistance in the computer analysis of the primary data. Supported in part by grants from the National Institutes of Health (AM 08476) and the American Cancer Society.

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Comparative Effects of Temperature Exposure on Mass and Oxidative Enzyme Activity of Brown Fat in Insectivores, Tupaiads and Primates[1]

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ABSTRACT

A study has been made to see if hyperplastic and biochemical responses similar to those which occur in temperature-exposed rodents also occur in insectivores, protoprimates and primates. Representative insectivores included the shrews *Suncus murinus* and *Cryptotis parva*. The representative protoprimate was *Tupaia chinensis*. The representative primate was *Macaca mulatta*. Both shrews show a striking thermogenic response in the brown fat with respect to increased specific activity of α-glycerophosphate oxidase and succinoxidase. *Suncus* also showed an increase in brown fat mass following cold exposure. In both shrews cold caused the brown fat to become darker in color. *Cryptotis* did not show a significant increase in brown fat mass, but this may be due to the fact that the cold exposure period was only four days. Thus the shrews appear to be rodent-like with respect to effects of cold on brown fat. In the Tupaiads the ratio of brown fat to body weight is not nearly as high as in rodents nor is there a striking hyperplasia with cold exposure. Thus the brown fat would seem to be of less importance to the Tupaiads than to rodents and shrews and the Tupaiads appear to be intermediate in this respect between the insectivores and the true primates. Adult *Macaca*, in spite of eight months of cold exposure at about 4-6 C, showed no hyperplasia of the brown fat and no increase in oxidative enzyme levels. Thus in adult primates so far studied, the evidence indicates that brown fat does not play an important role in homeothermy as it does in rodents.

INTRODUCTION

As part of an extensive study on biochemical thermoregulation in representative insectivores, protoprimates and primates, oxidative enzyme levels have been examined in the brown fat of the true shrews, *Suncus murinus* and *Cryptotis parva*, the protoprimate, *Tupaia chinensis* and the old world monkey, *Macaca mulatta*. Although this is not an exhaustive study of the biochemical acclimation characteristics of brown adipose tissue of these species, it is unique in providing the only such data available on these critically important phylogenetic groups.

The thermogenic role of brown fat has been well established in a number of adult mammalian species, especially rodents (1-7) and hibernators (3,8-14). However, earlier work on the squirrel monkey, *Saimiri sciurea* (15,16) indicates that biochemical changes seen in temperature acclimated rodents cannot be extrapolated in toto to other mammalian species such as primates. Thus the question which arises here is, does brown fat play a significant thermogenic role in other mammalian groups as it apparently does in rodents. In other words, upon exposure of an animal to cold does the brown fat increase in mass, distribution, oxidative enzyme levels and, therefore, potential heat production to such an extent that it could contribute significantly to maintaining the core temperature of the animal.

The choice of animals for this study was based on their phylogenetic relationships, the purpose being to determine the extent to which induction of tissue biochemical changes take place during temperature acclimation. Only brown fat will be considered in this communication. Furthermore, it was hoped that within the species selected there would emerge patterns of biochemical adaptation, intermediate between those seen in rodents and those seen in the squirrel monkey, which are related to their phylogenetic position.

The shrew chosen as a representative insectivore is *Suncus murinus*. This species is as large as a white mouse and thus not subject to

[1]One of nine papers to be published from the Symposium "Brown Adipose Tissue," presented at the AOCS-AACC Joint Meeting, Washington D. C. March, 1968.

extreme heat loss due to a large surface area to body mass ratio. *Cryptotis parva*, the least shrew, was studied because of its extremely small size and to see if brown fat development would be exaggerated in such an animal.

The Tupaiads were chosen as representative of primate-insectivore intermediate. *Tupaia* are considered by many taxonomists to be as close to the stem primate line as any animal living today (17). These animals are intermediate in size between a hamster and a white rat.

Since we have completed a study on a true primate representative of new world monkeys, *Saimiri sciurea* (15,16,18), the Rhesus was chosen as a representative of the old world monkeys. Studies on the latter species are preliminary in nature.

The level of α-glycerophosphate oxidase was determined because α-glycerophosphate might be derived from glycerol which may accumulate in lipolysis. Its possible role in the entrance of hydrogen from extramitochondrial reduced NAD and the dihydroxyacetone phosphate cycle have been considered in detail elsewhere (19,20). Succinoxidase was chosen as a flavoprotein-linked tricarboxylic acid-electron transport system (TCA-ETS) enzyme, whereas α-ketoglutarate oxidase was chosen as a representative of a NAD-linked TCA-ETS enzyme system.

MATERIALS AND METHODS

The acclimation regimen and the details of laboratory care varied considerably for the different species studied and will therefore be considered separately for each species.

Shrews

Cryptotis and *Suncus* were obtained from the colonies maintained at the University of Missouri by Professor Conaway. Adult animals of both sexes were used. The laboratory conditions, feeding and care of the control *Cryptotis* have been described elsewhere (21), with the exception that for this study all animals were caged singly. The lowest temperature which *Cryptotis* and *Suncus* could survive was about 6 C at a relative humidity of about 65%. Controls were maintained at 26-30 C. The animals were provided with a wooden box shelter in their cages and were exposed to 12 hr of light per day. Cold-exposed *Cryptotis* were kept at 6-8 C for four days. Because of their extremely small size (3.5-5.5 g) and consequently large surface to volume ratio, and because of their rapid metabolic rate, this was considered sufficient time to elicit a cold-acclimation response.

Suncus were maintained under conditions similar to those described for *Cryptotis*, but because of their larger size (20-35 g) they were cold-exposed (6-10 C) for five to eight weeks.

Tupaiads

Adult tree shrews (*Tupaia chinensis*), weighing from 120 to 250 g were obtained from Borneo through the Rider Animal Co., Brooksville, Florida. After arrival in Missouri they were quarantined for three weeks and checked for external injuries. Standard veterinary checks were made to insure reasonable health of the animals. The colony conditions and laboratory care of these have been described elsewhere (17,22).

Preliminary heat and cold tolerance tests were made on 20 animals. Ten were subjected to an increase of 2 C per day and 10 to a decrease of 2 C per day. These studies revealed that the temperature tolerance extremes for this species are 35 C and 12 C respectively. After four weeks at these temperatures, 8 of 10 animals survived in the cold, and 9 of 10 in the heat. Subsequently 90 animals were divided into three equal groups and caged individually in 20 x 20 x 18 in. cages. One group was placed at room temperature (24 C), another at 12.5 C and a third at 35 C. The elaborate diet required by various species of Tupaiads has been described elsewhere (17). At the end of two months of temperature acclimation, three animals (one from each group) were used per day for the enzymatic studies.

Monkeys

The conditions of acclimation of the squirrel monkeys (*Saimiri sciurea*) have been described elsewhere (15). They were found to be very sensitive to cold and therefore were kept at 14-15 C for cold acclimation and at 36-39 C for heat acclimation; the controls were maintained at 24 C.

Adult rhesus monkeys were acclimated by W. C. Kaufman (Holloman Air Force Base) at 4-6 C and left at that temperature for eight months. Controls were maintained at 22-28 C. Standard laboratory care was provided. Water and Purina monkey pellets were provided ad lib. and supplemented daily with fresh fruit.

Enzymatic Assays

At the end of the respective acclimation periods for each of the above species, one control and one acclimated animal per day were killed by decapitation or bleeding. The extent and distribution of the brown fat in the control and experimental animals were determined by exploratory dissection.

For enzymatic assays, brown fat was ex-

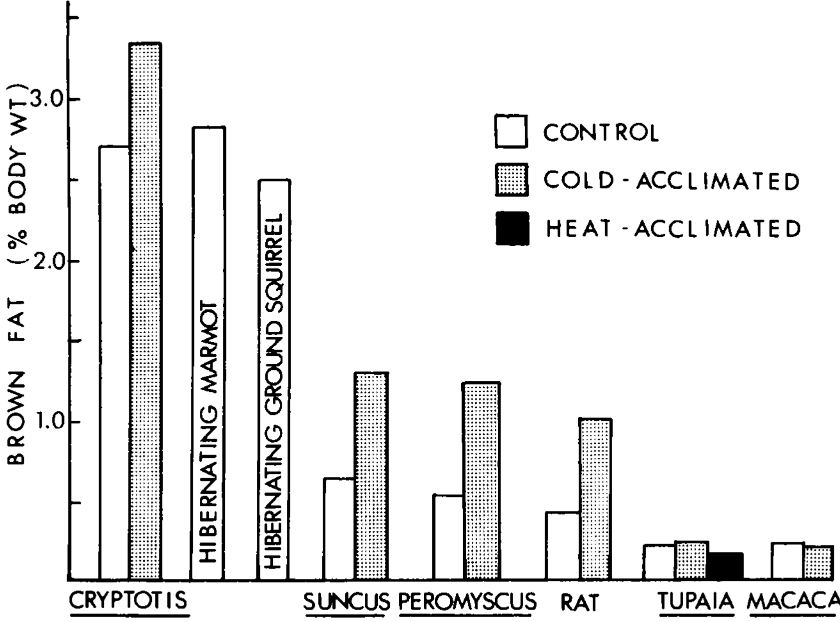

FIG. 1. Comparison of brown fat per cent of body weight in some representative rodents, insectivores, stem primates and primates. Data on hibernating marmot from Roberts et al. (unpublished data), hibernating ground squirrel (9), rat (4,7), *Peromyscus* (26).

tirpated and chilled on iced aluminum foil. The tissue was trimmed clean of associated white fat, muscle, connective tissue and, in the case of the monkeys, axillary nodes, and weighed to the nearest milligram. Homogenates of brown fat (10%) were prepared in 0.1 M phosphate buffer, pH 7.3. Succinoxidase activity was assayed polarographically by the method of Schneider and Potter (23) as modified by Chaffee et al. (15). Activities of other oxidative enzymes were assayed using modifications (8,15) of the methods of Copenhaver and Lardy (24).

RESULTS AND DISCUSSION

Quantity and Distribution of Brown Fat

A major portion of the brown fat of *Cryptotis* is found in two large pads which cover the whole dorsal and lateral aspect of the scapulae and rib cage from the mid-dorsal line and extend into the axillary region. Caudad they extend somewhat beyond the rib cage. If one were to cut across the mid-thoracic region, the combined cross sectional area of the brown fat at this level would probably exceed that of the thoracic cavity. Traces of brown fat are also found in the cervical region, but there appears to be little, if any, present along the dorsal aorta or in the thorax and no discernable amounts in the renal area. Brown fat in these animals is generally infiltrated with white fat.

In the cold-exposed *Cryptotis* the brown fat becomes darker, but by the fourth day there is still no significant increase in mass. In our dissections we were unable to remove all of the brown fat, thus the figures given in Table I are probably somewhat low. The brown fat to body weight ratio of these animals is considerably higher than that found in non-hibernating rodents and is as high as that found in the hibernating marmot (Roberts et al., unpublished data) and ground squirrel (9) (Fig. 1).

In *Suncus murinus*, as in *Cryptotis*, the brown fat is found in the axillary region and covering the dorsal scapular region. However, it does not extend as far laterally. In the interscapular region the bilateral fat pads make connections with a layer of infrascapular brown fat. A small isthmus, reminiscent of the wider membranous isthmus connecting the two lobes of the interscapular brown fat in rodents, connects the dorsal brown fat lobes. As in *Cryptotis*, there is apparently no aortic brown fat and little if any renal brown fat. Only traces are seen around the heart. Small depots of brown fat are found in the dorsal cervical musculature. The brown fat of *Suncus* appears to be less infiltrated with white fat than that of *Cryptotis*.

TABLE I

Brown Fat Weight Relative to Body Weight in Control, Cold- and Heat-acclimated Insectivores, Tupaiads and Primates

Species	Body Weight, g			Brown Fat, body wt			P Values for Brown Fat, % Body Wt
	Cold	Control	Hot	Cold	Control	Hot	
Cryptotis	3.73	4.17	---	3.35 (4)[a]	2.70 (8)	---	b
Suncus	33.35	32.55	---	1.29 (18)	0.63 (18)	---	<0.05
Tupaia	166.6	164.6	170.1	0.24 (30)	0.22 (30)	0.16 (30)	b
Saimiri[c]	806.0	801.4	855.0				b
Macaca	4169.0	4430.0	---	0.21 (2)	0.22 (2)	---	b

[a]In parentheses, number of animals.
[b]No significant differences.
[c]Data from Chaffee et al. (15).

As in *Cryptotis*, in our dissections of *Suncus* we did not remove 100% of the brown fat. In control *Suncus* the brown fat represented 0.63% of the body weight. This ratio increased to 1.29% of the body weight following cold acclimation. These values agree well with values of 0.65%-1.5% found by Buchalczyk and Korybska (25) for the shrew, *Sorex araneus*, studied at different times of the year.

In one *Suncus* exposed to cold for eight months, the final body weight was 35.5 g. Its brown fat, obtained from the combined dorsal interscapular and axillary regions was 0.9626 g. In a comparable animal kept in the cold for 34 days the brown fat weight was 0.3864 g and in a control of the same size the brown fat weighed 0.2087 g. In addition to the increased mass of axillary and interscapular brown fat in the long-term cold-acclimated *Suncus*, brown fat was also found in the inguinal region. Thus prolonged cold exposure seems to stimulate continued brown fat hyperplasia.

In the Tupaiads, brown fat is confined primarily to the axillary region although there is a thin layer of white fat over the dorsal region of the thorax and scapulae in which traces of pale brown fat appear. There are traces of perirenal brown fat, but here, as in the monkeys, there is a predominance of yellow fat. There is little, if any, aortic or mediastinal brown fat. There are some deposits of brown fat in the cervical region including traces in the perijugular region and subtrapezoid region. The distribution of brown fat in this species is more similar to that of the squirrel monkeys and rhesus monkeys than it is to that seen in the true shrews, *Suncus* or *Cryptotis*. The ratio of brown fat to body weight is much lower than that found in shrews (Table I) or than that previously reported in rats (4,7) or deer mice (26) (Fig. 1) and does not increase significantly when the animals are exposed to prolonged cold. During cold exposure, however, the amount of yellow fat increases and is found, among other places, in the interscapular region. In heat-acclimated animals, there is a decrease in the mass of both brown and yellow fat and both are paler than in the controls.

In the squirrel monkey, *Saimiri sciurea*, the brown fat is almost all axillary and surrounds the axillary nodes. There are traces of it in the perirenal area and in the cervical region around the jugular veins and the thyroid. In the cold the brown fat does not increase in mass, but becomes richer in protein content by about 40% (15) and becomes darker in color as is seen in the Tupaiads and rodents. In the heat-acclimated animals, on the other hand, the protein content decreases by about 50% and the tissue becomes pale in color (15). As in the Tupaiads, there are large quantities of yellow fat widely distributed in these animals. It seems to increase in quantity during cold-acclimation and to decrease or be converted to white fat in heat acclimation.

In *Macaca*, as in the squirrel monkey, the brown fat is primarily axillary; it surrounds and is intimately associated with the axillary nodes. There is also a large mass in the perijugular cervical region around the thyroid. No interscapular fat pads were evident in these animals. This distribution is similar to that described by Itoh and Hiroshige (27) in *Macaca fuscata*. Prolonged cold exposure did not cause an increase in the brown fat mass, nor was the brown fat consistently darker in the cold-acclimated compared to the control animals. In addition to brown fat, rhesus monkeys possess large amounts of yellow fat which increases considerably in the cold. Whether or not this tissue has a thermogenic role in these monkeys remains to be determined, but it no doubt has

TABLE II

Oxygen Uptake of Brown Fat Homogenates of Control, Cold- and Heat-acclimated Insectivores, Tupaiads and Primates

Species	Substrate	Assay Temp., C	Cold-acclimated	Control	Heat-acclimated	Units	P Values
Cryptotis	Succinate	34	15.12 (4)[a]	6.58 (8)	---	μl O_2/mg wet wt/hr	C>W, [b]$p<0.01$
Cryptotis	α-Glycero-phosphate	34	6.22 (4)	1.10 (8)	---	μl O_2/mg wet wt/hr	C>W, $p<0.001$
Suncus	Succinate	30	13.06 (18)	4.20 (18)	---	μl O_2/mg wet wt/hr	C>W, $p<0.001$
Suncus	α-Glycero-phosphate	30	4.81 (18)	1.44 (18)	---	μl O_2/mg wet wt/hr	C>W, $p<0.001$
Tupaia	Succinate	34	46.47 (30)	32.76 (30)	0 (30)	μl O_2/mg protein/hr	C = W>H, $p<0.001$
Tupaia	α-Glycero-phosphate	34	70.38 (30)	40.63 (30)	0 (30)	μl O_2/mg protein/hr	C>W<H, $p<0.02$
Saimiri[c]	α-Glycero-phosphate	26	30.93	20.99	5.45	μl O_2/mg protein/hr	C>W>H, $p<0.05$
Saimiri	α-Glycero-phosphate	26	6.18	3.03	0.427	μl O_2/mg wet wt/hr	C>W>H, $p<0.01$
Macaca	Succinate	34	5.82 (2)	6.46 (2)	---	μl O_2/mg wet wt/hr	
Macaca	α-Glycero-phosphate	34	3.83 (2)	4.55 (2)	---	μl O_2/mg wet wt/hr	
Macaca	α-Ketoglutarate	34	2.21 (2)	4.51 (2)	---	μl O_2/mg wet wt/hr	

[a]In parenthesis, number of animals.
[b]C, Cold-acclimated; W, Control; H, Heat-acclimated.
[c]Data from Chaffee et al (15).

some insulative value.

In comparing the brown fat to body weight data (Table I), it is of interest that in the *Tupaia*, the squirrel monkey and *Macaca* there is relatively less brown adipose tissue than in shrews or rodents, and there appears to be much less stimulation of growth in this tissue in response to cold.

An interesting possibility which bears investigation and apparently has hitherto not been examined is the question of the possible relationship of brown fat metabolism and the lymphatic system. The fact that in many species and especially in the primates and *Tupaia*, the brown fat is imtimately associated with the axillary nodes of the lymphatic system introduces the possibility of lacteal-lymphatic carriage of neutral fats directly from the intestine to the axillary brown fat. If such a system were functional, the size of the brown fat would not be as important as its metabolic output.

Enzymatic Results

In the cold-exposed shrews there are striking increases in oxidative enzyme levels per gram wet weight of brown fat (Table II) equal to or greater than those seen in rodents (4,8,26). In *Suncus*, the degree of brown fat hyperplasia following cold acclimation is about the same as it is in cold-acclimated rodents (Fig. 1) We have not as yet attempted to acclimate *Cryptotis* for a sufficiently long time to make this type of comparison. Thus in *Suncus* the increase in brown fat heat production potential following cold-exposure is of the same order of magnitude as that of rodents. The increase in oxidative enzyme levels seen in the brown fat of cold-exposed *Cryptotis* is as great or greater than that of non-hibernating rodents and is more like the increases seen in cold-exposed hamsters (8) or hibernating ground squirrels (9). Of particular interest is the large increase in α-glycerophosphate oxidase activity relative to the increase in succinoxidase upon cold-exposure. This relationship also occurs in hibernating rodents (8,9), but the reverse is true in a non-hibernating animal, such as the rat (Roberts et al., unpublished data). Since small decreases in ambient temperature demand relatively large amounts of compensatory heat production in an animal as small as *Cryptotis* and since in hibernators, arousal also requires a high rate of heat production, it may be significant that, in these species, the potentially calorigenic α-glycerophosphate oxidase system (19,20) is particularly enhanced.

In *Tupaia* exposed to cold the brown fat weight does not increase, but its specific activity with respect to succinoxidase and α-glycerophosphate oxidase does. Although the oxidative enzyme levels increase in the cold, the low relative brown fat weight and the failure of this to increase in the cold suggest that the brown fat of *Tupaia* may not have as great a thermogenic potential as that of the rat. It is also of interest that *Tupaia* cannot stand nearly as severe a cold stress as the rat. The fact that *Tupaia* is smaller than the rat, but shows much less brown fat hyperplasia in the cold seems to negate any hypothesis of an inverse relationship between per cent brown fat hypertrophy and animal size.

The evidence gained on studies of brown fat in heat-acclimated animals further implicates the relationship between environmental temperature and brown fat enzyme levels. In Tupaiads both succinoxidase and α-glycerophosphate oxidase activities are decreased to near zero values (Table II). This is similar to our findings in the squirrel monkey (15,16) and the heat-exposed hamster (28).

In adult *Macaca*, strangely enough, prolonged cold-exposure resulted in no measurable increase, and possibly a decrease, in the specific activity of the oxidative enzymes we studied (Table II). Since, in addition, there was no increase in brown fat mass, this would seem to indicate no change or possibly a decline in oxidative enzymatic potential in the brown fat of these animals. The lack of a change in brown fat mass is somewhat different from the results of Itoh and Hiroshige (27) who found that there was considerable brown fat in *Macaca fuscata*, exposed to the cold in northern Japan, while wild *Macaca cynomolgus* from the tropics had none. However, they were not comparing the effects of cold on the same species under laboratory conditions so it is difficult to correlate their very interesting findings with ours.

In the case of adult *Macaca* it would appear from our preliminary studies that brown fat is not playing a significant role in thermogenesis during cold-exposure and that other mechanisms make possible its survival in extreme cold. It may be that biochemical changes in other tissues account for at least part of the extra heat production required in the cold and coupled with purely physical thermoregulatory mechanisms, such as pelage changes and vasoconstriction, contribute to the maintenance of homeothermy. On the other hand, there is also the possibility that the *Macaca* is pre-adapted to cold with respect to the quantity and enzymatic content of brown fat, and that all that is required is catecholamine modulation (1,29,30) for the maintenance of homeothermy.

APPENDIX

Since presentation of the above paper, we have completed additional studies in Rhesus monkeys using smaller, younger animals weighing an average of 2.5 kg. Controls (n = 3) had more brown fat per gram body weight (0.45% body weight) than did the larger adult *Macaca* (Table I) and this increased significantly ($p < 0.01$) in the cold (0.76% body weight) and was apparently replaced by yellow fat in the heat. As in the adult *Macaca*, brown fat succin-oxidase and α-ketoglutarate oxidase did not change significantly in the cold-acclimated smaller *Macaca*. However, brown fat α-glycero-phosphate oxidase activity increased significantly ($p < 0.05$) in smaller cold-acclimated animals (11.97 μl O_2/mg wet weight tissue per hour) compared with controls (5.40 μl O_2/mg wet weight tissue per hour); a change which did not occur in the larger adult *Macaca* (Table II). It has been shown in a number of non-hibernating mammalian species, including guinea pigs (31), rats (32), mice (32), rabbits (33) and humans (34) that the relative amount of brown fat (which may be quite high in the newborn) either decreases with age or becomes highly infiltrated with white fat. It is, therefore, not surprising that this phenomenon is also seen in *Macaca*. The fact that adult *Macaca* appear to show neither a brown fat hypertrophy nor an increase in oxidative enzyme levels when exposed to cold, whereas young *Macaca* show both of these responses, indicates that in studying this tissue one must consider not only the species involved but also its age.

ACKNOWLEDGMENT

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Bioenergenetics of Brown Adipose Tissue[1]

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ABSTRACT

Examination of the effect of 2,4-dinitrophenol (DNP) in vivo on the brown adipose tissue of cold-exposed rats as well as the effect of DNP and dicumarol in vitro, indicates that brown fat does possess a functional electron transport-coupled phosphorylating system. Moreover, the fact that a norepinephrine-induced thermogenic response (in vivo) can be elicited from the brown fat after DNP administration implies that the effect of norepinephrine (NE) is not primarily due to stimulation of an adenosine triphosphatase system. Furthermore, since the magnitude of the NE-stimulated temperature increase is not diminished by prior treatment with DNP, it appears that the effect of NE is not achieved through any significant degree of uncoupling by the released fatty acids. Alternatively, our data suggest that under basal conditions (i.e., when the animal is not stimulated by cold stress or NE) the heat production (oxygen consumption) of the brown fat is limited by the availability of substrate rather than ADP. Conversely, it is proposed that under states of cold stress or NE infusion the thermogenic effect is induced through stimulation of lipolysis and consequent enhancement of substrate accessible for mitochondrial oxidation.

INTRODUCTION

Non-shivering thermogenesis develops as a primary physiological response of animals chronically exposed to cold; in the course of this, brown adipose tissue undergoes adaptive histological and cytological modifications. These changes involve: (a) transient regression of multilocular fat vacuoles (1); (b) repletion of these vacuoles at the expense of white fat depots (1); (c) depletion of the white fat cells in the brown fat areas (1); and (d) hyperplasia of brown fat (2) by cytogenesis de novo from progenitor stem cells (1), together with increased mitochondrial nitrogen (3) and functional vascularity of the tissue (4). Thus, during the altered steady state of cold acclimation, the metabolic potential of the brown fat is enhanced, a phenomenon which assumes great significance in view of the fact that the only known physiological function of this tissue is thermogenesis (cf. 5,6). Moreover, the brown fat is topologically arranged so that its heat is conveyed toward such areas as cervical spinal cord, heart, etc., which play a primary role in the maintenance of this steady state (7,8).

The thermogenic contribution of brown fat in the cold-adapted animals notwithstanding, it is now evident that this tissue is a site of significant heat production during the acute cold stress of many adult non-hibernators (9,10) and neonates (cf. 11,12), as well as during arousal from hibernation (6,13-16).

The control of the cold-induced thermogenesis of brown fat appears to depend upon an intact sympathetic innervation of the tissue (Fig. 1; cf. also 17,18), as is further substantiated by the work of several investigators relating norepinephrine (NE) administration to elevated brown fat temperatures and rates of oxygen consumption (19). Thus, any metabolic model for brown fat thermogenesis must account for the cold- or NE-induced stimulation of the oxygen consumption of the tissue.

In terms of current concepts holding electron transport-limited cellular respiration to be controlled by the ratio of ADP/ATP, an increased rate of oxygen utilization of brown fat can be explained by an increased availability of ADP, or by a dissociation (i.e., uncoupling) of cellular respiration from oxidative phosphorylation. Models involving both of these possibilities have been proposed to explain the action of NE on brown fat metabolism (see below).

In view of the known stimulatory effect of NE on triglyceride hydrolysis, it has been suggested that this hormone might elevate the qO_2 of the tissue by stimulating an adenosine triphosphatase cycle and thereby increasing the effective ADP/ATP ratio. As indicated in Figure 2, one such cycle involves hydrolysis of triglyceride to fatty acids and subsequent re-esterification (20), while the second model proposes oxidation of the fatty acids to acetyl CoA followed by resynthesis back to fatty acids (21). Additionally, it has been suggested that the norepinephrine-induced increased triglyceride hydrolysis might result in a physiological un-

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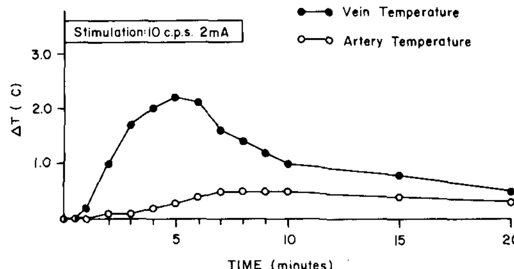

FIG. 1. Effect of stimulating the nerve bundle innervating the interscapular brown fat pad of cold-acclimated rats. ΔT represents temperature of blood minus temperature of colon. (Y. Imai, Unpublished observations.)

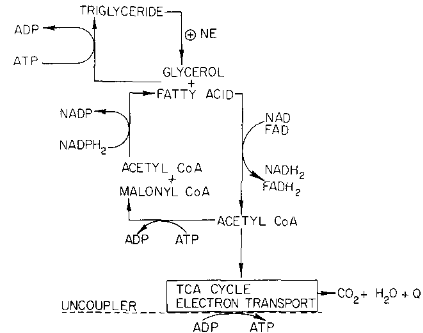

FIG. 2. Schematic of pathways involved in some proposed models for the norepinephrine-mediated thermogenesis of brown adipose tissue.

coupling of the brown fat mitochondria by virtue of the elevated levels of fatty acids within the brown fat cells (22).

With respect to this latter possibility, initial studies of the oxidative metabolism of isolated brown fat mitochondria indicated that these particulates did have a low efficiency of coupled oxidative phosphorylation (23-25). This apparent uncoupling, however, could be partially reversed by addition of bovine serum albumin (BSA) to the reaction flasks (Table I; 22,26-28). Furthermore, it was noted that these mitochondria were able to accumulate Ca^{++} via a respiration dependent pathway (29). These data therefore suggested that the site of the apparent uncoupling lay between the actual synthesis of ATP and the cation translocase-dependent high energy intermediate (29).

Additionally, the BSA-reconstitution of coupled oxidative phosphorylation has been explained as a protection from the uncoupling action of fatty acids released during the isolation procedure (22,27). It is also possible, however, that the effect of BSA may reflect a protection against or reversal of a physiological uncoupling situation (22,28). The present study was therefore undertaken to evaluate this possibility by examining the sensitivity of the brown fat in vivo and in vitro to 2,4-dinitrophenol and dicumarol.

MATERIALS AND METHODS

Young, adult, male Long-Evans rats were cold acclimated at 5 ± 1 C for a minimum of three weeks and were fed and watered ad libitum.

In Vivo Studies

Cold-adapted rats (300-450 g) were anesthetized with sodium pentobarbital (50 mg/kg) and the left jugular vein was cannulated. Copper-constantan thermocouples (TC) were inserted into the colon (minimum depth 6 cm), between the liver lobes and on the ventral aspect of each lobe of the interscapular fat pad. Either 2,4-dinitrophenol (DNP, recrystallized from ethanol) or L-arterenol bitartrate (NE) were infused i.v. for 2 min and the effects on the temperatures of the target areas were observed in the rats both at room temperature (23-24 C) and also during cold stress. The cold stress was induced by placing the animals on aluminum foil situated in crushed ice in such a way as to permit varying rates of body heat loss.

In Vitro Studies

Lightly etherized rats were exsanguinated and the brown fat from the interscapular, cervical, subscapular and thoracic regions was rapidly excised and placed in oxygenated Krebs-Ringer phosphate buffer (pH 7.4) at room temperature. The brown fat was cleaned of adhering muscle, white fat and connective tissue and then fragmented by passage through a stainless-steel Harvard press. The expressed fragments were roughly cylindrical with diameters of the order of 0.5 mm and weights less than 1 mg. These were then distributed into piles

TABLE I

Effect of BSA (18 mg/ml) on Brown Fat Mitochondrial P/O Ratios[a]

System	P/O
α-ketoglutarate (2mM ATP)	0.74
+ BSA (2mM ATP)	1.61
+ BSA (9mM ATP)	2.48
Succinate (2mM ATP)	0.19
+ BSA (2mM ATP)	0.89
+ BSA (9mM ATP)	1.53

[a]Modified from Hittelman (28).

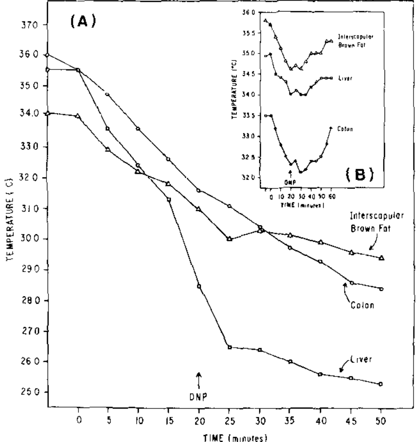

FIG. 3. Effect of DNP infusion on the temperature of the liver, colon and interscapular brown fat of cold-adapted rats (410 ± 5 g). Animals were exposed to severe cold stress (graph A) as well as a milder stress (B) (cf. Results).

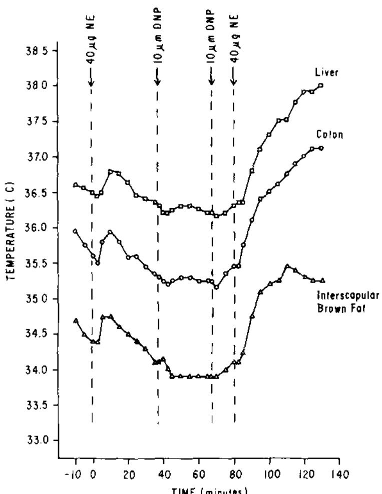

FIG. 4. Effect of infusion of DNP and NE on the temperature of the liver, colon and brown fat of a cold-adapted rat (400 g). Experiments were conducted at 23-24 C.

(10-20 mg), weighed and each placed in the main compartment of a 5 ml Warburg flask containing Krebs-Ringer phosphate (pH 7.4) and, when present, DNP (5 x 10^{-6} M, final concentration). Sodium succinate, 60 mM, was added to the sidearm; the center well contained 0.03 ml of 2 M KOH and a folded filter paper wick. The flasks were placed on the bath (37 C), gassed for 10 min with 100% oxygen and allowed to equilibrate another 7 min. Substrate was tipped in after the first 10 min reading and the readings were continued at 10 min intervals for 70-100 min. The oxygen consumption of the tissue was calculated as $\mu l\ O_2$/mg N/hr, the nitrogen being determined by a standard micro-Kjeldahl method.

Tissue homogenates were prepared by adding the cleaned brown fat to 9 vol of 0.25 M sucrose and homogenizing with 20 complete strokes in a Potter-Elvehjem teflon-glass homogenizer at room temperature. Aliquots of the preparation were added to Warburg flasks containing (final concentration): 12 mM KH_2PO_4 at pH 7.4, 6 mM $MgCl_2$, 250 mM sucrose and 60 mM Na succinate. When present, DNP (5 x 10^{-6} M) and dicumarol (5 x 10^{-5} M) were also placed in the main compartment. The final volume was 1.03 ml including 0.4 ml homogenate (0.4 - 0.6 mg N) and 2 M KOH in the center well.

Each experiment yielded qO_2 values with and without uncoupler (three replications per mean for the studies with DNP; four per mean for dicumarol). Separate analyses of variance were performed for the qO_2 values obtained 10 min and 30 min after substrate addition. The analyses of variance (three-way) considered the animals (i.e., each experiment) as well as the treatment (uncoupler) as main effects.

TABLE II

Effect of DNP on Brown Fat Fragments

Time, min	10	30
Control ($\mu l\ O_2$/mg N/hr)	557.5	516.0
DNP ($\mu l\ O_2$/mg N/hr)	638.6	604.2
S.E. (N=24)	17.2	15.5
Level of significance	$p<.05$	$p<.01$
$\frac{\text{DNP } qO_2}{\text{Control } qO_2}$	1.15	1.17

TABLE III

Effect of DNP and Dicumarol on Brown Fat Homogenates

Uncoupling agent	DNP		Dicumarol	
Time, min	10	30	10	30
Control ($\mu l\ O_2$/mg N/hr)	554.7	583.9	573.1	608.1
Uncoupler ($\mu l\ O_2$/mg N/hr)	602.7	623.1	746.0	722.8
S.E.	7.3	5.6	8.3	7.6
N	30	30	24	24
Level of significance	$p<.01$	$p<.01$	$p<.01$	$p<.01$
$\frac{\text{Uncoupler } qO_2}{\text{Control } qO_2}$	1.08	1.06	1.33	1.19

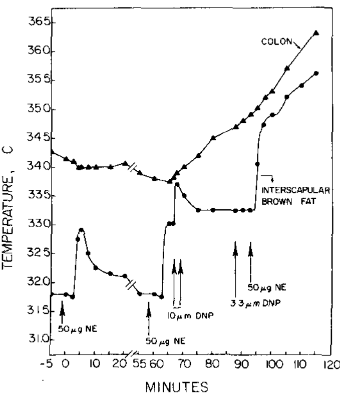

FIG. 5. Thermogenic response of brown fat to successive administration of NE and DNP (cf. Results). Experiment was conducted at 23-24 C.

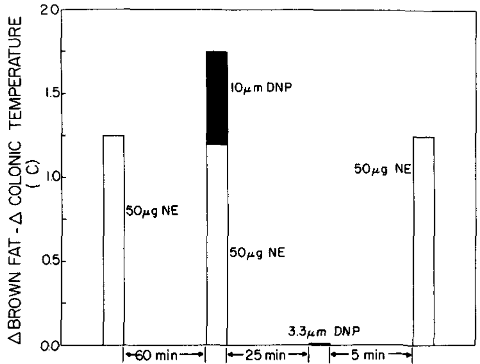

FIG. 6. Quantitative effect of NE and DNP on the temperature of the interscapular brown fat (from data of experiment described in Fig. 5).

RESULTS AND DISCUSSION

As illustrated in Figure 3, after the rat was placed in the ice pack the temperatures of the brown fat, liver and colon fell. During severe cold stress (i.e., the rat supine and separated from the ice by aluminum foil), infusion of 10 μmoles DNP markedly altered the heat exchanges of all three areas (Fig. 3A). Notably, DNP appeared to increase the temperature of the interscapular fat pad either before or at the same time as that of the colon and liver.

Similarly, under conditions in which the rats were subjected to a milder cold stress (by pronation and separation from the ice by paper towelling and aluminum foil), infusion of DNP also elicited a temperature response from the brown fat. Moreover, in these experiments, DNP actually reversed the cold-induced temperature decreases (Fig. 3B).

On the other hand, in studies conducted at room temperature, DNP produced little or no response in the brown fat, although infusion of NE (100 μg/kg) was followed by the expected increase in tissue temperature (Fig. 4). Characteristically, the effect of this dose of NE reached a maximum within 5 min after which the temperature of the tissue slowly returned to pre-injection levels. However, when NE was administered within a few minutes of DNP infusion (before or after), both the magnitude and duration of the temperature increases were greatly amplified (Fig. 4).

In view of the fact that the temperature of the brown fat appeared to be affected before that of the liver and colon, and since DNP has no direct cardiovascular effects (30), the in vivo results were viewed as indicating a direct action of DNP on the brown adipose tissue via the classically-defined pattern of uncoupling. However, to strengthen this interpretation, the effect of uncoupling agents on brown adipose tissue in vitro was examined.

Such studies demonstrated that in the presence of succinate (60 mM), the oxygen consumption of brown fat fragments (Table II) as well as homogenates (Table III) was significantly stimulated by both DNP (5 x 10^{-6} M) and dicumarol (5 x 10^{-5} M). Furthermore, the qO_2 of the homogenates was also enhanced by addition of an acceptor system (ADP or glucose-hexokinase).

Thus, it appears that mitochondria of brown fat do indeed exhibit an oxidative phosphorylating system which is electron transport-coupled and can be dissociated both in vivo and in vitro.

The finding that NE infused shortly after DNP administration was followed by an increase in temperature of brown fat greater than that obtained with either agent alone (Fig. 4) bears directly on the mechanism of the NE-induced brown fat thermogenesis. Specifically, the proposal that NE acts by stimulating an adenosine triphosphatase cycle (20,21) is not substantiated by the fact that NE still elicited a thermogenic response from the brown fat after DNP administration, i.e., after the dissociation of respiration from oxidative phosphorylation.

On the other hand, the apparently synergistic effect of DNP and NE observed here does not preclude the possibility that NE might contribute to a mitochondrial uncoupling; hence, this was examined in experiments conducted at room temperature wherein the dose of infused NE was such as to elicit a maximum thermogenic response from the brown fat. This response was defined as the maximum temperature change of the tissue minus the corresponding change in core temperature ($\Delta T_{brown\ fat}$ -

ΔT_{colon}). Fifty to 60 min after initial infusion of such a NE dose (125 μg/kg), a second dose was administered and followed by DNP (10 μmoles) at the time when the brown fat temperature had regained the previous maximum due to the NE (Fig. 5). It should be noted that at this point treatment with DNP further elevated the brown fat temperature (Fig. 5); that is, although the NE had elicited the maximum temperature increase from the brown fat, the tissue was still responsive to DNP. Additionally, despite the fact that the temperature of the brown fat was little affected by further treatment with DNP, a third dose of NE (125 μg/kg) still elicited a thermogenic response from the tissue (Fig. 5). Moreover, the effect of this last dose of NE was as great or greater than that obtained before the initial treatment with DNP (Fig. 6).

Hence, since the magnitude of the NE-induced elevation of brown fat temperature is not diminished after dissociation of electron transport from oxidative phosphorylation (i.e., after DNP administration) it appears that NE-stimulation of brown fat heat production does not denote any significant degree of uncoupling.

Indeed, the synergistic response noted between DNP and NE is most plausibly explained by a NE-induced increase in available substrate, which in turn is oxidized more rapidly as a result of the DNP uncoupling. This explanation implies that although the level of ADP/ATP affects the oxygen consumption of the brown fat when the tissue is stimulated during cold-stress or NE release, under basal conditions, the rate of oxygen utilization and therefore heat production is limited by the availability of substrate. This view is consistent with the finding that, at room temperature, DNP per se has little demonstrable effect on the brown fat temperature.

On the basis of the above considerations, it is therefore suggested that NE effects a thermogenic response from the brown fat by stimulating lipolysis, thereby increasing the amount of substrate available for mitochondrial oxidation.

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Cuticular Lipids of Insects: I. Hydrocarbons of *Leucophaea maderae* and *Blatta orientalis*

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ABSTRACT

The major hydrocarbons found in the surface lipids of the cockroaches *Leucophaea maderae* and *Blatta orientalis* have been identified as *n*-heptacosane, 11-methylheptacosane, 13-methylheptacosane and 3-methylheptacosane. The hydrocarbons from the two genera of cockroaches are qualitatively identical and quantitatively similar.

INTRODUCTION

The hydrocarbon constituents vary both qualitatively and quantitatively among insect species (1-10). Qualitative and quantitative differences in the principle haemolymph hydrocarbons among several genera of the same family of insects, the cockroaches, were demonstrated by Acree et al. (11), however, they did not identify the hydrocarbons. Baker et al. (12) identified the principle hydrocarbons in the haemolymph and a homogenate of the American cockroach, *Periplaneta americana*, L., as *n*-pentacosane, 3-methylpentacosane, and *cis,cis*-6,9-heptacosadiene, together with small quantities of heptacosane, nonacosane and C_{41}-C_{43} hydrocarbons. The present paper reports the qualitative identity and quantitative similarity of the cuticular lipid hydrocarbons of the cockroaches *Leucophaea maderae* and *Blatta orientalis*.

PROCEDURES AND RESULTS

All cockroaches used were adults with no distinction made as to sex. The cockroaches were killed by freezing at -29 C for 20 min. The surface lipids were removed by slurrying the whole roaches in chloroform (30 ml/roach) for 30 min. Preliminary studies showed that a 30 min slurry removed the surface lipids without removing appreciable internal lipids. All solvents were glass redistilled and all samples were stored under nitrogen at 4 C.

The total surface lipid samples were chromatographed on preparative silica gel (Adsorbosil 3) thin layer plates developed in hexane to isolate the hydrocarbon fraction from the minor components. The hydrocarbon fraction accounts for more than 80% of the surface lipid of *L. maderae* and *B. orientalis*. Chromatography of the hydrocarbons on silver nitrate-impregnated silica gel thin layer plates indicated that all the hydrocarbons were saturated.

Figure 1 is a gas chromatogram of *L. maderae* and *B. orientalis* hydrocarbons. Comparison of retention times to those of a known paraffin mixture indicated that the first major hydrocarbon was *n*-heptacosane and that the other major hydrocarbons had retention times less than that of *n*-octacosane. There is an indication that, in addition to the major hydrocarbon group, there are similar groups of hydrocarbons between pentacosane and hexacosane, between hexacosane and heptacosane, between octacosane and nonacosane, and between nonacosane and tricontane, with the relative intensities varying somewhat in the two roaches. Gas chromatographic retention time data indicate that each of these groups contains hydrocarbons similar in structure to the major hydrocarbons but with differing chain lengths. The quantitative similarity in the major hydrocarbon composition of the two cockroaches as determined by gas liquid chromatography (GLC) is shown in Table I.

Preparative GLC on an OV-17 column yielded sufficient quantities of the major hydrocarbons for mass spectrometry studies. Gas chromatographic-mass spectral data were also collected on the major hydrocarbons and the results were similar.

In order of their GLC appearance, the identification of the first major hydrocarbon as *n*-heptacosane was verified by comparison of the mass spectrum to that of known heptacosane. The significant fragments in the mass spectrum of the second and major GLC peak are at 168, 196, 224 and 252 (m/e) which are indicative of methyl branches at carbons 11 and 13 on hydrocarbons containing 28 total carbons. The mass spectrum is shown in Figure 2. Integration

TABLE I

Composition of Major Surface Hydrocarbons of *L. maderae* and *B. orientalis* (Area Per cent)

Hydrocarbon	*L. maderae*	*B. orientalis*
n-Heptacosane	14	12
11-Methylheptacosane and 13-Methylheptacosane combined	58	65
3-Methylheptacosane	18	17
Other hydrocarbons	10	6

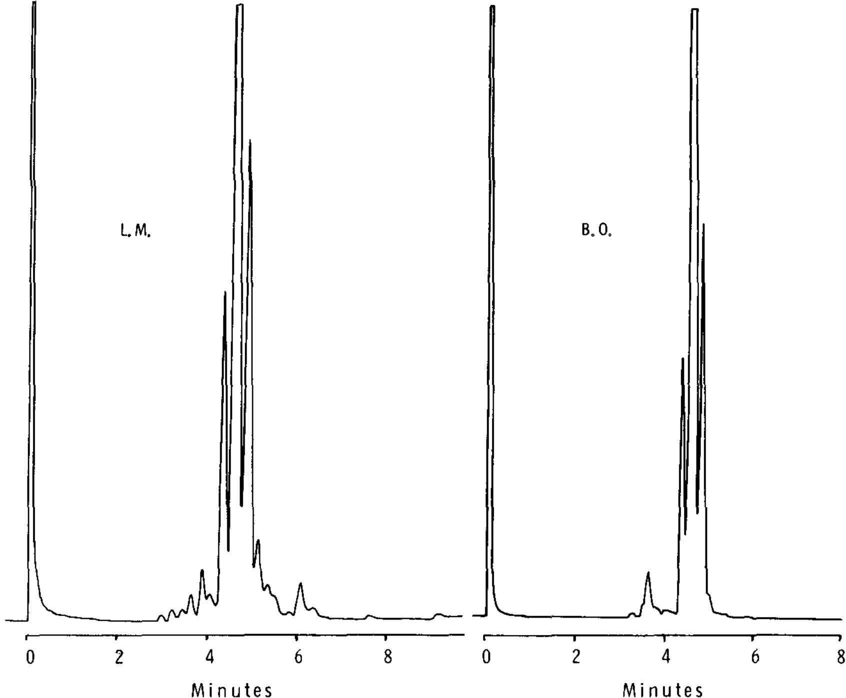

FIG. 1. Gas chromatograms of the hydrocarbons from the surface of *L. maderae* and *B. orientalis*. The column was 2% SE-54 (6 ft x 1/4 in. glass) operated at 200 C with a flow rate of 30 ml/min.

of the 60-mc NMR spectrum of the branched hydrocarbon mixture indicated that the ratio of methyl to methylene hydrogens was 1:5.5, which would allow for only one methyl branch on each hydrocarbon chain. The structures of 1 1-methylheptacosane and 13-methylheptacosane have, therefore, been assigned to hydrocarbons of the second major hydrocarbon peak. We were unable to separate 11-methylheptacosane and 13-methylheptacosane by GLC. The assignment of 3-methylheptacosane to the third major hydrocarbon is based on the mass spectrum showing a predominant peak at 365 (m/e), indicating a single 3-methyl branch in a hydrocarbon with 28 total carbons. The fragmentation pattern of this hydrocarbon compares with the fragmentation pattern of known 3-methyleicosane.

DISCUSSION

The hydrocarbons of *L. maderae* and *B. orientalis* are similar to the hydrocarbons of *P. americana* (12) in that, of the major hydrocarbons, one is normal and one is 3-methyl branched. The normal and 3-methyl hydrocarbons of *L. maderae* and *B. orientalis*, however, are two carbons longer than pentacosane and 3-methylpentacosane of *P. americana*. The third and approximately 65% of the hydrocarbon of *P. americana* is *cis,cis*-6,9-heptacosadiene (12), whereas approximately 60% of the hydrocarbon of *L. maderae* and *B. orientalis* surface lipid is internally branched, and none of the hydrocarbons of the latter two cockroaches is unsaturated. The American cockroach does not appear to have any internally branched hydrocarbons (12). This report of internally branched hydrocarbons on *L. maderae* and *B. orientalis* is the first report of internally branched hydrocarbons in the cuticular lipids of insects. Internally branched (13-, 15- and 17-methyl) hydrocarbons were observed in the homogenized extract of the common house cricket, *Acheta domesticus* L. (13), however, the most abundant internally branched hydrocarbons had an even number (34 and 36) of carbons in the normal chain. House cricket hydrocarbons also have 2-methyl alkanes in which the normal chain has an even number of carbons (28 and 30). The above mentioned cockroach branched hydrocarbons have an odd number of carbons in the normal chain.

The melting point of *L. maderae* and *B. orientalis* hydrocarbon mixture is somewhat higher (approx. 30 C) than the melting point of

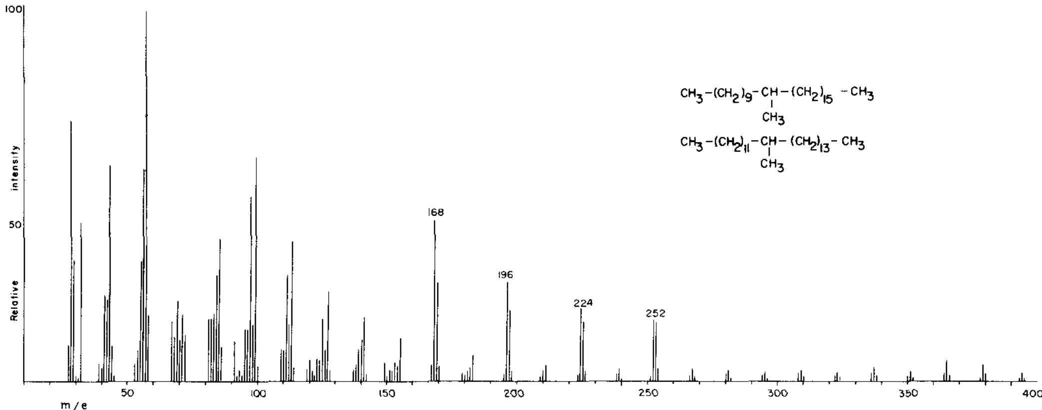

FIG. 2. Mass spectrum of the major hydrocarbon peak from *L. maderae* and *B. orientalis*.

P. americana hydrocarbon mixture (approx. 20 C). This increased melting point may be related to the climate inhabited by these cockroaches. The habitat of the American cockroach extends as far north as New York City, whereas the Oriental and Madeira cockroaches are found in West Africa, The West Indies, South and Central America and the southeastern United States. All three probably originated from Africa (14).

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Cuticular Lipids of Insects: II. Hydrocarbons of the Cockroaches *Periplaneta australasiae*, *Periplaneta brunnea* and *Periplaneta fuliginosa*

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ABSTRACT

The major cuticular hydrocarbons of the cockroaches *Periplaneta australasiae, Periplaneta brunnea* and *Periplaneta fuliginosa* are *n*-tricosane, *cis*-9-tricosene, 3-methyltricosane, 11-methyltricosane and 13-methylpentacosane. There are as yet unexplained quantitative differences between the hydrocarbon compositions of males and females of *P. australasiae* and *P. fuliginosa*, *cis*-9-tricosene being a major hydrocarbon of the males only. A series of mono-methyl internally branched hydrocarbons ranging in chain length from 23 to 26 carbons with the methyl branch on the 13th carbon from one end was observed. Minor quantities of other hydrocarbons have been identified.

INTRODUCTION

In the first paper in this series, we demonstrated that the four principal hydrocarbons in the cuticular lipids of the Madeira cockroach (*Leucophaea maderae*) and the Oriental cockroach (*Blatta orientalis*) are *n*-heptacosane, 11-methylheptacosane, 13-methylheptacosane and 3-methylheptacosane (1). The cuticular hydrocarbons from these two genera of cockroaches are qualitatively identical and quantitatively similar.

The principal hydrocarbons obtained from the hemolymph and the whole carcass of the American cockroach (*Periplaneta americana*) were reported by Baker et al. (2) as *n*-pentacosane, 3-methylpentacosane and *cis,cis*-6,9-heptacosadiene accounting for approximately 12%, 20% and 65% of the total hydrocarbons, respectively. They also observed small quantities of *n*-heptacosane, *n*-nonacosane and C_{41}-C_{43} hydrocarbons. Hydrocarbons represented 0.35% of the hemolymph in males and 0.68% in females. Acree et al. (3,4) reported that the total and the relative quantities of the principal hemolymph hydrocarbons in the American cockroach and in the German cockroach (*Blattella germanica*) are not only sex-dependent but also fluctuate with age and photoperiod.

The present paper reports the identity and quantitative similarity of the cuticular hydrocarbons of three cockroaches in the *Periplaneta* genus.

EXPERIMENTAL PROCEDURES

The Australian cockroaches (*Periplaneta australasiae*, Fabricius), southern brown cockroaches (*Periplaneta brunnea*, Burmeister) and smoky brown cockroaches (*Periplaneta fuliginosa*, Serville) used were all adults. The cockroaches were raised in 30 gal garbage cans affixed with an electric fence near the top to prevent escape. The lid was on the cans except for feeding and watering so the insects were in near darkness most of the time. The insects were fed ad lib with a diet of ground Gravy Train dog food (General Foods Corp., White Plains, N.Y.), 2217 g; vitamin diet fortification mixture in dextrose (Nutritional Biochemical Corporation, Cleveland, Ohio), 200 g; cholesterol, 6.0 g; and zinc chloride, 1.0 g. Water was also available at all times in the form of a 1% agar-99% water gel.

The cockroaches were killed by freezing at -29 C for 20 min. The surface lipids were removed by placing the whole roackes in a Buchner funnel with medium porosity fritted disc and covering the roaches with hexane, allowing the extract to pass through the fritted disc. Fresh hexane was added two more times. The hexane extracts were all combined. The roaches were then extracted with chloroform in the above manner. The solvent was removed under vacuum or under nitrogen sweep. All solvents were glass redistilled, and all samples were stored under nitrogen at 4 C.

The surface lipid extracts were investigated analytically on silica gel coated thin layer plates developed in hexane-diethyl ether-acetic acid (90:10:1 v/v/v) and compared to standards. The hydrocarbons were separated from other surface lipids by column chromatography. The columns were disposable Pasteur pipets stoppered with glass wool and filled with about 10 mg of silica gel per mg of lipid to be separated. The hydrocarbons were eluted with hexane and the other lipids eluted with chloroform. Purity of the fractions was verified using the above thin layer system.

Preparative thin layer chromatography (TLC) of the hydrocarbons on 5% silver

TABLE I

Characteristic Peaks in the Mass Spectra of the Internally Branched Cuticular Hydrocarbons of *P. australasiae*, *P. brunnea* and *P. fuliginosa*

Hydrocarbon	m/e	Fragment	m/e	Fragment
11-Methyltricosane	196(197)	$C_{14}H_{28}(H)$[a]	168(169)	$C_{12}H_{24}(H)$
12-Methyltetracosane	196(197)	$C_{14}H_{28}(H)$	182(183)	$C_{13}H_{26}(H)$
13-Methylpentacosane	196(197)	$C_{14}H_{28}(H)$	196(197)	$C_{14}H_{28}(H)$
13-Methylhexacosane	196(197)	$C_{14}H_{28}(H)$	210(211)	$C_{15}H_{30}(H)$

[a]Parenthesis signifies the presence of a labile hydrogen; the intensity of the peaks in parenthesis ranges from 60% to 100% of the other peak.

nitrate-impregnated silica gel thin layer plates developed in hexane separated the monoene hydrocarbon from the saturated and branched hydrocarbons. The position of the double bond in the monoene hydrocarbon was determined by osmium tetroxide oxidation (5), followed by periodate-permanganate oxidation (6) to form fatty acids. The fatty acids were methylated with diazomethane (7) and gas chromatographed on a 4 ft 2% SE-54 on Gas Chrom Z column comparing the retention times to the NIH Standard Mixture F.

The normal hydrocarbons were separated from the branched hydrocarbons by adding activated molecular seive 5A to a solution of the hydrocarbons in 2,2,4-trimethyl pentane (8). Preparative gas liquid chromatography (GLC) of the branched hydrocarbons on a 10 ft 5% OV-17 on Gas Chrom Z column yielded sufficient quantities of each branched hydrocarbon for mass spectrometry.

Analytical GLC of the hydrocarbons was carried out on a 6 ft 2% SE-54 on Gas Chrom Z column operated at either 200 C isothermal or programmed from 150 to 250 C in 10 min. The flow rate was 40 ml helium per minute. Quantitation was obtained by disc integration of the recorder trace.

Mass spectra were determined with a Hatachi RMU-6A instrument operating at ionizing voltage 75 ev, trap current 80μA. The samples were inserted directly into the ion source.

The infrared spectrum of *cis*-9-tricosene was taken by spreading the compound on a salt plate and taking the spectrum with a Beckman IR-20.

RESULTS AND DISCUSSION

The hexane extracts contained from 1.3 to 2.0 mg of cuticular lipid per cockroach, with the extracts from adult females having slightly more lipid than those from males. Adult females generally weighed on the order of 10% more than did their male counterparts. The hexane extract was composed of approximately 90% hydrocarbon, 7% triglyceride, 2% free fatty acids and 1% sterol (as estimated from preparative TLC). The chloroform extract of the previously hexane-extracted cockroaches contained only up to 0.15 mg per cockroach. Comparative TLC of the hexane extract and the chloroform extract showed that the chloroform extract had more triglyceride and some fatty acid methyl esters that the hexane extract did not have. It appears that chloroform might be penetrating into the internal lipid and extracting more triglyceride. In insects, the fatty acid methyl esters may be real or they may be artifacts of the chloroform extraction as shown by Sloan et al. (9). Since hexane extracts nearly as much lipid and without possible artifacts, only the hexane extract will be discussed further. Also, due to insufficient quantity of triglycerides, free fatty acids and sterols, we will limit our discussion to the cuticular hydrocarbons.

The structural assignment of the normal saturated hydrocarbons was made on the basis of their GLC retention time, their lack of complexing on silver nitrate-impregnated silica gel thin layer chromatography, and their inclusion in molecular seive 5A. Oxidation of the monoene hydrocarbon followed by methylation and gas liquid chromatography gave nonanoic methyl ester and myristic methyl ester in nearly equal quantities. The structure of the normal monoene was assigned as *cis*-9-tricosene. The *cis* assignment was based on the lack of a *trans* peak at 945 cm^{-1} and the presence of a peak at 730 cm^{-1} in the infrared spectrum.

The position of the methyl branch on the hydrocarbons that were not absorbed into molecular seive 5A was made on the basis of the mass spectral data. The mass spectra of the 3-methyl branched hydrocarbons were compared to mass spectra from 3-methyl (anteiso) hydrocarbons from other cockroaches (1,2) and from other sources (10,11). The principal 3-methyl hydrocarbon from these three *Periplaneta* is 3-methyltricosane, which is two car-

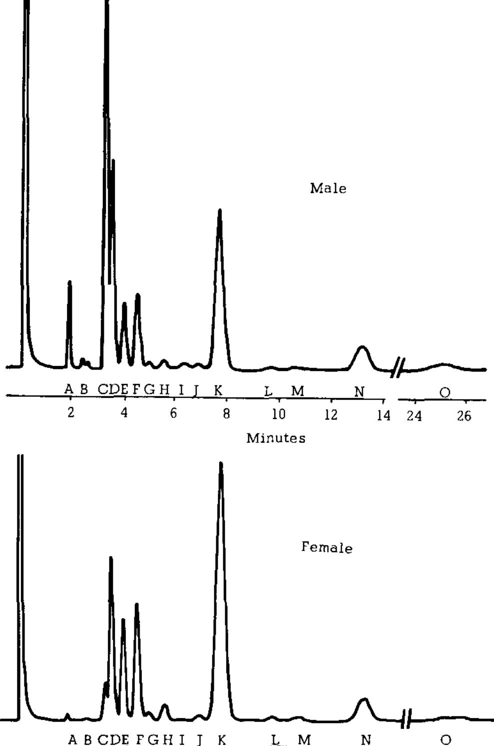

FIG. 1. Gas chromatogram of cuticular hydrocarbons from male and female *P. fuliginosa*. For identification of the hydrocarbons see Table II.

bons shorter than the 3-methylpentacosane from *Periplaneta americana* (2) and four carbons shorter than the 3-methylheptacosane from *L. maderae* and *B. orientalis* (1). The trace quantities of 3-methyltetracosane (a 3-methyl hydrocarbon with an even number of carbons in the chain) on the males of *P. australasiae* and *P. fuliginosa* are interesting and will be studied further, particularly in connection with the biosynthesis of 3-methyl hydrocarbons.

The presence of mono-methyl internally branched hydrocarbons in the cuticular lipids of cockroaches was first observed in *L. maderae* and *B. orientalis* cuticular hydrocarbons (1) and is again observed in the cuticular hydrocarbons of *P. australasiae, P. brunnea* and *P. fuliginosa*. The assignment of 11-methyltricosane, 12-methyltetracosane, 13-methylpentacosane and 13-methylhexacosane to the internally branched hydrocarbons is based on the mass spectral data in Table I. In the mass spectra of all four hydrocarbons there is a fragment of 196(197)m/e, indicating that these are of a homologous series with a characteristic CH_3-$(CH_2)_{11}$-$CH(CH_3)$-$(CH_2)_n$-CH_3 structure where n equals 9,10, 11 and 12. As is characteristic of other natural hydrocarbon isolates, hydrocarbons with an odd number of carbon atoms in the chain predominate, while even chains occur only in traces.

It is generally accepted that acetate units are incorporated into the hydrocarbons of insects (12-16), but beyond that the biosynthetic route to hydrocarbons is unknown. Of the possible biosynthetic routes, only two, the condensation and elongation mechanisms, are being considered seriously at present (for a review see 17). The elongation route consists of the addition of C_2 units derived from acetate to form a fatty acid of an appropriate chain length, then decarboxylation to the hydrocarbon. The condensation route is the condensation of two fatty acids followed by decarboxylation and reduction to the hydrocarbon. In both mechanisms fatty acids of 10 or more carbon atoms are proposed as the precursors to hydrocarbons. If the mono-methyl internally branched hydrocarbons of *P. australasiae, P. brunnea* and *P. fuliginosa* are biosynthesized via either of these routes, one might expect to find some precursor fatty acid with a methyl branch 13 carbons from the methyl end. If the 3-methyl hydrocarbons are also biosynthesized via either of the above routes, one might expect to find some precursor fatty acid with a methyl branch three carbon atoms from the methyl end. So far, the presence of either of these two types (ω-3 or ω-13) of fatty acids has not been observed in insects.

Analytical GLC of the cuticular hydrocarbons demonstrated quantitative differences in the hydrocarbon composition between male and female of *P. australasiae* and *P. fuliginosa* cockroaches (Table II), but not of *P. brunnea*. There are appreciable quantitative differences in the content of *cis*-9-tricosene, 11-methyltricosane, 3-methyltricosane and 13-methylpentacosane. In the major normal saturated hydrocarbon (*n*-tricosane), as well as the *n*-heptacosane and *n*-nonacosane, there is little compositional difference between sexes and among the three *Periplaneta* studied here. There is, however, some difference in the content of *n*-heneicosane, docosene and docosane in the cuticular hydrocarbons of *P. australasiae* and *P. fuliginosa*.

Quantitative differences in hydrocarbon content (2) and composition (3) have been observed in the hemolymph of cockroaches, however, this is the first time that such pronounced compositional differences have been

TABLE II

Cuticular Hydrocarbon Composition of Three *Periplaneta* Species of Cockroaches (Area Per Cent)

Hydrocarbon	*P. australasiae*		*P. brunnea*		*P. fuliginosa*	
	Female	Male	Female	Male	Female	Male
A *n*-Heneicosane	Trace[a]	7	ND[b]	ND	Trace	4
B Docosene and docosane	Trace	3	ND	ND	Trace	1
C *Cis*-9-tricosene	2	46	ND	ND	4	29
D *n*-Tricosane	16	14	11	12	14	15
E 11-Methyltricosane	3	2	20	18	10	6
F 3-Methyltricosane	12	3	15	15	13	8
G *n*-Tetracosane	Trace	Trace	Trace	Trace	Trace	Trace
H 12-Methyltetracosane	Trace	Trace	Trace	Trace	Trace	Trace
I 3-Methyltetracosane	ND	Trace	ND	ND	ND	Trace
J *n*-Oebtacisabe	Trace	Trace	Trace	Trace	Trace	Trace
K 13-Methylpentacosane	54	9	48	46	45	24
L *n*-Hexacosane	Trace	Trace	Trace	Trace	Trace	Trace
M 13-Methylhexacosane	Trace	Trace	Trace	Trace	Trace	Trace
N *n*-Heptacosane	4	5	1	2	6	6
O *n*-Nonacosane	3	4	2	2	2	2

[a]Trace equals less than 1% but greater than 0.2%.
[b]ND equals not detectable at the 0.2% level.

observed in cuticular hydrocarbons between sexes. The differences might be related to the reproductive processes, be under hormonal control, be related to a precursor supply or be related to a sex-linked compositional control mechanism. We have no evidence to support or refute any of these suggestions.

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Cholesterol Esters of Milk and Mammary Tissue[1]

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ABSTRACT

The fatty acid composition and metabolic activity of cholesterol esters in milk and mammary tissue (cow, sow and goat) were investigated. Cholesterol esters of freshly secreted milks incubated at 40 C for 2 hr showed no change in fatty acid composition and no incorporation of 1-^{14}C-palmitate. The fatty acid composition of cholesterol esters in the milk of all three species exhibited elevated levels of a unique group of fatty acids when compared to other milk ester lipid classes. This group was comprised of monounsaturated acids and acids with odd numbers of carbons. Tissue cholesterol esters contained lower levels of these acids. Evidence from experiments in which an odd carbon acid (C_{15}) was infused into the lactating mammary gland indicated that the group of unique acids is preferentially retained in the cholesterol ester fraction which is secreted with milk. These infusion experiments also provided evidence that cholesterol esters accumulate in developing milk fat globules in a manner paralleling triglyceride accumulation, and that acyl moieties of cholesterol esters may be desaturated in the form of the intact ester. Our results are compatible with the hypothesis that acyl moieties of cholesterol esters in lactating mammary tissue are turning over rapidly.

INTRODUCTION

The cholesterol of milk and milk products has attracted much attention, however little is known about its metabolism in the lactating mammary gland. Cholesterol of milk fat originates both from de novo synthesis from acetate within the mammary gland and by transfer from blood into the mammary gland (1-4). A high level of metabolic activity has been observed for the cholesterol esters of milk. Using the technique of intramammary infusion of fatty acids through the teat canal, McCarthy and Patton (5,6) found that uptake of these acids by the cholesterol ester fraction was more intense and more rapid than for glycerides or phospholipids. The possibility of a precursor-product relationship between fatty acids of cholesterol esters and those of triglycerides and phospholipids led McCarthy and Patton (5) to speculate that cholesterol esterification serves to transfer fatty acids away from the fatty acid pool to the site of glyceride assembly. Data have not been presented to confirm this hypothesis.

The amount of cholesterol in the milk lipids is small (0.25% to 0.40%) of which about one tenth is in the ester form (6,7). Since the milk lipids are 98% to 99% triglycerides, such glycerides represent a fatty acid pool several thousand times the size of that involved in the cholesterol esters. So far as tracer experiments are concerned the difference in the size of these two pools should by itself rather strongly influence specific activity data. However, within the tissue the ratio of the two fatty acyl pools appears to be smaller. Patton and McCarthy (8) found the cholesterol esters to represent as much as 1.0% of mammary tissue lipids as compared with tissue glyceride levels ranging from 50% to 80% of the total lipids.

The fatty acid composition of the cholesterol esters of milk appears to be complex although published data are limited (6). Work reported here attempts further investigation of this composition, especially as it relates to cholesterol ester metabolism of the mammary gland.

EXPERIMENTAL PROCEDURES

Freshly secreted milk samples were collected in a manner described previously (9). Milk lipids were recovered by the Mojonnier modification (10) of the Roese-Gottlieb solvent extraction. Mammary tissue lipids were obtained from two sows, a goat and a cow. Immediately after death, the udder was removed from each animal and active mammary tissue was recovered. Tissue samples were cut into 1 in. cubes and squeezed under cold running tap water to remove residual milk. To extract lipids, wet tissue was homogenized in 6 to 8 vol of chloroform-methanol (2:1 v/v). Oxidative and other changes were minimized by the use of vacuum or a stream of nitrogen to remove solvent, followed by prompt analysis. Samples requiring storage were held under solvent in sealed vials at 5 C.

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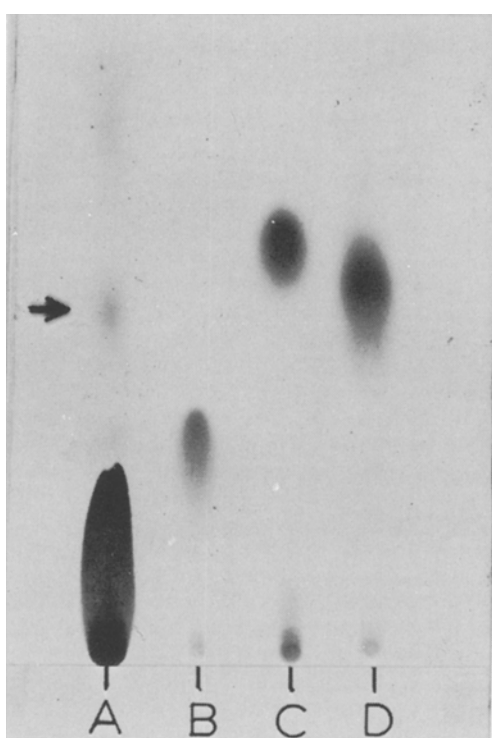

FIG. 1. TLC analysis of lipids on precoated Silica Gel F-254 plates (Merck) with benzene-hexane 50:50 solvent. A, 10 μl of a 20% (w/v) solution of bovine mammary tissue lipids in chloroform. B, 1 μl of a 5% (w/v) solution of methyl esters (milk fat) in hexane. C, 10 μl of authentic cholesterol stearate solution, 1% (w/v) in benzene. D, 10 μl of bovine mammary tissue cholesterol ester solution (chloroform) derived by preparative TLC on Silica Gel HR (Merck) from sample A in the area indicated by the arrow.

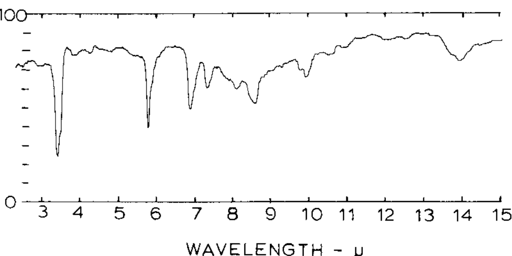

FIG. 2. Infrared spectrum (KBr pellet) of cholesterol esters isolated from bovine mammary tissue (Sample D, Fig. 1).

Cholesterol ester, triglyceride, and diglyceride fractions were separated from crude lipid extracts by silicic acid column chromatography (11). Purity of fractions obtained were routinely assayed by thin layer chromatography (TLC) (8). Where necessary, further purification of these fractions was achieved with TLC. Identity and purity of the cholesterol ester fractions was confirmed by TLC analysis of the saponifiable and unsaponifiable materials from this fraction. In some cases free fatty acids were removed from crude lipid extracts by the method of McCarthy and Duthie (12). Fatty acid composition of cholesterol esters was determined by gas chromatography (GLC) as previously described (13). Methyl esters were prepared by transesterification of samples with 1% H_2SO_4 in methanol (v/v). In some cases cholesterol esters were saponified, the unsaponifiables removed, and the fatty acids then methylated with the methanolic H_2SO_4. Methyl esters were identified by log plotting and by comparison of their retention times with authentic reference compounds on both polar (diethylene glycol succinate) and nonpolar (Apiezon L) stationary phases. The identity of unsaturated acids was confirmed by analysis both before and after hydrogenation. Radioactivity in lipid classes was assayed in a liquid scintillation counter.

To determine changes in fatty acid composition of cholesterol esters in milk on standing, freshly secreted milk samples were obtained from goats 1 hr after normal milking. A portion of each sample was extracted immediately after collection and another portion was incubated for 2 hr at 40 C before extraction. Cholesterol ester fatty acids from these samples were then analyzed by GLC. To study incorporation of fatty acids into cholesterol esters of milk, either potassium pentadecanoates (5 mg) or a trace level (200,000 cpm) of $1\text{-}^{14}C$-potassium palmitate (10 μc/μM) was dissolved in water and added to freshly secreted milks at the time of collection. These samples were then incubated for various intervals at 40 C before recovery and analysis of cholesterol esters. Incorporation of pentadecanoate was determined by GLC and $1\text{-}^{14}C$-palmitate by liquid scintillation counting.

Two in vivo experiments were conducted to study metabolism of a fatty acid containing odd numbers of carbons in the cholesterol ester fraction of milk. In these experiments potassium pentadecanoate solution was infused into the lactating gland and uptake of the acid in the cholesterol esters and other lipid classes of the subsequently secreted milk was analyzed. One gram of pentadecanoic acid was converted to the potassium salt by refluxing with the required amount of KOH in methanol. After removing methanol, the soap residue was dissolved in 20 ml of water and this solution was infused into one half of the udder of a lactating

TABLE I

Fatty Acid Composition of Cholesterol Esters of Freshly Secreted Milk at the Time of Collection and After Incubation at 40 C

Fatty acid	Fatty acid composition (wt %)			
	Goat A		Goat B	
	0 hr[a]	2 hr[b]	0 hr	2 hr
10:0	5.9	5.9	4.5	4.0
10:1	Trace[c]	Trace	Trace	Trace
12:0	4.6	5.0	3.9	2.9
12:1	0.2	0.6	1.7	1.4
13:0	Trace	Trace	Trace	0.1
13:1	0.7	0.5	1.1	0.7
14:0	9.4	10.0	9.0	8.3
14:1	0.3	0.8	2.4	1.5
15:0	1.5	1.2	0.9	0.8
15:1	Trace	Trace	Trace	Trace
16:0	40.6	43.8	38.1	38.2
16:1	Trace	Trace	Trace	Trace
17:0	Trace	Trace	Trace	Trace
18:0	8.6	9.0	9.3	11.4
18:1	26.7	22.5	26.4	28.7
18:2	1.5	0.7	2.7	2.0

[a]Sample collected 1 hr after initial milking of an individual goat. Lipids extracted immediately after collection.

[b]Sample incubated for 2 hr at 40 C before lipid extraction.

[c]Trace, less than 0.1%.

goat through the teat canal. In the first experiment milk from the infused side was collected for analysis just before and at intervals for 36 hr after infusion. The uninfused side was milked at these times but not analyzed. Previous results have shown that infused metabolites do not enter the uninfused half of the udder to any significant extent (6). Fatty acid compositions of cholesterol esters and triglycerides of these milk samples were analyzed by GLC. In the second experiment milk from the infused side was collected 12 hr after a similar infusion into a second goat and the level of pentadecanoate in the various ester lipid classes determined.

RESULTS AND DISCUSSION

Cholesterol ester fractions isolated from bulk lipid samples had Rf values by TLC corresponding closely to those obtained with authentic cholesterol esters (Fig. 1). On recovery from thin layer plates, the cholesterol ester fractions yielded highly authentic infrared spectra (Fig. 2). On saponification, only cholesterol was detected in the unsaponifiable material of these fractions. After extraction of the unsaponifiables, only fatty acids (and a small amount of residual cholesterol) were detected by TLC analysis of extracts of the acidified solution. Other sterols have not been found in milk in any amount approaching the concentration of cholesterol. With reference to the unsaponifiables of milk fat, Schwartz et al. (14) stated that cholesterol accounts for well over 90% of the weight of this fraction. In later work Schwartz et al. (15) reported that lanosterol and dihydrolanosterol occur in milk fat in unesterified form only at about 1/60 the concentration of cholesterol. In view of these results, there seems to be little doubt that the sterol ester fraction of milk fat is composed largely, if not exclusively, of cholesterol esters.

In order to determine the stability of the cholesterol esters in milk, analyses on fresh and aged samples were conducted. The data (Table I) show little evidence for changes in fatty acid composition of goat milk cholesterol esters during a 2 hr incubation at 40 C. Repeated analyses of other portions of these samples showed essentially the same patterns as those in Table I. Although minor differences in the percentages of each fatty acid can be noted comparing the fresh and 2 hr samples, in the main the fatty acid composition of samples was essentially constant for each animal. From these data it can be concluded that if cholesterol esters are being metabolized in freshly secreted milk, their fatty acid pattern remains relatively constant.

To examine the incorporation of fatty acids into cholesterol esters, potassium pentadecanoate and 1-^{14}C-potassium palmitate were dissolved in water and added to freshly secreted milks (cow and goat) in separate experiments. In the pentadecanoate trials, no increase in the content of this acid could be detected in cholesterol esters after 1 and 2 hr of incubation. Cholesterol esters did not incorporate appreciable levels of labeled palmitate under these same conditions. The specific activity of cholesterol esters from cow's milk was 40-60 cpm/mg after 2 hr incubation and there was no detectable activity in 1 mg of goat's milk cholesterol esters after 2 hr incubation. These milk samples contained active acylases (9), as evidenced by the incorporation of pentadecanoate (an increase of from 0.5% to 1% of the total fatty acids) and 1-^{14}C-palmitate (about 140 cpm/mg) into triglycerides. In the experiments with labeled palmitate, 15% to 20% of the added radioactivity was recovered in neutral esters. This information indicates that if cholesterol esters turn over in freshly secreted milk, they do so at a rate much too slow to appreciably affect their fatty acid composition.

Throughout this study a number of odd

TABLE II

Comparison of Total Lipid, Triglyceride, Diglyceride, Free Acid and Cholesterol Ester Fatty Acids From an Individual Cow's Milk Sample

	Fatty acid composition (wt %)				
Fatty acid	Total fat	Cholesterol esters	Triglycerides	Diglycerides	Free acids
10:0	5.2	1.6	5.1	5.6	2.5
10:1	ND[a]	0.8	ND	ND	ND
12:0	6.1	3.4	5.9	4.6	5.1
12:1	Trace[a]	0.4	Trace	ND	ND
13:0	Trace	0.6	Trace	Trace	Trace
13:1	ND	0.4	ND	ND	ND
14:0	15.3	11.5	15.0	14.0	14.0
14:1	1.3	0.6	1.3	0.5	0.8
15:0	1.1	2.1	1.1	1.3	1.2
15:1	ND	0.7	ND	Trace	ND
16:0	32.5	27.6	32.2	37.0	34.8
16:1	0.9	6.0	1.1	1.3	1.5
17:0	Trace	1.1	Trace	Trace	Trace
17:1	ND	1.1	ND	ND	ND
18:0	12.5	13.6	13.1	12.8	14.8
18:1	22.8	28.0	23.1	21.7	24.0
18:2	2.2	0.6	2.1	1.3	1.2

[a]Trace, less than 0.1%; ND, not detected.

carbon chain length and monounsaturated fatty acids in milk cholesterol esters was evident (Tables I-III). These esters contain significant amounts of several acids not normally detected when total milk fat methyl esters are analyzed. This complexity was evident in milk cholesterol esters from the cow (Tables II and III) and sow (Table III) as well as the goat (Tables I and III). For comparative purposes milk total lipid, triglyceride, diglyceride, free fatty acid and cholesterol ester fatty acid compositions from a sample from an individual cow are given in Table II. These data, which are representative of several samples, show the occurrence of measurable quantities of several more fatty acids in cholesterol esters than in other lipid classes. That the compounds detected on GLC analysis were all acids was confirmed by saponification of the cholesterol ester fraction and subsequent extraction of the unsaponifiable material prior to methylation and analysis. Although complete characterization of all of these acids will require further work, it is obvious that some of the minor acids found in milk occur in higher concentrations in cholesterol esters than in other neutral lipid classes. With the exception of 14:1, higher relative levels of all monounsaturated acids detected were found in cholesterol esters compared to the other lipid classes analyzed (Table II).

The observed fatty acid composition of milk cholesterol esters led to a consideration of the comparative composition of mammary tissue and milk cholesterol esters. Since mammary tissue lipids are mainly milk lipids in process of formation, such a comparison should clarify the reasons for the fatty acid complexity of cholesterol esters of milk. Mammary tissue lipids were secured from two sows, a goat and a cow. Immediately before death, milk samples were drawn from the glands from which tissue samples were obtained. Data obtained from analyses of the fatty acid compositions of cholesterol esters from these sources are summarized in Table III. Mammary tissue cholesterol esters had detectable quantities of nearly all of the fatty acids found in milk cholesterol esters. However, mammary tissue cholesterol esters contained higher quantities of the normal milk lipid fatty acids (defined as 10:0, 12:0, 14:0, 16:0, 16:1, 18:0, 18:1 and 18:2) and correspondingly lower levels of the other or trace fatty acids. For the two sow samples, mammary tissue cholesterol esters were composed of 95% and 92.5% normal acids while the corresponding milk cholesterol esters contained only 89% and 82%, respectively, of the normal acids. For the cow and goat, the tissue cholesterol esters were composed of 91% and 86% normal acids while those from the milks were composed of 83% and 82% normal acids, respectively. One can reason from this that fatty acid composition of cholesterol esters in the gland changes during synthesis and secretion of the milk or that the secretion process preferentially selects certain cholesterol esters to a greater degree than others. The two experiments in which pentadecanoate was

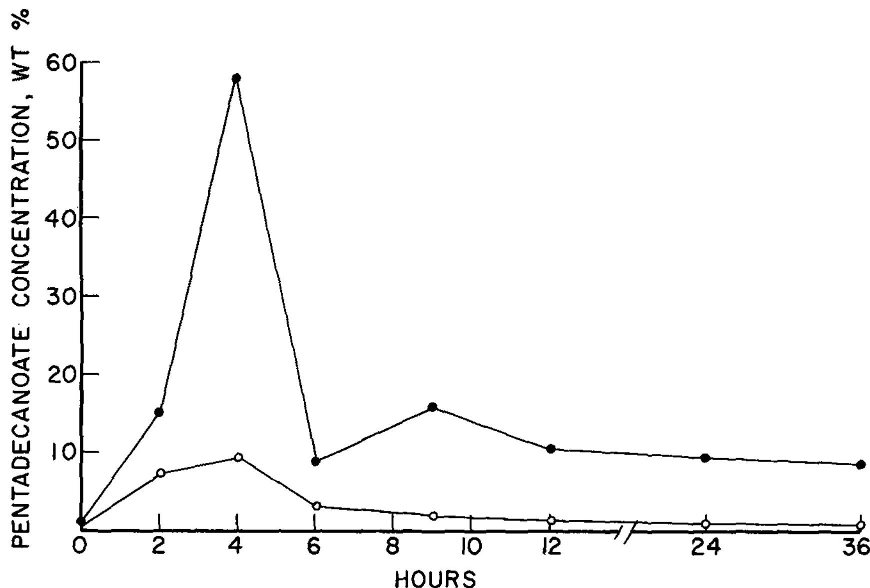

FIG. 3. Changes in pentadecanoate content of goat milk lipid classes after intramammary infusion of potassium pentadecanoate; ●————● cholesterol esters, ○————○ triglycerides.

TABLE III

Comparison of Milk and Mammary Tissue Cholesterol Ester Fatty Acid Compositions

Fatty acid	Fatty acid composition (wt %)							
	Sow A		Sow B		Cow		Goat	
	Milk	Tissue	Milk	Tissue	Milk	Tissue	Milk	Tissue
10:0	10.7	8.1	9.6	2.9	2.9	3.4	8.2	3.1
10:1	ND[a]	ND	1.5	ND	0.3	ND	Trace	Trace
12:0	5.3	4.0	6.0	3.4	4.1	4.5	4.4	4.1
12:1	Trace	Trace	0.3	0.8	0.2	0.2	0.6	Trace
13:0	0.4	ND	3.8	Trace	Trace	ND	0.6	0.6
13:1	1.3	1.2	6.8	0.9	11.0	3.1	1.9	2.8
14:0	10.2	9.7	7.9	7.5	6.9	8.1	8.9	10.1
14:1	0.5	0.4	1.8	0.6	0.5	0.5	1.2	Trace
15:0	1.9	1.4	1.5	1.2	2.1	2.1	6.3	6.1
15:1	2.6	2.0	0.8	0.4	2.6	2.8	0.6	ND
16:0	31.1	33.8	28.1	33.3	26.9	27.7	26.0	39.5
16:1	10.6	11.9	8.4	11.8	11.9	13.1	3.8	6.1
17:0	3.9	Trace	1.1	3.5	Trace	Trace	3.8	4.1
17:1	ND	ND	ND	ND	ND	ND	3.2	ND
18:0	5.3	6.7	7.2	8.9	6.7	8.9	13.3	8.1
18:1	14.7	18.5	13.4	19.4	13.7	15.5	13.9	12.8
18:2	1.5	2.3	1.7	5.3	10.1	10.1	3.2	2.6
Total normal acids[b]	89.4	95.0	82.3	92.5	83.2	91.3	81.7	86.4
Total 18 carbon acids	21.5	27.5	22.3	33.6	30.5	34.5	30.4	23.5

[a]ND, not detected; Trace, less than 0.1%.

[b]Defined as 10:0, 12:0, 14:0, 16:0, 16:1, 18:0, 18:1 and 18:2.

TABLE IV

Pentadecanoic Acid in Lipid Classes of Goat Milk Before and After Intramammary Infusion of the Acid[a]

Fraction	Weight %[b]	
	Before	After (12 hr)
Cholesterol esters	1.2	8.8[c]
Triglycerides	0.5	2.3
Diglycerides	0.5	2.4
Phospholipids	0.2	1.0
Phosphatidyl ethanolamine	0.1	0.4
Lecithin	0.3	3.2
Sphingomyelin	0.2	0.5

[a]One gram as the K salt in 20 ml H_2O.

[b]Calculated from peak areas of gas chromatograms.

[c]Also 5.3% in $C_{15:1}$ or a total of 14.1% in C_{15} acids.

infused into the mammary gland shed further light on this matter. Results from the first experiment (Fig. 3) which compared incorporation of the odd carbon acid into the cholesterol esters and triglycerides in a series of milkings show a dramatic uptake of the acid, especially in the cholesterol ester fraction. As previously mentioned there is a large difference in the apparent pool size of the two classes of lipids which could account for the much higher concentration achieved in the cholesterol esters. The fact that the content of the acid reaches a maximum in both cholesterol esters and triglycerides at the 4 hr milking suggests involvement of both lipids in the same process, namely milk fat globule formation (triglyceride accumulation). Since globules exposed to the pentadecanoate pool the longest should contain the greatest level of the acid, it can be deduced coincidentally that globule formation required about 4 hr. The data suggests that the cholesterol esters are contained mainly within the core of fat globules, along with triglycerides, which would explain their compositional stability in milk. The uniformly higher level of pentadecanoate in the cholesterol esters as compared to the triglycerides over the entire milking period suggests preferential retention of this acid in the cholesterol ester fraction.

In the second infusion experiment in which only one milking at 12 hr was collected and analyzed, substantial pentadecanoate (8.8%) incorporation into the cholesterol esters again occurred (Table IV). A further point of interest was the presence of a significant level (5.3%) of monounsaturated pentadecanoate in the cholesterol esters. The absence of this acid in the other lipid classes suggests selective metabolism of it as the cholesterol ester and perhaps intact desaturation as the ester. Kinsella (16) has suggested similarly that cholesterol stearate is converted intact to cholesterol oleate by cultures of mammary cells. No unsaturated pentadecanoate was observed in the first infusion experiment. This may be attributed to retention of all the pentadecanoate in the gland for 12 hr in the second experiment, whereas most of it would have been removed at the 2 hr and subsequent milkings in the first experiment.

We conclude that the fatty acid composition of cholesterol esters in milk does not change significantly after secretion and this composition differs from that of the total milk lipid, mainly triglyceride, in containing higher levels of unique fatty acids, some of which are monounsaturated and some of which are odd numbered in carbon chain lengths. The reason for this unique cholesterol ester composition apparently results from preferential retention of these acids in the cholesterol ester fraction that is secreted in milk.

When the data of our present research on pentadecanoate incorporation into cholesterol esters (Fig. 3) are compared with earlier findings from this laboratory (5,6) on incorporation of 1-^{14}C-palmitate into cholesterol esters under similar conditions, it is noted that the specific activity maximum had already been passed at 2 hr with the palmitate whereas the mass incorporation with pentadecanoate did not peak until 4 hr. While this observation as well as others herein will bear further investigation, the findings suggest a rapid turnover of the acyl moiety of cholesterol esters in lactating mammary tissue quite apart from the small mass of these esters that is secreted in the milk.

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Free Fatty Acids in Cultured Cells

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ABSTRACT

A method for isolation and quantitation of cellular free fatty acid has been developed. When this method was used to quantitate the free fatty acid content of various cells and tissues, their levels of free fatty acids were found to vary over a wide range. In comparing tissue culture cells having different levels of free fatty acid, it was demonstrated that the conditions of culture and the type of serum in the medium are not responsible for the difference in levels. Isotopic studies have shown that the cellular free fatty acid is not biosynthesized, but is derived from the free fatty acid of the medium. Preliminary studies on the fate of the intracellular free fatty acid and a discussion of possible factors controlling the level of this compound in cells are presented.

INTRODUCTION

Previous studies on the lipids of human diploid cell strain WI-38 and the transformed line WI-38VA13A (1,2) have indicated that in both types of cells about 20% of the neutral lipid is present as free fatty acid. Unesterified fatty acids cannot be strictly referred to as neutral lipids, but here we are using the commonly accepted terminology of referring to non-phospholipid material as neutral lipid. Very little data on the free fatty acid content of tissues can be found in the literature. Most of the earlier studies on the quantitation of lipids in cells or tissues relied on column chromatography for the separation of lipid classes; free fatty acids trail in these systems and are difficult to quantitate. In the available data, values reported for liver (3,4), thymus (5) and L cells (6), are much lower than those found in WI-38 or WI-38VA13A cells. On the other hand, Yamakawa et al. (7) report 36-42% free fatty acid in the nonphosphatide lipid of Ehrlich Carcinomas and Sarcoma 180 of mice, and Tuna and Mangold (8) found large areas corresponding to free fatty acid in the course of thin layer chromatography (TLC) of several rabbit tissues.

Little is known about the factors that affect cellular free fatty acid concentrations. Spector and Steinberg (9,10) have shown that the uptake of free fatty acids by suspensions of Ehrlich ascites cells depends upon the ratio of free fatty acid to albumin (ν) in the extracellular environment. The intracellular free fatty acid concentration was shown to rise as ν increased, but oxidation and esterification were very rapid and the levels never reached those found in our studies. In subsequent studies (11) they also found that the presence of glucose can increase esterification and thus result in a decrease in the intracellular free fatty acid levels. Higgins and Green (12) also showed an elevation of free fatty acid content of rat liver cells in the presence of free fatty acid-albumin complexes, but they were rapidly depleted by oxidation and esterification. Recently, Ontko (13) has shown that increasing the calcium concentration in suspensions of isolated liver cells or liver homogenates increases the cellular free fatty acid because it inhibits oxidation; but again the levels reported were below those found in this study.

All of the above studies were performed for short periods of time under conditions very different from those employed during cell culture. Consequently, we set out to investigate more thoroughly the free fatty acid content of cultured cells. The first step was the development of a suitable method for the direct quantitation of the cellular free fatty acid. A variety of cultured cells and rat tissues were then studied to determine the range of free fatty acid generally present. After this, studies were conducted to determine the source and function of the cellular free fatty acid, and attempts were made to determine what factors control the cellular level of this compound.

MATERIALS AND METHODS

Cells and Tissues

Cultures of WI-38, a human diploid cell strain, WI-38VA13A, a line derived from WI-38 by SV_{40} virus transformation, muscle fibroblasts, HeLa, L, L-5178Y cells, and skin fibroblasts were used. The WI-38, WI-38VA13A, skin and muscle fibroblasts and HeLa cells were cultured as monolayers on Eagle's basal medium using methods described previously (1). The L cells

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were cultivated both as a monolayer and in suspension, and they could be routinely alternated from one method of culture to the other; L-5178Y cells were cultivated only in a suspension culture. For the suspension cultures, cells were agitated in Joklik's modification of Eagle's basal medium (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 10% calf serum and 50 μg/ml aureomycin. In certain of the studies fetal calf serum (Microbiological Associates, Bethesda, Md.) was used in place of calf serum (Flow Laboratories, Rockville, Md.) and the concentrations were varied from 2% to 10%. Cells were harvested and washed using procedures described previously (1), except that the trypsin was omitted in harvesting the suspension cultures.

Rat tissues were obtained from 200 g male Wistar rats which had been fed a normal diet.

Isolation and Quantitation of Free Fatty Acids

The procedure developed for the isolation and quantitation of free fatty acids is the following. An aliquot of the harvested cells was taken for the determination of dry weight and cell protein. The remainder was extracted with 20 vol of warm chloroform-methanol (2:1) for 15 min. This total lipid extract was washed with 1/5 vol of 0.05 N NaCl according to the procedure of Folch et al. (14). Phospholipids were removed from the lipid extract by the method of Bloor (15). The total lipid extract was dried under a stream of N_2 and redissolved in 2 ml of cold petroleum ether in a centrifuge tube. Seven ml of cold acetone and three drops of a saturated solution of $MgCl_2$ in ethanol were added, and the tubes were placed in an ice bath for 5 min. The samples were then centrifuged for 10 min at high speed in an International centrifuge and the precipitate was washed once with 2 ml acetone. The efficiency of the precipitation was assayed by adding a mixture of ^{14}C-labeled phospholipids to the cell lipid extract, and the results indicated that more than 96% of the phospholipid was precipitated.

The free fatty acids were then separated from the other cellular neutral lipids by forming a hydrophilic salt. The combined supernatants from the phospholipid precipitation were dried under N_2 and the neutral lipid dissolved in 15 ml petroleum ether; 20 ml 50% ethanol and 0.5 ml 50% KOH were added and the solution stirred for 1 hr in the cold. The free fatty acids were found in the ethanolic KOH layer. When ^{14}COOH-tripalmitin was subjected to the isolation procedure, 99% of the glyceride was found in the petroleum ether layer. The experiments using labeled triglyceride also indicated that there was no saponification of the triglyceride during the extraction procedure. The fatty acids were then removed from the aqueous phase by acidification to pH 2 with concentrated HCl and three extractions with 20, 15 and 10 ml ethyl ether. This last partition, coupled with the original aqueous wash procedure of Folch, assures complete separation of the fatty acids from other cellular acids such as lactate and pyruvate.

When the free fatty acid was to be isolated from serum, the serum was acidified with HCl to pH 2 and extracted with 20 vol of chloroform-methanol (2:1). Five volumes of water were then added. After removal of the aqueous phase the resultant lipid extract was carried through the same procedure as the cellular lipid.

Rat tissues were minced and a weighed aliquot taken for the determination of dry weight. Another weighed aliquot was placed in 20 vol of warm chloroform-methanol (2:1) and subjected to the same procedures as described for the cellular lipid extract.

The efficiency of the extraction procedure for fatty acids was followed by adding ^{14}C-labeled stearic acid to the total lipid extract at the beginning of the procedure. Typically, less than 3% of the free fatty acids are lost in the acetone precipitate, 2-3% are lost in the petroleum ether fraction, and 3-5% are not removed from the aqueous layer. Recoveries range from 85-95% of the original free fatty acid. After the free fatty acids were isolated, they were quantitated by the colorimetric method of Duncombe (16). This method involves making the cupric salt of the fatty acid and then assaying the copper using diethyl-dithiocarbamate. The possibility that the isolation procedure would interfere with the colorimetric method was assessed by adding a known amount of stearic acid to the cellular lipid extract at the beginning of the procedure. Ninety-eight per cent of the stearic acid was recovered in the colorimetric determination.

Quantitation of Protein, Glucose and Triglyceride

Protein was measured by the method of Lowry et al. (17), and glucose was quantitated enzymatically by the glucose oxidase procedure (Glucostate-Worthington Biochemicals, Freehold, N.J.). Triglycerides were measured by the method of van Handel and Zilversmit (18).

Isotopic Studies

Sodium-1-^{14}C-acetate, U-^{14}C-glucose, ^{14}COOH-tripalmitin and sodium-1-^{14}C-palmitate were obtained from New England Nuclear Corp., Boston, Mass., and the tri-

TABLE I

Free Fatty Acid Contents of Cultured Cells and Rat Tissues[a]

Cell or tissue	μmole Free fatty acid mg dry wt	μmole Free fatty acid 10^8 cells	μmole Free fatty acid mg lipid	Per cent total lipid
WI-38	.032	1.60	.149	4.2
WI-38VA13A	.038	0.90	.120	3.4
HeLa	.027	0.61	.166	4.7
Skin fibroblasts	.026	3.40	.125	3.5
Muscle fibroblasts	.073	1.50	.149	4.2
L	.010	0.36	.035	1.0
L5178Y	.010	0.14	.072	2.0
Liver	.008	---	.052	1.5
Muscle	.008	---	.023	0.7
Skin	.015	---	.046	1.3
Lung	.030	---	.180	5.1

[a]Average of four determinations.

palmitin and palmitic acid were purified by TLC. Isotopic dilution experiments using these isotopes to determine the source of the cellular free fatty acid were conducted according to methods described elsewhere (Howard and Kritchevsky, in preparation). (Known specific activity of precursor was added to the medium at the time of subcultivation, the glucose and acetate as aqueous solutions and the palmitate and tripalmitin as emulsions. The specific activity of the cellular free fatty acid at the end of the cultivation time was then determined and compared to that of the precursor used). Radioactivity was measured in a Packard Tri-Carb scintillation spectrometer which was equipped with an external standard. The lipid samples were dried under a stream of N_2 and then dissolved in 15 ml of Bray's solution (19). Aqueous samples in less than 1 ml amounts were added directly to 15 ml Bray's solution. Samples were counted long enough to reduce the counting error to 1%. When necessary, they were corrected for quenching, using the external standard. In one experiment, radioactivity of lipids on a TLC plate was assayed using a Packard radioactive scanning unit. The Geiger tube had a linear response, and the relative amounts of activity in each lipid were proportional to the area of each peak recorded graphically.

RESULTS

Comparison of the Free Fatty Acid Contents of Various Cultured Cells and Rat Tissues

After the development of a suitable technique for measuring cellular free fatty acid, the method was used to measure directly the free fatty acids of WI-38 and WI-38VA13A cells and to compare them to several other tissue culture lines and strains. Several rat tissues were also assayed in order to compare results obtained by this method to others reported in the literature and to compare the content of free fatty acids in cultured cells with those of tissues in vivo. The data obtained are shown in Table I.

The data are best expressed as μmoles/mg dry weight. The values obtained by this method for the rat liver compare favorably to those reported by Ontko (13) and others (3,4). It can be seen that the various tissues and cultured cells vary greatly in the content of free fatty acid. The WI-38 and WI-38VA13A contain .035 μmole/mg dry weight, and this value is much higher than that contained in the L and L-5178Y cells or in rat liver, skin and muscle. However, the free fatty acid contents of the WI-38 and WI-38VA13A cells are not unusual; the HeLa cells, skin fibroblasts and rat lung contain similar levels of free fatty acid, and the amount in the muscle fibroblasts is even higher. When the data are expressed as per cent of total lipid, the WI-38 and WI-38VA13A cells have approximately 4% free fatty acid, which corresponds well to values of approximately 5% derived from previous studies (1,2) in which the free fatty acids were measured by a nonspecific acid dichromate oxidation method. When expressed in this manner, the cells and tissues again vary in free fatty acid levels. The skin and muscle fibroblast-like strains, WI-38, HeLa, WI-38VA13A cells and the lung tissue have about 4% free fatty acid, whereas in the L and L-5178Y cultures and the other rat tissues only approximately 1-2% of the cellular lipid is free fatty acid. It is also convenient to express the data for the cultured cells in terms of μmoles of fatty acid/10^8 cells. In this case the L and L-5178Y cells are again clearly lower than the others, although the values obtained for HeLa and WI-38VA13A cells are lower than those for

TABLE II

Per Cent of Free Fatty Acid Derived From Cellular Biosynthesis

	U-^{14}C-Glucose	U-^{14}C-Glucose	1-^{14}C-Acetate	1-^{14}C-Acetate
Precursor (per ml medium)				
cpm	205,638	249,540	138,530	85,720
mg	1.08	0.83	0.10	0.10
sp. activity $\left(\frac{cpm}{mg}\right)$	190,000	300,000	1,380,000	857,000
Cellular free fatty acid				
cpm	609	260	875	220
mg	0.256	0.177	0.262	0.222
sp. activity $\left(\frac{cpm}{mg}\right)$	2,380	1,470	3,340	991
Per cent biosynthesized $\left(\frac{\text{sp. act. cell. free fatty acid}}{\text{sp. act. precursor}}\right)$	1.2%	0.49%	0.24%	0.12%

the fibroblast-like cells, perhaps because the cells are smaller.

Source of Free Fatty Acid in WI-38 Cells

The extent of biosynthesis of free fatty acids in WI-38 cells was determined by growing the cells in the presence of U-^{14}C-glucose or sodium = 1-^{14}C-acetate. The specific activities of the precursor in the medium and of the free fatty acid present in the cells after confluency was reached were determined as described above. The ratios of specific activities are a measure of the percentage of free fatty acid synthesized from precursor. The results, which are presented in Table II, indicate that very little free fatty acid is biosynthesized from either glucose or acetate under the present growth conditions. The low level of cellular fatty acid biosynthesis suggests that fatty acid is being supplied to the cells. Since serum is present in the growth medium of WI-38 cells, experiments were conducted to determine which serum lipids served as the source of cellular free fatty acid. Since triglyceride is the most abundant neutral lipid in serum, the contribution of serum triglyceride to cellular free fatty acid was first assessed. Tripalmitin-^{14}COOH was added to the medium and the experiment was conducted in the same way as those for glucose and acetate. In this case the ratios of specific activities are an indication of the percentage of free fatty acid derived from uptake of serum triglycerides. The data (Table III) show that only 1-2% of the cellular free fatty acid is derived from serum triglyceride. The results of these experiments thus indicate that serum triglyceride is not the source of cellular free fatty acid. It was also observed that little of the radioactive triglyceride was absorbed intact.

TABLE III

Per Cent of Free Fatty Acid Derived From Serium Triglyceride

	Experiment 1	Experiment 2
Triglyceride (per ml medium)		
cpm	11,972	25,730
μg	210	345
sp. act. $\left(\frac{cpm}{\mu g}\right)$	57.2	74.4
Cellular free fatty acid		
cpm	185	722
μg	284	440
sp. act. $\left(\frac{cpm}{\mu g}\right)$	0.654	1.64
Per cent derived from serum triglyceride $\left(\frac{\text{sp. act. free fatty acid}}{\text{sp. act. triglyceride}}\right)$	1.3%	2.2%

The contribution of serum free fatty acid to the cellular pools was now measured. This was accomplished through an isotopic dilution experiment similar to the others, using 1-^{14}C-palmitate. The results, shown in Table IV, are calculated on the basis of the free fatty acid in the medium at both the beginning and the end of the experiment. This was done because it was noted that the specific activity of the free fatty acid in the medium dropped during the course of the experiments; the radioactivity of the free fatty acid fraction decreased, but the actual amount of free fatty acid in the medium remained constant. We have found that hydrolysis of the serum triglyceride replenishes the free fatty acid during culture and that this hydrolysis accounts for the decrease in specific activity of the free fatty acid fraction during the course of the experiment (2).

The results summarized in Table IV show a close correlation between the specific activities of the free fatty acid in the cells and of the medium at the end of the experiment. These

TABLE IV

Derivation of Cellular Free Fatty Acid From Serum Free Fatty Acid

	Experiment		
	1	2	3
Free fatty acid - medium, day 0			
cpm	4810	8358	9231
μmole	1.17	1.02	0.78
sp. act.$\left(\frac{cpm}{\mu mole}\right)$	4100	8180	11,880
Free fatty acid - medium, day 7			
cpm	2139	5160	5073
μmole	1.08	1.02	0.66
sp. act.$\left(\frac{cpm}{\mu mole}\right)$	1980	5060	7680
Free fatty acid - cells			
cpm	2020	6410	17,400
μmole	1.05	1.2	2.1
sp. act.$\left(\frac{cpm}{\mu mole}\right)$	1940	5330	8280
Per cent derived from serum free fatty acid			
$\frac{\text{sp. act. cells}}{\text{sp. act. medium, day 0}}$	47%	65%	70%
$\frac{\text{sp. act. cells}}{\text{sp. act. medium, day 7}}$	98%	105%	107%

findings suggest that the cellular free fatty acid is derived from the serum free fatty acid, but that the cellular pool is in rapid equilibrium with the medium. If the intracellular free fatty acid pool were static, its specific activity would be expected to reflect an average of the medium specific activity at the beginning and end of the experiment.

The Effect of Serum on the Free Fatty Acid Levels of Cultured Cells

Since it had been established that the free fatty acid level of WI-38 cells was consistent and that the fatty acid was derived from the serum free fatty acid, experiments were initiated to compare WI-38 with one of the cell cultures with lower levels of free fatty acid in an effort to determine what factors influence cellular free fatty acid levels. The L cell was chosen because much of the work reported on the lipids of cultured cells has been derived from this line. Since the L cells in the previous study had been cultured in the presence of fetal calf rather than calf serum, and since fetal calf serum contains much less lipid than calf serum (Baltimore Biological Laboratories, Md.), it seemed possible that the difference in cellular free fatty acid levels might be due to a difference in the fatty acid contents of the two sera. Therefore, cultures of WI-38 cells were grown in fetal calf serum for 1-2 months, and cultures of L cells (monolayers) were cultured on calf

TABLE V

Free Fatty Acids of WI-38 and L Cells Grown on Calf and Fetal Calf Sera[a]

Cell	Type of serum	μmoles FFA mg protein	μmoles FFA 10^8 cells
WI-38	calf	.106	2.10
	fetal calf	.103	1.90
L	calf	.023	0.57
	fetal calf	.025	0.57

[a]Average of four determinations.

serum for an equal length of time. The free fatty acid contents of these cultures were analyzed and compared with those found in WI-38 and L cells grown in calf and fetal calf sera, respectively. The data obtained are shown in Table V. It is evident that the type of serum used in the growth medium does not affect the free fatty acid level found in each cell.

Effect of Different Conditions of Growth on the Free Fatty Acid Levels of WI-38 and L Cells

Another difference between the cells in Table I, in addition to free fatty acid levels, was that the L and L-5178Y cells were suspension cultures whereas the others were monolayers. Since this difference would necessarily represent alterations in the surface configuration of the cells, it seemed possible that the differences in free fatty acid content might be correlated with the different modes of cultures. Alterations in size and shape of the cells would alter surface-volume ratios and could influence nutrient exchange with the medium and other metabolic parameters. These changes might affect the free fatty acid spectrum of the cells. No techniques have been developed for the growth of WI-38 cells in suspension culture. L cells, however, can be cultivated under both conditions. Therefore, both monolayer and suspension cultures of L cells were established using identical serum levels, and their fatty acid levels were compared. As seen in Table VI, the proportion of free fatty acids (μmoles/mg protein) is the same in L cells grown either in suspension or as monolayers. When the data are expressed as μmoles/10^8 cells there appears to be less free fatty acid in the suspension culture, but this could be a reflection of differences in cell size between the two cultures. The data suggest that the difference in free fatty acid levels between L and WI-38 cells cannot be attributed to the method in which they are cultured.

Another difference between the WI-38 and L cells is that cells harvested from the WI-38 cultures have reached confluency and are not

TABLE VI

Free Fatty Acids of L Cells in Monolayer and Suspension[a]

Type of Culture	μmoles FFA / mg protein	μmoles FFA / 10^8 cells
Monolayer	.024	.57
Suspension	.023	.40

[a]Average of four determinations.

TABLE VII

Free Fatty Acid Content of WI-38 During the Growth Cycle

Day	Per cent confluency	μmoles FFA / mg protein	μmoles FFA / 10^8 cells
3	25	.076	4.0
5	66	.054	1.6
10	100	.051	1.9

rapidly dividing, whereas the L cells, because of the lack of contact inhibition, are actively proliferating at the time of harvest. Therefore, it seemed possible that the WI-38 cells might have a lower free fatty acid content during the early days of culture when they are proliferating rapidly.

The results of an analysis of the fatty acids during the growth cycle are shown in Table VII. When the data are expressed either as μmoles/10^8 cells or as μmoles/mg protein, no decrease in free fatty acid level is seen in the cells during the early days of culture. In fact, the data for μmoles/10^8 cells show a very high value for the cells when they are 25% confluent. The significance of this elevation is hard to interpret without further experiments. Nevertheless, the results of this experiment indicate that the difference in free fatty acid levels between WI-38 and L cells is not due to their different rates of proliferation.

DISCUSSION

The method employed in this study for the quantitation of cellular free fatty acid yields more satisfactory results than the two procedures generally employed for plasma free fatty acids (16,20). Both of these are subject to error in the presence of acidic phosphatides and acidic metabolic intermediates, such as pyruvate and lactate, and additional extraction procedures (21,22), are not completely effective in eliminating these cellular contaminants (23).

Since our procedure was developed, two reports have appeared in the literature on the quantitation of free fatty acids. One, by Anstall and Trujillo (24), employs a phospholipid precipitation similar to the one above. They do not then partition the free fatty acids from the other neutral lipids but assay the supernatant directly for fatty acids. In the other report, Ibrahim (25) excludes phospholipids by adsorption on silicic acid and then assays the free fatty acid by titration. His data indicate that the silicic acid completely removes cephalins which are the phospholipids mainly responsible for interference with titration. Although Ibrahim assayed only plasma free fatty acids, Rose et al. (26) utilized a similar adsorption of phospholipids on silicic acid before titrating liver-cell free fatty acids. The removal of phospholipids by silicic acid would seem to be quicker than precipitation, but the effect of the silicic acid on the colorimetric procedure would have to be assessed.

The data obtained in this study indicate that cultured cells and tissues can vary greatly in their content of free fatty acid. WI-38 cells have higher free fatty acid levels than do cell lines, such as L and L-5178Y, and tissues, such as liver, skin and muscle. However, WI-38 and WI-38VA13A cells are not unusual, and other cultured cells such as HeLa and certain fibroblast-like strains have been shown to have similar free fatty acid levels. The determination of the source of cellular free fatty acids by means of isotopic dilution experiments was based on methods developed for a more extensive study of the source of total cellular lipid in cells during culture (Howard and Kritchevsky, in preparation). The results of the isotope studies indicate that under the present conditions of culture the existing cellular free fatty acid of WI-38 cells is not biosynthesized de novo, but is derived solely from the free fatty acid of the serum. The observation that biosynthesis of fatty acid is minimal could mean that the cells are not able to biosynthesize fatty acid; alternatively, it could mean that there is a feedback inhibition or that a repression mechanism is operative, since Bailey (27) has shown that L cells can synthesize fatty acid, but that synthesis is inhibited when the cells are grown in the presence of a serum.

The experiments in which cells were cultured using calf and fetal calf sera indicated that the observed differences in free fatty acid level in cells like WI-38 and L cells are not due to the composition of the different sera used in the culture of the cells. Although there is much less free fatty acid per ml in the fetal calf serum, the ratio of free fatty acid to albumin (ν) is similar in the two sera. The calf serum

used in these studies contained 0.72 μmoles of free fatty acid and 0.49 μmoles of albumin per ml (ν = 1.4) and the fetal calf serum contained 0.29 μmoles of free fatty acid and 0.28 μmoles of albumin per ml (ν = 1.0). We determined the free fatty acid content by the method described above and information concerning the albumin content was obtained from the supplier, Baltimore Biological Laboratories, Md. Thus the fact that free fatty acid remains constant in each cell during growth in the two sera, although the levels of free fatty acid in the two sera are so different, is consistent with the work of Spector et al. (9) who demonstrated that the rate of uptake of free fatty acid varies with the ratio of free fatty acid to albumin (ν) in the extracellular environment. The results of our experiments also suggest that the differences in free fatty acid levels between cells are probably not due to physical factors of cell culture, such as differences between monolayer and suspension methods, nor do they reflect differences in the division cycle or stages of growth of cell cultures.

Preliminary experiments tracing the fate of the free fatty acid taken up by WI-38 cells indicate that the cellular free fatty acid is both oxidized and converted to phospholipids and glycerides. Thus many possibilities exist as explanations for the differences observed in the intracellular free fatty acid levels of cells. The cells could vary in their rate of uptake of free fatty acid, or the level of uptake could be the same in all cells but the rates of excretion could vary. Finally, differences in enzymatic capacities for oxidation and esterification could result in a more rapid depletion of the pools in some cells. Experiments are presently being conducted to compare the rates of uptake, oxidation, esterification and excretion in cultured cells to determine which is most important in the regulation of intracellular free fatty acid levels and the different levels in different cells.

ACKNOWLEDGMENTS

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Ceramide Phosphorylglycerol Phosphate A New Sphingolipid Found in Bacteria

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ABSTRACT

Ceramide phosphorylglycerol phosphate (CPGP) has been identified in the lipid extract of the anaerobic bacterium *Bacteroides melaninogenicus*. To our knowledge this is the first report of this lipid in biological material. The ceramide derivative contains two phosphates, an amide linked fatty acid and a dihydrosphingosine long chain base. Glycerol diphosphate (PGP) identified by paper and column chromatography can be isolated after mild acid hydrolysis of the ceramide derivative. Inorganic phosphate is liberated quantitatively on treatment of the PGP from the ceramide derivative with alkaline phosphatase. The proportions of the fatty acids found linked to the amide of the dihydrosphingosine (LCB) differ from those esterified to cardiolipin in this organism. The long chain base appears to consist of part of an homologous series of branched and normal LCB containing from 17 to 21 carbon atoms. Previous work has indicated that ceramide phosphorylethanolamine and ceramide phosphorylglycerol (CPG) are present in the lipid extracts of *B. melaninogenicus*. By analogy with phosphatidylglycerol synthesis, CPGP is postulated to be an intermediate in the synthesis of CPG.

INTRODUCTION

The anaerobic bacterium *Bacteroides melaninogenicus* has been shown to contain phosphate containing sphingolipids which account for half the extractible lipid phosphate (1). Sphingolipids are exceedingly rare in eubacteria (2). Ceramide phosphorylethanolamine (CPE), a rare sphingolipid previously reported in insects, protozoa and certain snails, and ceramide phosphorylglycerol (CPG), a lipid not previously reported, make up the major portion of the sphingolipid of *B. melaninogenicus* (1). A trace of a third phosphate containing sphingolipid was detected. In this study the trace ceramide has been identified as ceramide phosphorylglycerol phosphate (CPGP), a lipid not previously described in nature.

MATERIALS AND METHODS

Materials

The strain of *B. melaninogenicus*, the cultural conditions, harvesting procedures and methods for insuring cultural purity have been described in previous work (1,3). $H_3{}^{32}PO_4$ was supplied in plastic bottles by Tracerlabs, Waltham, Mass.

Column Chromatography

Fatty acid methyl esters were separated from ceramides or LCB on 1 g silicic acid columns (11 X 50 mm, Unisil, 100-200 mesh). The fatty acid methyl esters were eluted in 5 ml of chloroform. The dihydrosphingosine long chain bases (LCB) or ceramides were eluted with 5 ml of chloroform-methanol, (1:1) followed by 5 ml of methanol.

Glycerol phosphate esters derived from the lipids were eluted from 0.4 X 81 cm columns of Dowex-1 8X (200-400 mesh) in the formate form prepared as described (4,5). The esters were eluted with an ammonium formate-sodium borate gradient (4) or with 0.3 M ammonium formate pH 9.5 (Lester, unpublished method). The esters were desalted with Dowex-1 (100-200 mesh) as described in the text.

Paper Chromatography

Lipids were separated on silica gel loaded paper (Whatman SG-81) using solvents of chloroform-methanol-diisobutylketone-acetic acid-water (23:10:45:25:4 v/v), Solvent 1 in the first dimension and chloroform-methanol-diisobutylketone-pyridine-0.5 M ammonium acetate pH 10.4 (30:17.5:25:35:6 v/v), Solvent 2. Lipids were eluted from the silica gel loaded paper with a solvent of chloroform-methanol-19 mM ammonium hydroxide (20:20:1) by soaking the paper in 3 ml of solvent for 1 hr. The paper was then rinsed in three 1 ml portions of solvent. The recovery was quantitative.

Glycerol phosphate esters were separated on acid washed amino-cellulose paper (Whatman AE-81) (7). Solvents were 0.4% pyridine in 3 M formic acid and modified Wawszkiewicz solvent (5). This solvent contains 1.15 M ammonium acetate with 11.8 mM ethylenediaminetetra-

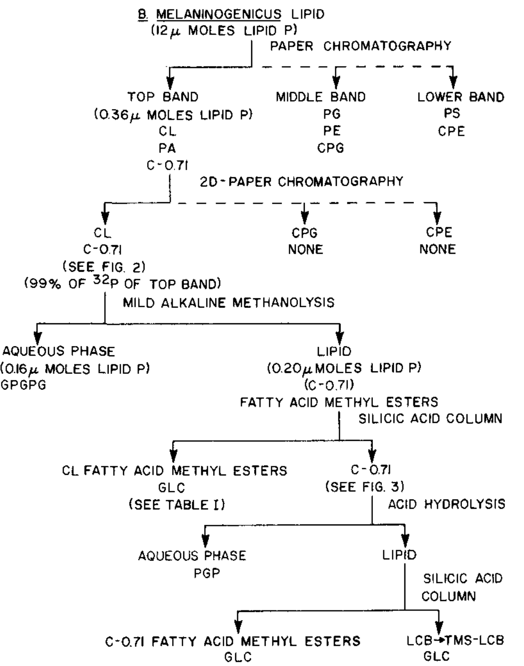

FIG. 1. Flow sheet for the purification of the unknown ceramide derivative.

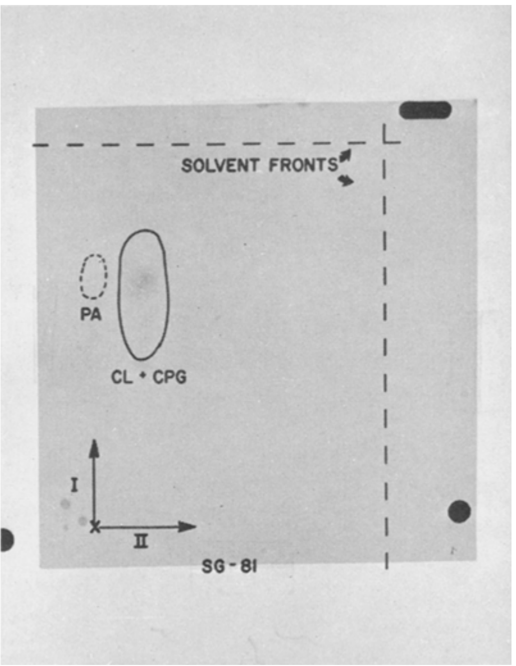

FIG. 2. Radioautogram of the chromatographic separation of the unknown ceramide derivative and CL from *B. melaninogenicus* grown with ^{32}P. See Materials and Methods and Reference 7.

acetic acid made to pH 5.0 with acetic acid and diluted 3 to 7 with 95% ethanolic 0.25 M ammonium hydroxide. Schleicher and Schuell 589 acid washed paper was used with ascending paper chromatography with the modified Wawszkiewicz solvent. The lipids were detected with the Hanes-Isherwood reagent for phosphate (4) or by periodate treatment followed by *o*-toluidine (4).

Gas Chromatography

Fatty acid methyl esters were prepared and separated on ethylene glycol succinate or SE-30 columns under the conditions described previously (8). Trimethylsilyl ether derivatives (TMS) of the LCB were prepared and analyzed as in an earlier study (1).

Measurement of Radioactivity

^{32}P was counted on paper disks in a scintillation spectrometer (7). Radioautograms were prepared with Kodak no-screen x-ray film (7). Illustrations of radioautograms were prepared by drawing the figures, copying the figure on a mylar sheet with the Xerox copier, then superimposing the developed film and the mylar sheet properly. The sheet and superimposed film were then photographed on a glow box.

Extraction and Analysis of the Lipid

Lipids were extracted from the bacteria by a modified Bligh and Dyer procedure (9). A 30 ml suspension of bacteria in 50 mM phosphate buffer pH 7.6 containing about 200 mg dry weight of cells was mixed with 75 ml of methanol and 37.5 ml of chloroform and shaken vigorously. The one phase system was allowed to stand overnight. Then 37.5 ml of chloroform and 37.5 ml of 1.0 M KCL solution containing glacial acetic acid (0.4% v/v) was added and the mixture shaken. After several hours the mixture separated into two phases. The lower layer containing the lipid was filtered through a 4 cm piece of Whatman No. 12 filter paper.

Purification of the Lipid

A flow chart of the purification of the unknown lipid is illustrated in Figure 1. A total of 12 μmoles of lipid phosphate isolated from cells grown in the presence of ^{32}P was spotted near the bottom edge of two silica gel impregnated papers. The lipids were separated into three bands by ascending chromatography in a solvent of chloroform-methanol-diisobutylketone-acetic acid-water (23:10:45:25:4 v/v). The bands were located by radioautography

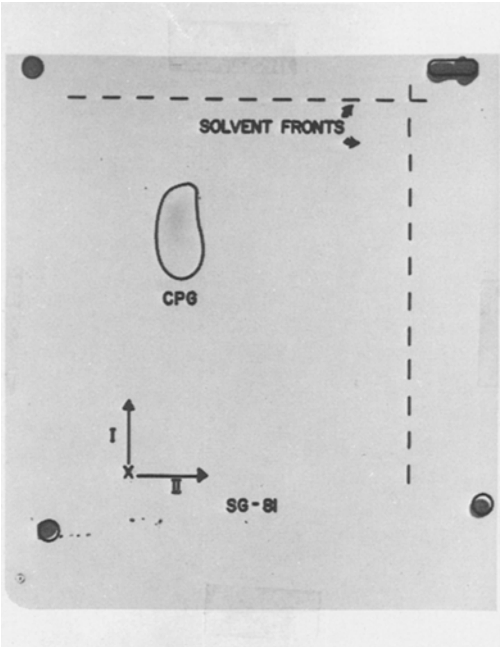

FIG. 3. Radioautogram of the unknown ceramide derivative after mild alkaline methanolysis which removes the CL. See Figure 2.

TABLE I

Distribution of Fatty Acids Between the Amide of Ceramide Phosphorylglycerol Phosphate and the Esters of Cardiolipin in *Bacteroides melaninogenicus*[a]

Fatty acid	Amide of CPGP	Ester of Cl
12:0	3.7	---
13:0, Br	2.8	0.9
14:0, Br	8.0	---
14:0	---	1.6
15:0, Br	25.8	64.6
15:0	10.5	---
16:0, Br	2.9	---
16:0	12.8	8.2
17:0, Br	---	8.6
18:0, Br	11.5	1.3
19:0, Br	0.8	5.4
19:0	0.3	7.3
20:0, Br	8.1	---
20:0	---	2.1
21:0	2.5	---

[a]Fatty acid methyl esters determined from the areas of response after GLC on ethylene glycol succinate columns. The data are given as the percentage of the total fatty acids recovered from the amide or the ester linkage.

and the lipids recovered. The top band contained lipids with the chromatographic mobility in other solvent systems (7) of cardiolipin (CL), phosphatidic acid (PA), and the trace ceramide derivative (R_f value 0.71). The middle band contained lipids with the chromatographic mobility in other systems of phosphatidylglycerol (PG), CPG and phosphatidyl ethanolamine (PE) (R_f value 0.52) and the lower band contained CPE and phosphatidylserine (PS) (R_f value 0.43). The lipids from the top band were recovered from the silica gel impregnated paper and spotted on sheets of this paper again. Two-dimensional chromatography separated the CL and ceramide derivative from the phosphatidic acid (Fig. 2). The CL plus ceramide derivative were recovered and accounted for 99% of the ^{32}P from the top band. Mild alkaline methanolysis was performed at 0 C for 2 hr and the KOH neutralized with Biorex 70 (a weakly acidic cation exchange resin (7). The water soluble glycerol phosphate ester derived from the diacyl lipid was then separated from the fatty acid methyl esters and the ceramide derivative using a sequence of three extractions with diethyl ether and a final extraction with chloroform to minimize emulsion formation (1). The mild alkaline methanolysis is complete in 2 hr (1).

Lipid phosphate was analyzed after digestion of the samples in perchloric acid (4); 0.16 µmoles of radioactive phosphate were recovered as a water soluble ester which co-chromatographed with unlabeled authentic diglycerol phosphorylglycerol (GPGPG) in two dimensions on aminocellulose paper (7). The remainder of the ^{32}P (0.20 µmoles lipid phosphate) was recovered in the organic solvent after the mild alkaline methanolysis.

The organic phase from the mild alkaline methanolysis contained fatty acid methyl esters derived from the CL. The fatty acid esters were separated from the ceramide derivative by silicic acid chromatography. The fatty acid esters were then analyzed by gas liquid chromatography (GLC). The ceramide derivative recovered from the silicic acid column was chromatographed in two dimensions on silica gel-loaded paper (Fig. 3). The ceramide derivative had identical chromatographic mobility before and after mild alkaline methanolysis (Fig. 2 and 3). This suggests the ceramide derivative contains no ester linked fatty acids. No fatty acyl esters were detected in ceramide phosphorylethanolamine (CPE) in this organism (1). The ceramide derivative did not react with periodate (4) before or after mild alkaline hydrolysis.

The ceramide derivative was hydrolyzed in methanolic 2 N HCl containing water (9.5% v/v) at 100 C for 2 hr (1,10). This procedure quantitatively liberates the amide linked fatty

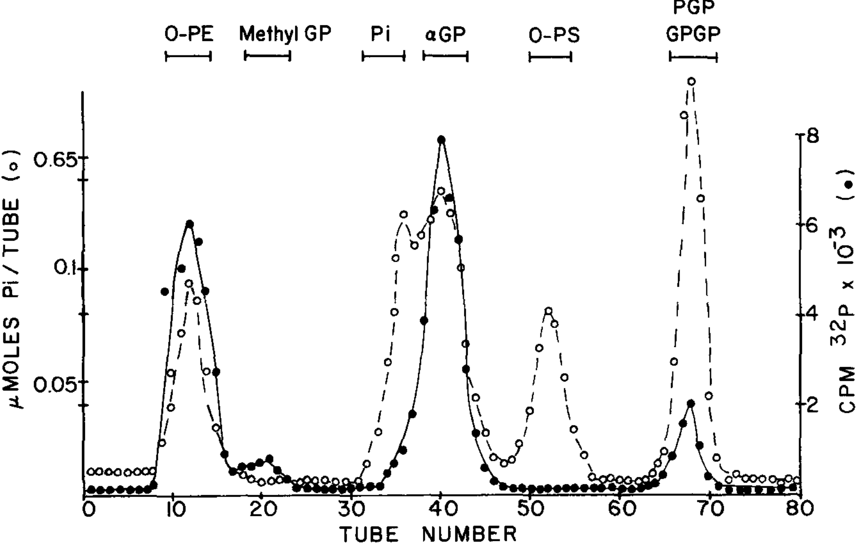

FIG. 4. Column chromatography of the water soluble products of the mild acid hydrolysis of the ceramides of *Bacteroides melaninogenicus* grown in the presence of ^{32}P. Fractions of 2.65 ml were collected, a portion removed to assay the ^{32}P and the remainder digested for the phosphate determination. The elution volumes of authentic compounds are illustrated at the top of the Figure. The recovery of ^{32}P was quantitative.

acid, the LCB and water soluble phosphate derivative (1). The fatty acids derived from the amide of the LCB were separated from the LCB by silicic acid chromatography of the organic phase of the hydrolysis mixture. These fatty acids were methylated (8) and separated by GLC. A comparison of the proportions of fatty acid components from the amide of the LCB and the fatty acids in ester linkage to CL is given in Table I. The fatty acids derived from the amide contain less 15:0, Br and more of the longer branched fatty acids than are found in the ester linkage. The LCB fraction was recovered from the silicic acid column and TMS derivatives prepared (1,11). TMS derivatives of the LCB had retention times corresponding to 16:0 (1.5%), 17:0,Br (39%), 18:0 (18%), 20:0, BR (4%), 20:0 (20%) and 21:0 (15%). The total detector response corresponded to 0.10 μmoles of TMS-dihydrosphingosine. This indicates that the molar ratio of LCB to phosphate in the ceramide was 1.00 to 2.05. The total response of the amide-linked fatty acid corresponded to 0.09 μmoles of methyl palmitate for an amide fatty acid to phosphate molar ratio of 0.92 to 2.00.

Identification of the Water Soluble Product of Acid Hydrolysis

To collect a large amount of the water soluble hydrolysis product of the unknown ceramide derivative for identification, the total lipid was deacylated by mild alkaline methanolysis and the fatty acids separated from the ceramide derivatives on a silicic acid column. The ceramide derivatives were hydrolyzed in 1 ml methanolic 2 N HCl containing 6.5 M water for 1 hr at 100 C. After cooling, 1 ml of water was added and the mixture extracted with two 2 ml portions of petroleum ether. The aqueous phase was then made to pH 10 with KOH and the petroleum ether extraction repeated twice. The aqueous phase was desalted by passing through a 5 X 300 mm column of Dowex 50-8X, 200-400 mesh, in the acid form and the ^{32}P recovered quantitatively. The HCl was removed in a stream of nitrogen. The ^{32}P labeled hydrolysis products were combined with authentic *o*-phosphorylethanolamine (*o*-pE), L-α-glycerol phosphate (αGP), inorganic phosphate (P_i), glycerol diphosphate (PGP) and *o*-phosphorylserine (*o*-pS) in 20 mM sodium borate pH 9.5 and loaded on a Dowex-1 column. The esters were then eluted from the column with an ammonium formate-sodium borate gradient (4). Esters containing ^{32}P were detected at the elution volumes of *o*-pE (43.3% of the ^{32}P), methyl-GP (4.5%), αGP (43.6%) and GPGP or PGP (8.5%). This is illustrated in Figure 4. The fractions corresponding to PGP or GPGP were combined and diluted to eight times their volume with distilled water. The sample was then pumped onto a 0.4 X 15 cm column of Dowex-1 -8X (100-200) mesh in the

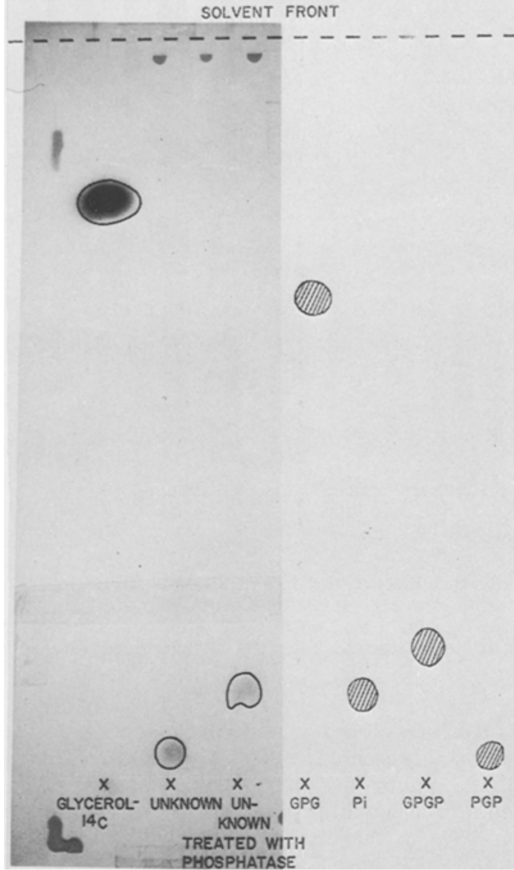

FIG. 5. Radioautogram of the separation of the water soluble glycerol phosphate esters derived from the trace ceramide of *Bacteroides melaninogenicus* grown in the presence of ^{32}P. The PGP fraction from the column illustrated in Figure 4 as desalted and chromatographed before and after treatment with alkaline phosphatase as described in Materials and Methods.

formate form. The column was washed with 3 column vol of 10 mM formic acid to remove the borate. The glycerol phosphate ester was then eluted with 0.3 M ammonium carbonate pH 8.0. The ^{32}P was quantitatively recovered. The ammonium carbonate was removed from the fraction containing the ^{32}P by boiling to dryness in a stream of nitrogen.

A portion of the ^{32}P containing ester was dissolved in 25 μl of 20 mM ammonium acetate pH 8.0. This was treated with 25 μl of alkaline phosphatase (1 mg/ml) from *Escherichia coli* (Worthington) for 3 hr at 25 C (5). The products of enzymatic hydrolysis and unhydrolyzed ester were applied to paper and subjected to ascending chromatography on acid washed paper with the modified Wawszkiewicz solvent (5). In this system the R_f values were glycerol, 0.80; glycerol phosphorylglycerol (GPG), 0.64; GPGP, 0.18; P_i, 0.15; and PGP, 0.05. A radioautogram of the ^{32}P-containing ester and its hydrolysis product together with a chromatogram of authentic standards is illustrated in Figure 5. The ^{32}P-containing ester had the chromatographic mobility of PGP. Only $^{32}P_i$ could be detected after alkaline phosphatase treatment. As a further identification the ester corresponding to the ^{32}P spot in Figure 5 was eluted from the paper with water, mixed with authentic P_i, GPGP and PGP and applied to a Dowex-1 8X (200-400 mesh) column. The esters were eluted with 0.3 M ammonium formate pH 9.5 as illustrated in Figure 6. The ^{32}P containing ester is clearly not GPGP.

RESULTS

Separation of the Unknown Ceramide Derivative

The lipid isolated by the procedure illustrated in Figure 1 was separated from CL. This is confirmed by the different proportions of fatty acids from the ceramide derivative and CL (Table I). Chromatography of the water soluble portion from the mild alkaline methanolysis performed on aminocellulose paper (7) indicated that there was no ceramide derivative in the GPGPG. The radioautograms illustrated in Figures 2 and 3 indicate that no CPE or CPG contaminate the unknown ceramide derivative. After mild alkaline methanolysis for 2 hr at 0 C the unknown ceramide derivative migrates as a single component in two dimensional paper chromatography (Fig. 3). Authentic CL is completely deacylated in 1.5 hr at 0 C and the phosphate can be quantitatively recovered in the aqueous phase (4,7).

Separation of the Ceramide Derivatives

A second purification procedure was used to confirm the results derived from the lipid isolated as in Figure 1. The total lipid extract from *B. melaninogenicus* grown with $H_3{}^{32}PO_4$ was subjected to mild alkaline methanolysis, the ceramide derivatives and fatty acid methyl esters from the diacyl lipids were recovered in the organic phase. The fatty acid methyl esters and ceramide derivatives were separated with a silicic acid column. The ceramide derivatives were then separated by chromatography on silica gel-loaded paper. The ceramide derivatives in the lipid sample accounted for 48% of the lipid phosphate. A radioautogram of the separated ceramide derivatives is illustrated in Figure 7. The two major ceramide derivatives correspond to CPE and CPG which have been identified previously (1). The distribution of

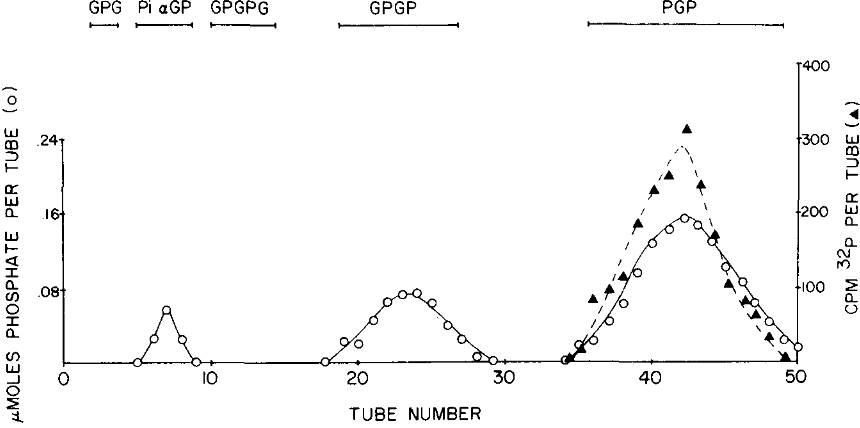

FIG. 6. Chromatography of the PGP isolated from the trace ceramide of *Bacteroides melaninogenicus* grown with ^{32}P. PGP was eluted from the paper chromatogram used for Figure 5 and chromatographed on Dowex-1 as described in Materials and Methods. See Figure 4. Each fraction contained 5.0 ml. The recovery of the ^{32}P was quantitative. The elution volumes of authentic glycerol phosphate esters are given at the top of the Figure.

^{32}P in the three ceramide derivatives was CPE, 45%, CPG, 51% and unknown 4%. The unknown ceramide derivative has the same chromatographic mobility when recovered and the chromatography repeated as that illustrated in Figure 3.

Water Soluble Product of Acid-Hydrolysis of the Unknown Ceramide Derivative

The ^{32}P-containing derivative obtained after hydrolysis of the unknown ceramide derivative cochromatographed with PGP in the paper chromatographic system illustrated in Figure 4 and the column chromatographic system illustrated in Figure 5. This was true with the unknown ceramide separated from other lipids chromatographically and then from CL by mild alkaline methanolysis as in Figure 1, from the unknown ceramide derivative separated from the other ceramide derivatives after mild alkaline methanolysis of the total lipid extract as in Figure 7, or from the hydrolysis product of the ceramide derivative mixture separated as in Figure 4.

The elution from a Dowex-1 column of the ^{32}P-glycerol ester recovered from the hydrolysis of the ceramide derivative is not exactly coincident with authentic PGP (Fig. 6). Perhaps there are two glycerol diphosphate esters in the lipid or one is an artifact of the hydrolysis. The column does indicate that the ester is not GPGP.

The absence of reactivity with periodate before or after mild alkaline methanolysis (Fig. 2, 3 and 7), the chromatographic mobility on paper (Fig. 5) and columns (Fig. 4,6) and the release of $^{32}P_i$ and only $^{32}P_i$ by alkaline phosphatase indicate that PGP is the ester liberated after acid hydrolysis of the minor component of the ceramide derivatives.

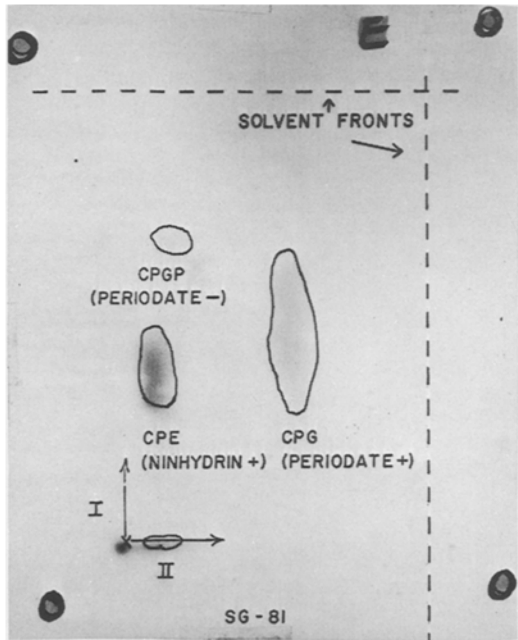

FIG. 7. Radioautogram of the chromatographic separation of the ceramides of *Bacteroides melaninogenicus* grown in the presence of ^{32}P as in Figure 2. The paper was then dipped in periodate solution followed by *o*-toluidine (4). A second paper was dipped in ninhydrin reagent (4).

DISCUSSION

The ceramide derivative described in this study contains two phosphates, an amide-linked fatty acid and one dihydrosphingosine homologue per molecule. The phosphate is probably linked to the 1 position of the LCB as in the other lipids in this organism (1). This ceramide derivative cannot be separated from cardiolipin by chromatography in two dimensions and its chromatographic mobility on silica gel-loaded paper is not affected by deacylation. No vicinyl hydroxyl groups can be detected before or after deacylation of the ceramide by mild alkaline methanolysis. After mild acid methanolysis PGP was recovered in the aqueous phase. The PGP was identified chromatographically. All the ^{32}P in $^{32}PG^{32}P$ was liberated as inorganic ^{32}P after treatment with alkaline phosphatase. It would appear that the structure of this ceramide derivative is ceramide-1-phosphoryl-1′-*sn*-glycerol-3′-phosphate. To our knowledge this lipid has not been described previously.

The ceramide derivative described in this study is homologous with phosphatidyl glycerol phosphate. A ceramide derivative homologous with phosphatidyl glycerol has also been detected in this organism (1). The presence of these two lipids suggests that the biosynthesis might also parallel the synthesis of phosphatidyl glycerol as described in *E. coli* (12). The CPG is present in about 10 times the concentration of the CPGP in *B. melaninogenicus*.

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Oxidation Products of α-Tocopherol Formed in Autoxidizing Methyl Linoleate

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ABSTRACT

The oxidation products of ^{14}C-α-tocopherol formed by heating with methyl linoleate in an air atmosphere at 60 C or 100 C were investigated. The products included a dimer, trimer and dihydroxy dimer, α-tocopherol quinone and unidentified degradation compounds. The dimer and trimer constituted the major products present after heating for 70 hr at 60 C. After 70 hr at 100 C most of the ^{14}C-α-tocopherol had been converted to degradation products; part of the ^{14}C originally present in the 5-methyl group was recovered as $^{14}CO_2$ and $^{14}CH_3OH$.

INTRODUCTION

Previous experiments in this series have shown that ^{14}C-*d*-α-tocopherol is oxidized in rat liver to α-tocopherol quinone (1), a dimer (2), trimer and small amounts of dihydroxy dimer (3). In addition, it was found that these compounds were formed by mild oxidation of α-tocopherol with alkaline $K_3Fe(CN)_6$, and it was proposed that they arise in vivo as a result of reactions with lipid free radicals or peroxides.

The present study was designed to characterize the oxidation products of α-tocopherol formed in the presence of autoxidizing methyl linoleate. The results demonstrate that, under appropriate conditions, the products are analogous to those produced in vivo and by mild inorganic oxidizing agents.

EXPERIMENTAL PROCEDURES

Experiments With Unlabeled α-Tocopherol

Three mixtures were prepared to contain the following amounts of pure *d*-α-tocopherol (α-T) and methyl linoleate (ML), respectively: 1-20 + 40 mg; 2-25 + 100 mg; 3-1 + 2 g. The samples were incubated in open beakers at 60 C in an air oven. After 70 hr, Sample 1 was dissolved in twice-distilled petroleum ether (Skellysolve F, bp 30-60 C) and applied to a 1.5 X 15 cm column of Bio-rad neutral alumina (6% H_2O added). The column was developed with mixtures of diethyl ether in petroleum ether (3).

After being heated at 60 C for 20 days, Sample 2 was extracted with 50 ml of petroleum ether and the residue from the soluble fraction was saponified in the presence of pyrogallol by standard procedures. The unsaponifiable fraction (12 mg) was fractionated on Silica Gel G thin layer chromatograms. Three solvent systems were used to resolve the oxidation products of α-T: benzene-hexane (95:5); hexane-benzene (55:45); cyclohexane-methanol (97:3). Pure standard samples of α-T, dimer, trimer, tocopherol-*p*-quinone (α-TQ) and dihydroxy dimer (DHD) were prepared by $FeCl_3$ or $K_3FE(CN)_6$ oxidation of α-T (2,3). The developed chromatograms were sprayed with 3.5% phosphomolybdic acid reagent which gives a brown color with the trimer and a blue-green color with α-T, α-TQ and the dimer.

Sample 3 was heated at 60 C for 70 hr, then extracted twice with 50 ml of 87% ethanol to remove peroxides and other polar lipids. The residue was dissolved in petroleum ether, washed three times with 87% ethanol and evaporated to dryness in vacuo under N_2. Aliquots of the residue (total weight 1.2 g) and of the combined ethanolic extracts were examined for tocopherol compounds by thin layer and column chromatography.

Experiments With ^{14}C-d-α-Tocopherol

For easier detection of the oxidation products of α-T in the autoxidized mixtures, subsequent experiments were carried out with the use of *d*-α-tocopherol-5-methyl-^{14}C (^{14}C-α-T). Twenty milligrams of ^{14}C-α-T (3.3 X 10^6 dpm)

TABLE I

R_f Values of Components of the Autoxidized α-Tocopherol-Methyl Linoleate Mixtures

	TLC (Silica Gel G)		
	Hexane-benzene		Cyclohexane-CH_3OH
Fraction	95:5	55:45	97:3
α-T[a]	.71	.47	.58
Dimer	.85	.41	.95
Trimer	.92	.91	.95
α-TQ	.11	.10	.31
ML	.80	.78	.95
MLHP	.15	.00	.16

[a]Abbreviations: α-T, α-tocopherol; α-TQ, α-tocopherol quinone; ML, methyl linoleate; MLHP, methyl linoleate hydroperoxide.

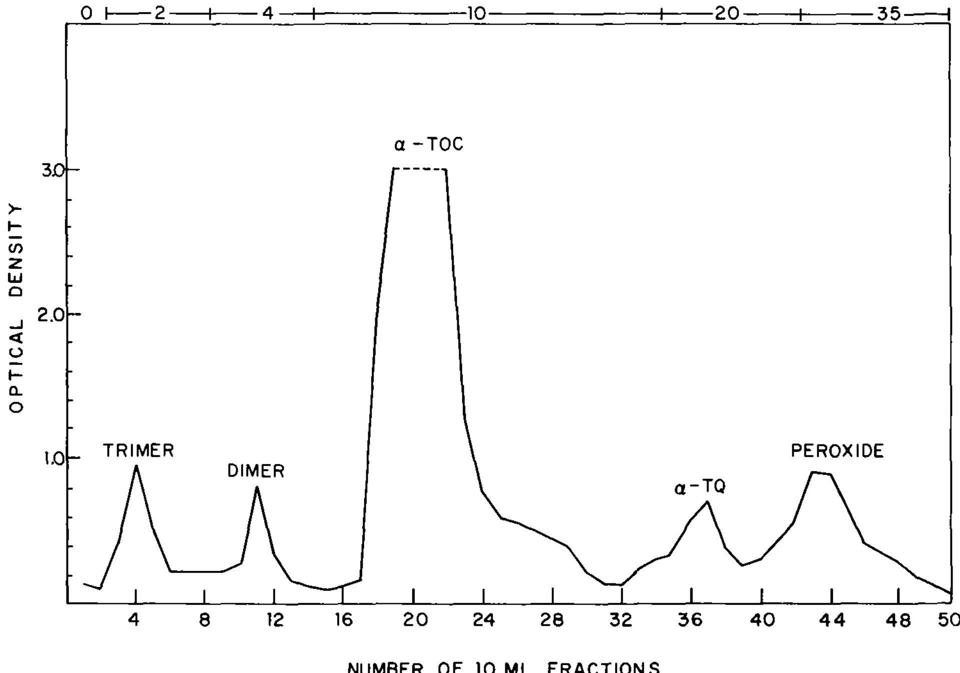

FIG. 1. Column chromatography of ML/α-T (2:1) mixture heated in an air oven at 60 C for 70 hr. See text for details.

were heated with 40 mg of ML in air at 60 C and a sample of ^{14}C-α-T alone was maintained under identical conditions as a control. After 70 hr both samples were dissolved in petroleum ether, about 3 mg of synthetic trimer, dimer, DHD and α-TQ were added as carriers and the mixture was chromatographed on an alumina column. The radioactivity in a 1 ml aliquot of each 10 ml fraction was plotted against the optical density measured at the absorption maximum of the individual carriers.

For the detection of possible volatile degradation products of α-T, another series of ^{14}C-α-T-ML mixtures was heated in round bottom flasks immersed in an oil bath. The flasks were continuously flushed with CO_2 free air and the effluent was passed through two 50-ml hyamine traps in series. Samples containing 20 mg ^{14}C-α-T (2.1 x 10^6 dpm) + 40 mg ML were incubated under the following conditions: 70 hr at 60 C in 10 ml isooctane; 70 hr at 60 C in the dry state; 70 hr at 100 C in the dry state. After incubation each sample was dissolved in petroleum ether and chromatographed on an alumina column with appropriate standards. The radioactivity in aliquots of the hyamine solutions was determined by liquid scintillation spectrometry.

The volatile ^{14}C-compound(s) detected in the above experiments were collected in a 2 liter evacuated flask (0.02 mm Hg) which was attached to the sample flask. The volatile effluent produced by heating a sample containing 20 mg ^{14}C-α-T + 40 mg ML at 60 C for 70 hr under CO_2 free air was collected every 24 hr for seven days. The collection flasks were connected to a high vacuum line fitted with dry ice-acetone and liquid nitrogen traps, and the ^{14}C-labeled material in each trap was collected in hyamine for counting. The identity of the volatile compounds collected during the first four days was established by gas chromatography (Lorenco, 20% Carbowax on Chromosorb W, 60 C, helium flow 75 ml/min).

RESULTS

Experiments With Unlabeled α-Tocopherol

The elution pattern of the autoxidation products of Sample 1, which was heated at 60 C for 70 hr, is shown in Figure 1. The position of the main compound was characteristic of α-T and its identity was confirmed by TLC in three systems (Table I), its reaction to Emmerie-Engel reagent and its UV absorption maximum at 298 mμ in isooctane. The first

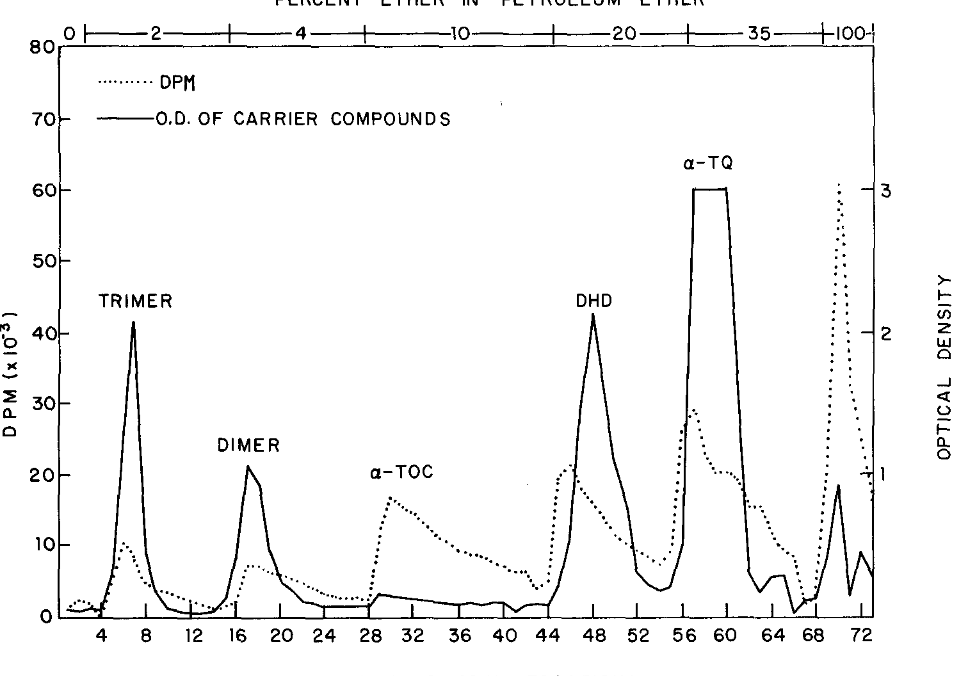

FIG. 2. ^{14}C elution pattern obtained by column chromatography of ML/^{14}C-α-T (2:1) mixture heated in an air oven at 60 C for 70 hr. DPM, disintegrations per minute. See text for details.

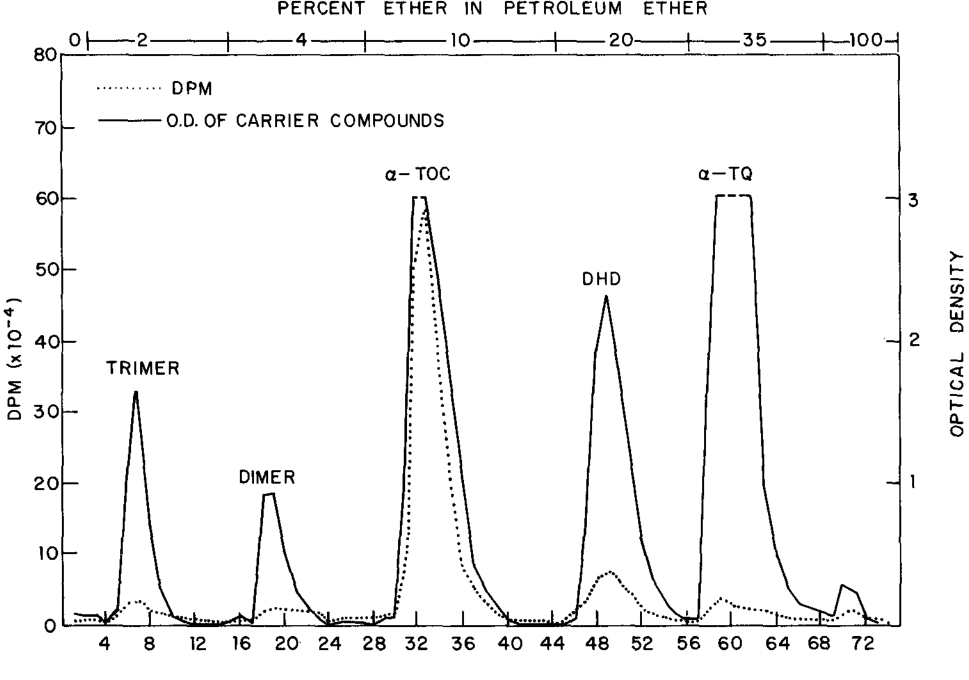

FIG. 3. ^{14}C elution pattern obtained by column chromatography of ^{14}C-α-T heated in an air oven at 60 C for 70 hr. See text for details.

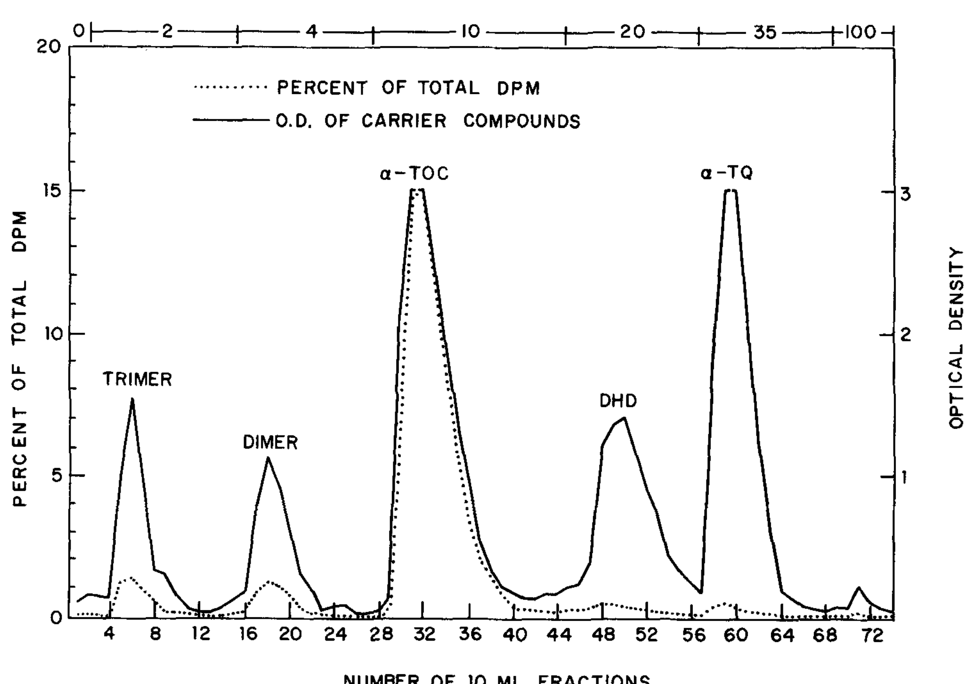

FIG. 4. Distribution of radioactivity obtained by column chromatography of ML/^{14}C-α-T (2:1) mixture heated in isooctane at 60 C for 70 hr. Details given in text.

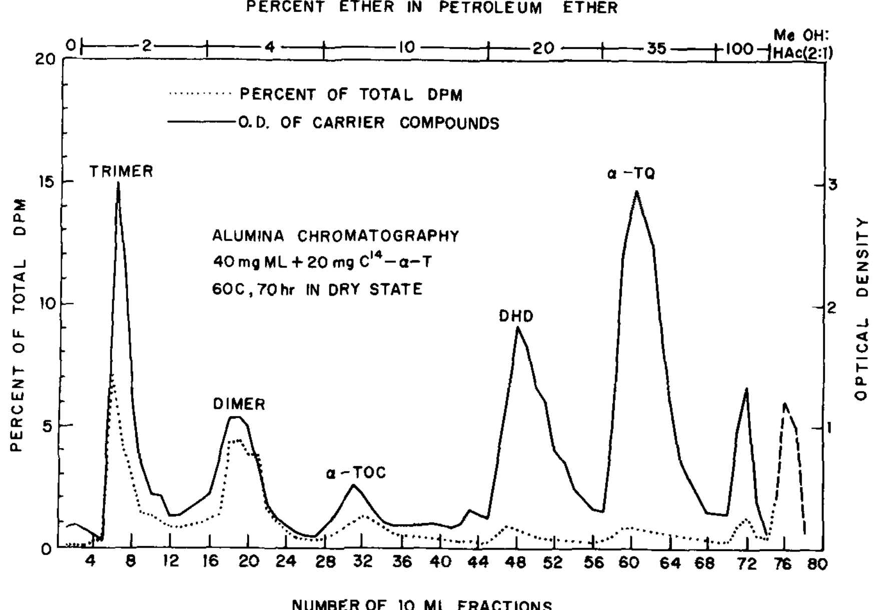

FIG. 5. Column chromatography of ML/^{14}C-α-T (2:1) mixture heated in 60 C oil bath under CO_2 free air for 70 hr. Details given in text.

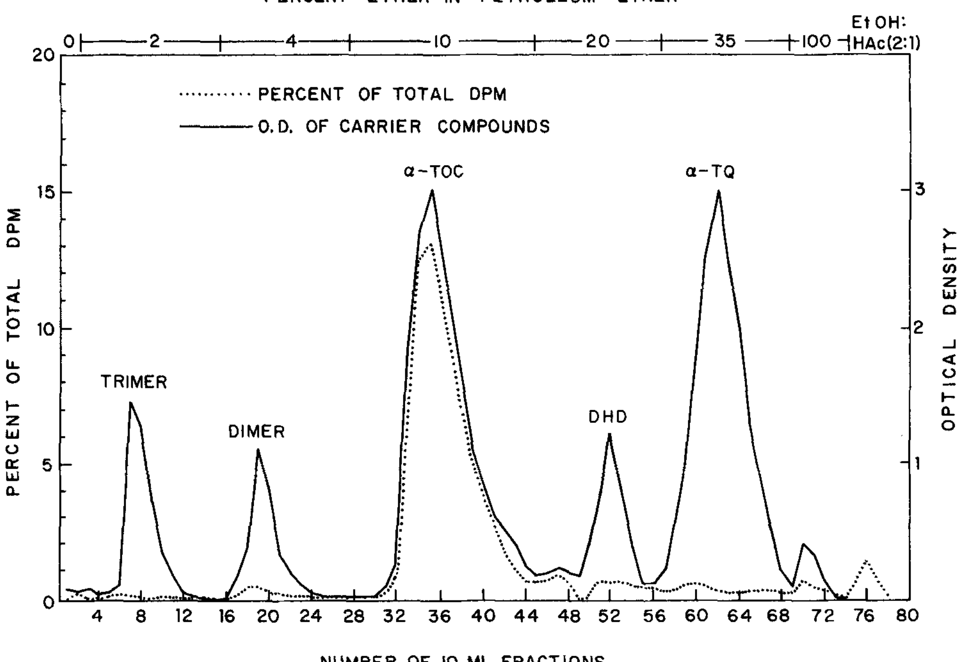

FIG. 6. Elution profile of ^{14}C-labeled compounds obtained by column chromatography of ^{14}C-α-T heated in a 60 C oil bath under CO_2 free air for 70 hr.

peak is indicative of the trimer (3) and its identity was supported by the results of TLC against a pure standard (Table I). After removal of ML by TLC in the hexane-benzene (55:45) system the UV absorption maximum of the purified compound corresponded to that of synthetic trimer (295 mμ).

The second peak was characteristic of the dimer (3) but also contained substantial amounts of unoxidized ML. After purification by TLC using benzene-hexane (95:5) and hexane-benzene (55:45), spots were observed which corresponded to synthetic dimer (2) and a UV absorption maximum corresponding to that of the dimer was observed at 300 mμ. The residue from Fraction 37 (Fig. 1) was purified by TLC using cyclohexane-methanol (97:3). A band which coincided with standard α-TQ (R_f 0.30) was eluted with ether and, after further purification in the hexane-benzene (55:45) system, was found to display absorption in the 260-270 mμ region indicative of this quinone. A spot also was present at R_f 0.16 which corresponded to standard methyl linoleate hydroperoxide. The 35% ether fraction (Fig. 1) contained primarily oxidation products of methyl linoleate which exhibited a strong band at 232 mμ attributed to conjugated double bonds.

Purification of the unsaponifiable fraction of Sample 2 revealed the presence of α-T and small amounts of dimer, trimer and α-TQ, but only 60% of the weight of the original tocopherol was recovered in the unsaponifiable fraction. In previous work (5) it was found that after heating 0.2 mg of ^{14}C-α-T with 10 ml cod liver oil for 10 days, only 7% of the radioactivity was recovered in the unsaponifiable fraction. It is apparent that under these conditions α-T is converted to polar compounds which remain in the aqueous phase when the autoxidized sample is saponified.

By use of larger amounts of α-T and ML in Sample 3 and extraction of the oxidized mixture with 87% ethanol to remove the bulk of linoleate oxidation products, preparations of pure dimer, trimer and α-TQ which exhibited authentic spectra were isolated by column and thin layer chromatography. The ethanolic extract was found to contain α-T but no significant amounts of its oxidation products.

Experiments With ^{14}C-α-Tocopherol

The effect of heating 20 mg ^{14}C-α-T with 40 mg ML at 60 C for 70 hr is reflected in the ^{14}C elution pattern shown in Figure 2. Peaks corresponding to α-tocopherol and the trimer, dimer, DHD and α-TQ standards are in evidence, in addition to an unidentified peak in the 100%

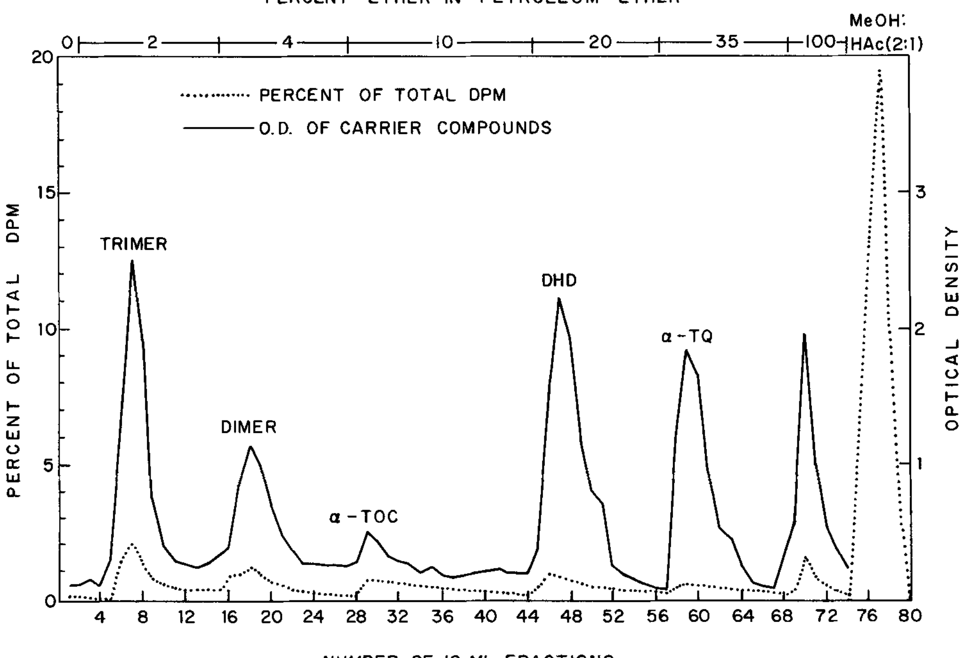

FIG. 7. Chromatography of ML/^{14}C-α-T (2:1) mixture heated in 100 C oil bath under CO_2 free air for 70 hr. See text for details.

ether fractions. The fact that the recovery of radioactivity from the column was only 22.4%, however, suggested that much of the ^{14}C-α-T has been converted to highly polar or volatile compounds. A subsequent experiment demonstrated that most of the remaining radioactivity could be recovered by eluting with methanol-acetic acid (2:1).

Figure 3 illustrates the extent of oxidation of ^{14}C-α-T in the absence of ML when heated in air at 60 C for 70 hr. While traces of oxidation products were formed, 68.0% of the recovered counts were present as unchanged ^{14}C-α-T, and 92.1% of the total ^{14}C was recovered from the column in the ether-petroleum ether fractions. In contrast, after heating ^{14}C-α-T in the presence of ML, only 4.6% of the radioactivity applied to the column was recovered in the α-T peak (Fig. 2).

Heating a 1:2 mixture of ^{14}C-α-T and ML in isooctane solution at 60 C for 70 hr resulted in little oxidation of α-T (Fig. 4). Of the total counts recovered from the column, 79.8% were present in the α-T peak. In contrast, when the solvent was removed before incubation, virtually no ^{14}C-α-T remained (Fig. 5). Instead, most of the counts were distributed among the trimer, dimer and methanol-acetic acid fractions. In the absence of ML, heating ^{14}C-α-T in the dry state caused only limited oxidation, 82.8% of the radioactivity being recovered in the α-T fractions (Fig. 6). Increasing the incubation temperature to 100 C resulted in a shift in the distribution of ^{14}C toward the methanol-acetic acid fraction, which now contained 50.0% of the counts recovered from the column (Fig. 7).

No radioactivity attributable to volatile ^{14}C compounds was generated by heating the control and isooctane samples, but 0.6% and 5.9%, respectively, of the original 5-methyl-^{14}C was recovered in hyamine when the dry samples were heated at 60 C and 100 C. This observation indicates that when α-T is subjected to severe heat in the presence of unsaturated lipids it undergoes not only oxidation of the heterocyclic ring structure but extensive decomposition of the molecule. A summary of the distribution of ^{14}C among the oxidation products of α-T formed under various conditions of incubation is presented in Table II.

Daily monitoring of the atmosphere above the dry mixtures of ^{14}C-α-T and ML revealed that significant amounts of volatile labeled material were produced which could be partitioned between the dry ice-acetone and liquid nitrogen traps. Figure 8 shows the relative amounts of volatile compounds produced by

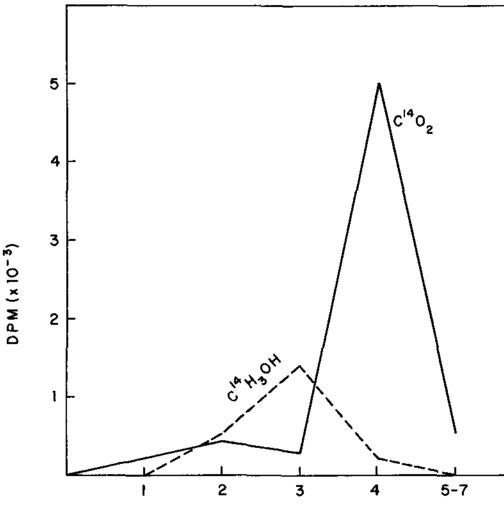

FIG. 8. $^{14}CO_2$ and $^{14}CH_3OH$ produced by heating α-T-5-methyl-^{14}C with ML at 60 C for seven days.

oxidation of the 5-methyl group of α-T when this compound was heated in a 1:2 mixture with ML for seven days at 60 C in an atmosphere of CO_2-free air. Peak recovery of activity in the liquid nitrogen trap was obtained on the fourth day, whereas the activity in the dry ice-acetone trap was maximal on the third day.

Over 99% of the volatile material collected during incubation of ^{14}C-α-T and ML at 60 C for four days was found to be H_2O. However, a chromatographic peak was observed at a retention time which coincided with that of methanol. The identity of the latter component was confirmed by passing it through a catalytic hydrogenation tube containing 1% Pd on Chromosorb W with H_2 (20 ml/min) as gas phase. When the effluent was chromatographed on a Chromosorb W column coated with 5% squalene a peak for methane was obtained. As expected, the material frozen out at liquid nitrogen temperature exhibited a single peak for CO_2 (silicic acid column, helium flow 100 ml/min, 70 C).

DISCUSSION

Although α-TQ has been generally regarded as the main product of α-T oxidation in autoxidizing lipids, it is apparent that the dimer and trimer constituted major products under some of the conditions employed in this study. For example, when the dry ^{14}C-α-T-ML mixture was heated at 60 C for 70 hr, approximately half of the radioactivity recovered from the

TABLE II

Distribution of ^{14}C-labeled Compounds Among the Oxidation Products of ^{14}C-*d*-α-Tocopherol Formed in Autoxidizing Methyl Linoleate (%)

Fraction	Autoxidation conditions			
	^{14}C-α-T:ML (1:2) in isooctane, 60 C, 70 hr	^{14}C-α-T:ML (1:2), dry, 60 C, 70 hr	^{14}C-α-T:ML (1:2), dry, 100 C, 70 hr	^{14}C-α-T alone, dry, 60 C, 70 hr
α-T[a]	72.4	7.6	6.2	80.6
Dimer	5.0	23.5	5.7	1.6
Trimer	5.0	20.1	7.9	0.7
α-TQ	2.2	6.8	4.0	3.2
DHD	3.7	4.6	5.4	4.8
100% ether	0.4	3.3	3.7	1.9
MeOH:HAc (2:1)	0	18.6	50.0	4.5
Hyamine	0	0.5	5.4	0
Total[b]	90.7	90.5	90.8	97.4

[a]Abbreviations: α-T, α-tocopherol; α-TQ, α-tocopherol quinone; DHD, dihydroxy dimer; ML, methyl linoleate.

[b]Disintegrations per minute (dpm) recovered in alumina column eluates and hyamine as a per cent of original dpm in sample.

alumina column was present in the dimer, dihydroxy dimer and trimer fractions (Table II). The amount of α-TQ formed under this condition is difficult to estimate, as the activity in the 100% ether and MeOH:HAc fractions may have arisen from decomposition of this compound, but the amount must have been less than that of the combined dimers and trimer. If ^{14}C-α-T oxidation proceeded by the same mechanism at 60 C and 100 C, it can be inferred that the increased amounts of polar compounds recovered in MeOH:HAc at the higher temperature (Table II) were formed by decomposition of the dimer and trimer which predominated at the lower temperature.

The products of tocopherol oxidation formed under relatively mild conditions of autoxidation (60 C in ML without catalyst) therefore resemble those formed by weak inorganic oxidizing agents such as ferricyanide. Somewhat stronger agents such as $FeCl_3$ and $AuCl_3$ yield a preponderance of α-TQ. Oxidation of α-tocopherol with the free radical initiator azobis-isobutyronitrile or the stable free radical *t*-butylphenyoxyl yields a dimer and no α-TQ (6,7), whereas the *p*-quinone is formed by oxidation with benzoylperoxide (8). Studies analogous to those reported here have shown that incubating α-tocopherol with pure methyl linoleate hydroperoxide leads to the formation of the dimers, trimer and *p*-quinone (Mei Chiu et al., unpublished results). The finding that the oxidation of α-tocopherol in vivo leads to a similar mixture (3) indicates that its metabolism in animals proceeds by way of reactions with lipid free radicals or peroxides.

ACKNOWLEDGMENTS

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Metabolic Fate of Epoxycholesterol in the Rat[1]

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ABSTRACT

The rate of disappearance of intubated epoxycholesterol from the rat gastrointestinal tract has been determined. The loss of this sterol is accompanied by the appearance of a sterol metabolite. This was isolated by preparative GLC and TLC and identified by mass spectrometry as 5α-cholestan-3β-5α,6-β-triol.

INTRODUCTION

Previous experiments have shown that epoxycholesterol (5α-cholestan-5α,6α-epoxy-3β-ol) is toxic when fed to rats at the 1.5% level in their diets for 90 days (1). Analysis of the lipids from the various tissues and from the serum of these animals failed to reveal the presence of any of this sterol (2). In that preliminary study only about 50% of the ingested epoxycholesterol could be accounted for in the fecal lipids when it was intubated as a 10% solution in monoolein.

In order to obtain more information about the metabolic pathway of epoxycholesterol, the rate of disappearance was determined. Also a search was made for metabolites which might help to explain the observed biological activity (1). In lieu of a more elaborate radiotracer technique, it was hoped that this simple direct approach would provide some clues to the toxicity of the sterol. The disappearance of epoxycholesterol from the gastrointestinal (GI) tract with time after intubation was accompanied by the development of increasing amounts of an unknown sterol metabolite. This sterol has been isolated by preparative TLC and GLC and identified by mass spectrometry.

EXPERIMENTAL PROCEDURES

Sprague-Dawley male rats weighing 400-600 g were used for the studies described. Doses of 10% epoxycholesterol in monoolein equivalent to 1, 1.5, 2.0 and 2.5 g/kg were administered interperitoneally (IP); the animals were then observed during 14 days for acute effects. The same 10% solution was used in the oral administration for the LD_{50} determination. The range in this case was 0.5 to 4 g/kg and the animals were retained for 21 days. The results were evaluated statistically by probit analysis.

The rate of absorption study was done with Cesarean derived Sprague-Dawley rats weighing from 400 to 600 g. The animals were intubated with 1-1.2 g of a 10% solution of epoxycholesterol in monoolein. They were subsequently killed in pairs either immediately or 1, 3 or 5 hr after intubation. The entire GI tracts were then removed, cut open lengthwise, and macerated in a Waring Blendor with 100 ml of benzene-methanol (2:1) containing 20-40 mg of 7-ketocholesterol which served as the internal standard. The resulting mass was filtered and then reextracted twice with 100 ml of benzene. The combined filtrates were washed three times with 50 ml of 10% NaCl. The organic layer was then analyzed by thin layer chromatography (TLC).

TLC was done using commercially available Silica Gel G plates (Analtech, Inc.). After 25-50 μl of extract had been spotted at the origin, the plates were developed with petroleum ether-ethyl ether-acetic acid (2:1:1). This system is similar to that used by Kritchevsky and Tepper (3). Plates were then charred by spraying with chromic sulfuric acid (10) and subsequently heating at 180 C for 30 min.

For the gas liquid chromatographic (GLC) analyses, 2 ml of extract was dried thoroughly by repeated evaporation at 50-60 C under a stream of nitrogen, diluting the residue each time with 2 ml of benzene. Then trimethylsilyl ether derivatives (TMS) were prepared by adding 2 ml of silylating mixture (4). The derivatives were allowed to stand at room temperature in a desiccator for 72 hr prior to analyses.

An F&M 810 gas chromatograph equipped with dual flame ionization detectors was used. The 6 ft x 1/8 in. o.d. stainless steel column was packed with 10% UC-W98 on 80/100 Gas Chrom Q (Applied Science Laboratories, Inc.). The column was held at 310 C; the injection port and detector at 340 and 390 C, respectively. The helium flow rate was approximately 60 ml/min.

Prior to preparative TLC, the benzene-methanol tissue extract was saponified. This was done on 3.4 g of lipids from the extraction of the 5 hr sample. It was refluxed for 2 hr with 50 ml of 0.5N NaOH in methanol. Most of the alcohol was then evaporated and, after the addition of 100 ml of water, the mixture was extracted three times with 50 ml of benzene. The extracts were combined and washed three times with 50 ml of 10% NaCl. The salt water

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[1]Presented in part at the AOCS Meeting, San Francisco, April 1969.

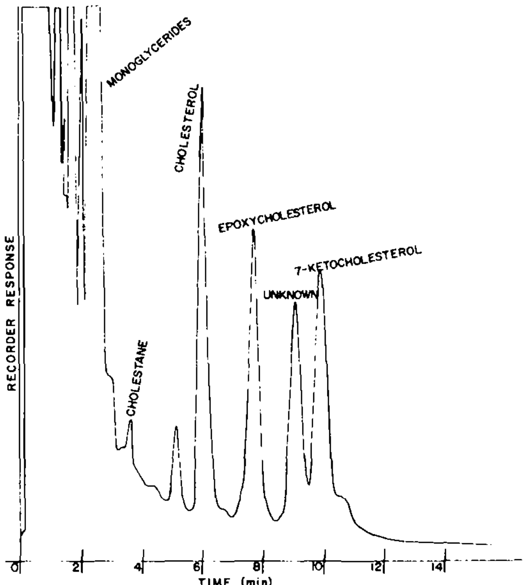

FIG. 1. GLC of sterol-TMS derivatives from rat GI tract. Five hour intubation sample.

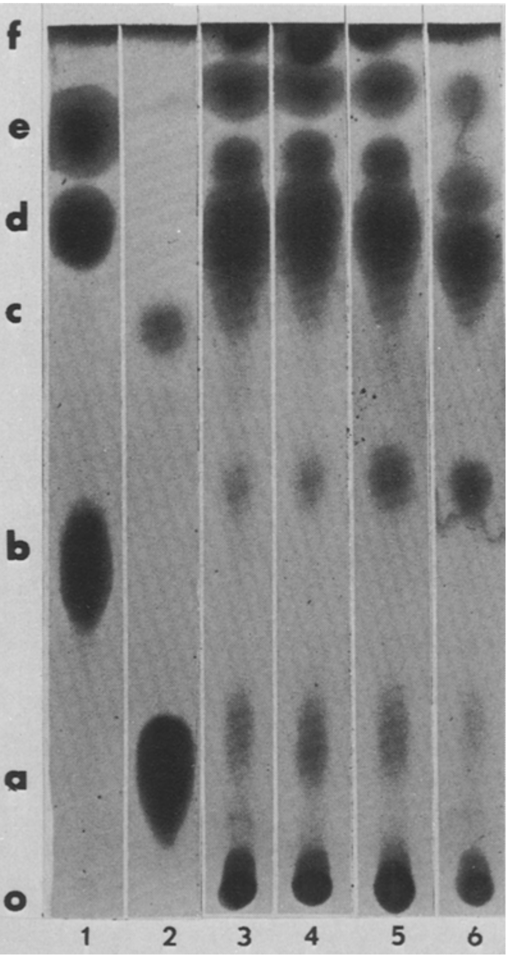

FIG. 2. TLC of lipids from rat GI tract. Solvent, petroleum ether-ethyl ether-acetic acid (2:1:1). o, origin; a, cholic acid; b, 5α-cholestan-3β,5α,6β-triol; c, monoolein; d, 7-ketocholesterol (internal standard); e, epoxycholesterol; and f, cholesterol or diglycerides. Lane 1, 50 μg each cholestane triol, 7-ketocholesterol, and epoxycholesterol; Lane 2, 100 μg cholic acid; Lanes 3-6, 50 μl each of 0 time, 1, 3 and 5 hr GI extract, respectively.

was combined with the residual soaps from the saponification and was extracted with ethyl ether for 24 hr in a liquid-liquid extractor. After concentration to about 10 ml, a portion of this ether extract was silylated. Ten to 50 μl of the silylated mixture was then injected into a Perkin Elmer Model 900 gas chromatograph equipped with a 15:1 ratio stream splitter. The conditions used were identical to those described previously. The injection was repeated about 20 times, until droplets of condensate of the desired component were visible on the sides of the collection capillary tube.

One half of the above condensate was analyzed by high resolution mass spectrometry. The instrument used was a Consolidated Electrodynamics Corp. 21-110 C equipped with a combination detector system and an electronic peak-matching accessory for precise mass measurements. The samples were introduced into the ion source by the direct introduction probe technique. The ion source was held at 210 C, and the mass spectra were recorded on plates at probe temperatures of 150-160 C. Mass measurements were made using a "Projectina" (Optical Works, Ltd., Switzerland) optical precision micrometer.

The remaining half of the sample was eluted with benzene and analyzed by GLC. The preparative TLC was done on ChromAR 500 sheet, 25 cm in length (Mallinkrodt Chemical Works). Two milliliters of the concentrate from the liquid-liquid extractor was streaked on the sheet manually and was then developed using petroleum ether-diethyl ether-acetic acid (20:80:5). After the solvent front had travelled about 20 cm, a 2 cm strip was cut and charred to locate the various bands. The band containing the unknown metabolite was extracted with methanol.

The chlorohydrin derivative of epoxycholesterol was prepared by bubbling gaseous HCl through a 3.3% solution of epoxycholesterol in chloroform for 2 hr at 30-50 C. When the solution was cooled, the product precipitated. It was recrystallized from ethyl acetate-

TABLE I

Relative Retention Time of Silylated Sterols

Sterols	Relative retention time[a]		
	10% UC-W98	10% OV-225	10% OV-17
5α-Cholestane	1.00 (3.7)	1.00 (1.4)	1.00 (2.3)
7α-Hydroxycholesterol	1.51	1.21	1.26
Cholesterol	1.68	1.71	1.65
Cholestanol	1.73	1.71	2.13
3,5-Cholestadiene-7-one	1.78		
7β-Hydroxycholesterol	1.89	1.64	
Epoxycholesterol	2.11	2.93	2.52
6β-Chlorocholestan-3β,5α-diol	2.14		
Lanosterol	2.24	2.57	2.69
β-Sitosterol	2.30	2.43	
5α-Cholestan-3β,5α-diol-6-one	2.49		
Cholestane triol[b]	2.54	2.14	2.78
6-Ketocholesterol	2.62		
7-Ketocholesterol	2.70	6.86	3.83
25-Hydroxycholesterol	2.81	2.87	2.96

[a]Based on the recorded retention time of 5α-cholestane, which is given in parenthesis (min).

[b]Di-TMS derivative.

methanol (1:1), and then dried in a vacuum at 70 C; mp 168-171 C, literature 173-174 C (8).

RESULTS AND DISCUSSION

It is known that some fatty epoxides fed to rats survive the stomach acid and are deposited in the tissue (2,5). In a past study we had observed that the oxirane moiety of epoxycholesterol is more resistant to acid cleavage than the aliphatic, internal epoxides (6). It was expected, therefore, that some epoxycholesterol would also pass through the stomach unchanged but the extent of this survival was not known until this study had been carried out. The TLC results on lipids extracted from the GI tract of rats are shown in Figure 2. These animals had been intubated with a 10% solution of epoxycholesterol in monoolein. The animals were then killed at intervals, the first immediately after intubation and the others after 1, 3 and 5 hr. The TLC plate shows indications of a decreasing level of epoxycholesterol in the tissue with time after intubation. Also a new material having an R_f equal to that of 5α-cholestan-3β,5α,6β-triol appears (Fig. 2, b); it increases in amount with time after intubation.

In order to quantify these results, some preliminary gas liquid chromatographs were first obtained on mixtures of known silylated sterols. Table I gives relative retention times for such a series on three different substrates. Also used but not shown were the substrates SE-30 and OV-1. These gave results similar to OV-17. Previously used QF-1 (6) was abandoned because of its poor stability at 230 C. The sterols of interest can be chromatographed without silylation (11) but this results in loss of peak symmetry and partial adsorption or decomposition, or both, which renders quantitation difficult. Only in the case of 10% UC-W98 was it possible to successfully separate epoxycholesterol from cholestane triol.

TABLE II

Disappearance of Epoxycholesterol From the Rat Gastrointestinal Tract

Epoxycholesterol intubated, mg	Time, hr	Cholesterol, mg	Epoxycholesterol recovery, %	Unknown mg
105	0	43	77	Trace
121	0	50	75	Trace
129	1	52	59	3
120	1	47	68	7
124	3	56	52	24
110	3	39	54	28
100	5	39	45	44
111	5	51	55	30

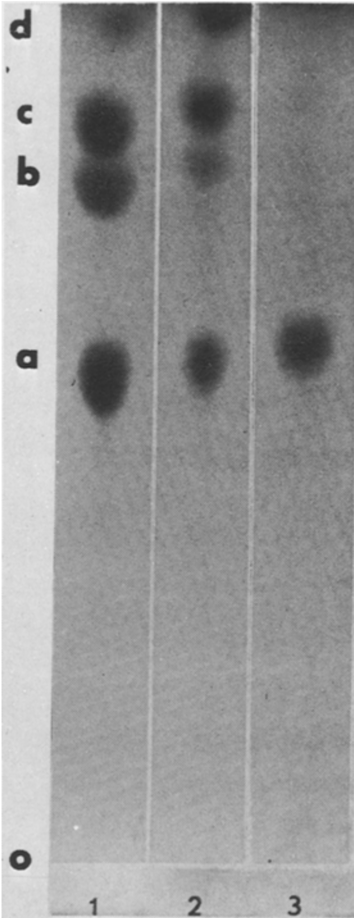

FIG. 3. TLC of unknown metabolite. Solvent, same as in Figure 2. a, 5α-cholestan-3β,5α,6β-triol; b, 7-ketocholesterol; c, epoxycholesterol; and d, cholesterol. Lane 1, mixture of a-d; Lane 2, rat GI tract unsaponifiable concentrate; and Lane 3, triol region from preparative TLC plate (in methanol).

Subsequent separations with this substrate were then made on the sterol mixture from the 5 hr sample of rat GI tract. The results achieved are illustrated in Figure 1. Note that epoxycholesterol is separated completely from the unknown peak which falls between it and the internal standard, 7-ketocholesterol. Small amounts of cholestane and lanosterol (12) were also found in the extracts from all of the GI tracts. Peak areas were measured by triangulation. The results shown in Table II clearly indicate a gradual loss of epoxycholesterol over the 5 hr period. The recovery immediately after intubation of only 75-77% cannot be fully explained. Although low, this value is reproducible since in a second experiment involving five rats the average recovery was 74% with a mean deviation of 5% and a range of 68-80%. Part of this loss is no doubt due to the emulsifying action of monoolein which prevents epoxycholesterol from being completely extracted by the organic phase. Intubation of the internal standard along with the epoxycholesterol might have solved this problem. Grundy et al. (9) took this route and used β-sitosterol as an internal standard to correct for cholesterol losses in sterol balance studies.

This sterol could not be used here because it could not be resolved from epoxycholesterol on any of the GC columns tried. On the other hand, 7-ketocholesterol could not be intubated because its fate in the rat GI tract is unknown, therefore, it was added externally, immediately prior to solvent extraction. In spite of this low recovery, it is apparent that the amount of epoxide is decreasing with time and that this is accompanied by an increase in the size of the unknown sterol peak.

The intraperitoneal injections demonstrated that up to 2.5 g/kg no lethal or gross toxic effects are produced by epoxycholesterol. By oral administration, on the other hand, toxicity of this sterol was shown by an LD_{50} value of 1.82 g/kg. The results of these studies suggested that a metabolic intermediate (or intermediates) is the toxicant. This fact made the isolation and identification of the unknown metabolite all the more pertinent.

The isolation step was complicated by the polar character of the unknown. Saponification was used to effect an initial separation from monoolein and other tissue glycerides. Extraction of the residue from saponification with benzene yielded large amounts of cholesterol, epoxycholesterol and the added 7-ketocholesterol, but only trace amounts of the metabolite. Also present in the benzene layer in substantial amounts was 3,5-cholestadien-7-one which results from the alkaline dehydration of 7-ketocholesterol (7). It was subsequently found that the bulk of the metabolite remained with the soap and could be salted out and extracted with diethyl ether.

Various attempts to purify the crude sterol metabolite contained in this ether extract by preparative TLC were unsuccessful. These failed because the unknown, as well as the known cholestane triol, could not be visualized by the usual nondestructive methods; e.g., with iodine, dichlorofluorescein, or an ultraviolet phosphor. As was mentioned in the previous section, ChromAR sheet can easily be cut into narrow strips which can be charred for location of the various bands. The unknown band was subsequently cut from the sheet and extracted with methanol. The white residue from the methanol

TABLE III

Mass Spectral Data

Sample	Molecular ion (M^+)	Principal ions
5α-Cholestan-3β,5α,6β-triol	420	402[a]; 418,403,385, 384,369,348,331,271, 262,247,244,229
Rat metabolite isolated by TLC	420.3588[b]	402[a];403,385,384, 369,348,331,271,262, 248,247,244,229
Rat metabolite (TMS) isolated by by GLC	564.4450[c]	546.4315[a,d],531,475, 456,403,385

[a]Base peak (M^+-H_2O).
[b]Calculated for $C_{27}H_{48})_3$ = 420.3603.
[c]Calculated for $C_{27}H_{46}O_3Si_2$ $(CH_3)_6$ = 564.4390.
[d]Calculated for $C_{27}H_{44}O_2Si_2$ $(CH_3)_6$ = 546.4284.

melted at 218-220 C. On the same Kofler hot stage, the commercial triol (Steraloids, Inc.) had a melting point of 221-224 C. Figure 3 illustrates the effectiveness of ChromAR preparative TLC as a means of purifying the unknown metabolite.

To rule out the possibility that the metabolite might be a chlorohydrin derived by ring opening with HCl in the rat stomach, a sample of this material was prepared. By GLC the material (both before and after silylation) was found to have a retention time almost identical to that of epoxycholesterol on all columns used. TLC showed it to have an R_f equal to that of 7-ketocholesterol. From this data, the possibility that the metabolite formed was the chlorohydrin was ruled out.

On the assumption that the unknown metabolite was 5-α-cholestan-3β,5α,6β-triol some high resolution mass spectrometry was carried out. A sample of the known triol, and both the TLC and GLC preparations, were analyzed. As shown in Table III when the TLC preparation was analyzed, it showed a molecular ion (M^+) of 420; the loss of two molecules of water; i.e., M^+-18 and M^+-36 gave peaks equivalent to 402 and 384 molecular weights. These fragments were also observed in the known triol. The prominent peaks from the preparative GLC (silylated) sample include M^+-72 and M^+144 indicating that the triol forms a di-silyl derivative and that the hindered 5-α-OH group did not react under the conditions used.

The mass spectral data given in Table III unquestionably identifies the metabolite as a cholestane triol. Consideration of its precursor, and its dehydration and silylation behavior, make it almost certain that the sterol is 5α-cholestan-3β,5α,6β-triol. An attempt to further confirm this identification by NMR was unsuccessful because of the limited amount of sample available.

The fact that epoxycholesterol is converted mainly to cholestane triol in the rat GI tract has been established. The relationship of this conversion to the observed toxicity of epoxycholesterol is not clear at the present time.

The work now under way is designed to clarify this point and should also help in the interpretation of the results from the chronic toxicity study now in progress.

ACKNOWLEDGMENTS

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Relation of Cholanate Structure to Inhibition of ^{14}C-26-Cholesterol Oxidation by Mitochondria from Rat Liver

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ABSTRACT

Eight bile acids and 20 of their derivatives, of known purity, were compared for inhibitory effect upon oxidation of cholesterol in vitro by rat liver mitochondria. All inhibited oxidation of ^{14}C-26-cholesterol; none inhibited oxidation of ^{14}C-1-octanoate. The α-monohydroxy- and α-dihydroxycholanic acids were more potent inhibitors than α-trihydroxycholanic acid and trioxocholanic acid. Most were more potent than their derivatives. In general, the relative inhibitory potency of derivatives was: methyl esters > free alcohols > glycine conjugates > taurine conjugates. Mitochondria from rats subjected to 4 hr alternate light-dark periods were less susceptible to the inhibitory action of cholanate conjugates than were mitochondria from rats under normal day-night conditions. These experiments with compounds of known purity show that the hydroxylation pattern is the determining factor in cholanate inhibition of the cholesterol oxidation.

INTRODUCTION

While the ability of a commercial grade of bile salts to inhibit oxidation of cholesterol by hepatic mitochondria was observed some time ago (1,2), systematic study of this inhibitory action began only recently. Lee and Whitehouse (3) compared taurine conjugates of a number of bile acids at a single level (0.5 mM) and were able to show marked differences in inhibitory power. Dean and Whitehouse (4) used an improved parameter (concentration for 50% inhibition) in comparing inhibitory action of selected cholanates upon the oxidation of three cholesterol derivatives. However, in none of these studies was the purity of the bile acids or their derivatives stated. Pope et al. (5) and Dietschy (6) have observed that the majority of physiological effects of commercial bile salts are not caused by the bile salts themselves but rather by their impurities. Since the kinds and amounts of cholanates which supply the enterohepatic circulation may influence the formation (7-10) as well as the degradation (4) of cholesterol, further systematic comparison of compounds of known purity for their inhibitory action on cholesterol oxidation appears timely.

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MATERIALS AND METHODS

Purity of Bile Acids and Their Derivatives

All of the bile acids and their derivatives were examined by thin layer chromatography (TLC) and gas liquid chromatography (GLC) analysis and in most cases were subjected to prior purification. The observed percentage impurity is shown parenthetically.

Cholic acid, from Nutritional Biochemicals, Inc., was recrystallized from 70% ethanol several times (impurities < 0.5%).

Deoxycholic acid sodium salt, Calbiochem, was purified by washing 200 ml of the 5% aqueous solution twice with 100 ml petroleum ether in a separator. The aqueous solution was acidified with 25 ml of 1 N HCl and extracted with chloroform. The chloroform extract was washed twice with 100 ml of saturated NaCl, dried over anhydrous sodium sulfate and evaporated to dryness. The residue was dissolved in hot 70% ethanol and recrystallized at 4 C overnight (chenodeoxycholic acid and trace of an unidentified substance, total 2%).

Lithocholic acid sodium salt, Calbiochem, was partially purified as described for deoxycholic acid and further purified by preparative TLC on Silica Gel G with chloroform-acetone-acetic acid (80:20:1) before recrystallization (impurities < 0.5%).

The following commercial products were tested for purity and were used without further purification: chenodeoxycholic acid, Baker Chemical Co. (deoxycholic acid, 2%); 3β-cholanic acid, Mann Research Labs. (no impurities detectable); 3α,6α-cholanic acid, Mann (chenodeoxycholic acid, 2.5%); 3α,7β-cholanic acid, Mann (chenodeoxycholic acid, 2.5%); 3,7,12-trioxocholanic acid, Calbiochem, (cholic acid, 1.5%). Glycodehydrocholic acid sodium salt, Calbiochem (glycocholate + dehydrocholate, 5%); taurodehydrocholic acid sodium salt, Calbiochem (taurocholate + dehydrocholate, 5%).

The taurine and glycine conjugates of lithocholic, deoxycholic, chenodeoxycholic and cholic acids were prepared by treatment with

TABLE I

Effectiveness of Hydroxy Cholanic Acids and Their Derivatives as Inhibitors of Hepatic Mitochondrial Oxidation of ^{14}C-26-Cholesterol[a]

	Concentration (μM) for 50% inhibition				
5β-Cholanate	Free acid	Methyl ester	Glycine conjugate	Taurine conjugate	Free alcohol
3α	10	10	36	32	---
3β	14	27	---	---	---
3α,6α	25	28	---	---	---
3α,7α	9	12	83	170	56
3α,12α	8	16	43	93	32
3α,7β	12	12	---	---	---
3α,7α,12α	33	49	310	320	34
3,7,12-trioxo	68	70	---	640	---

[a]Mitochondrial preparations from livers of male rats were incubated with ^{14}C-26-cholesterol and cofactors to which four or more levels (1 to 1000 μM) of the cholanate were added in Tween 80 suspension. After 4 hr, $^{14}CO_2$ was collected for scintillation counting and the 50% inhibition point was obtained from a semilog plot (4). Before use, the cholanates were purified by crystallization and TLC.

mixed carboxylic acid anhydrides (11). After crystallization the conjugates were separated from unreacted bile acids by preparative TLC on Silica Gel G. The developing solvent for the taurine conjugates was butanol-acetic acid-water (10:1:1) and for the glycine conjugates was chloroform-acetone-acetic acid (70:20:10). The conjugate band was scraped from the TLC plate, eluted with methanol-water (10:2) and recrystallized from ethanol at 4 C (impurities < 2%).

To prepare methyl esters the bile acids were reacted with diazomethane (12). Completeness of methylation was verified by TLC on Silica Gel G with chloroform-acetone (80:20) for methyl lithocholate, chenodeoxycholate and deoxycholate, and with chloroform-acetone-acetic acid (70:20:5) for methyl cholate (impurities < 1%).

The methyl cholanates were reduced to alcohols by lithium aluminum hydride in ether (13). After acidification the reaction mixture was washed three times with saturated NaCl, dried over Na_2SO_4, and the cholanyl alcohols were separated from unreacted methyl esters by preparative TLC on Silica Gel G. Developing solvents were chloroform-acetone (80:10) for lithocholate, chloroform-acetone (80:20) for chenodeoxycholate and deoxycholate and chloroform-acetone-acetic acid (70:30:5) for cholate.

Acetylation of the hydroxyl groups in these compounds produced derivatives which were so insoluble as to prevent their comparison as inhibitory substances.

Preparation of Hepatic Mitochondria

Mitochondrial preparations were obtained from the livers of male Wistar rats. The animals (200-300 g) were killed by cervical dislocation, the livers quickly excised and placed in ice cold 0.25 M sucrose. All subsequent operations were completed at 4 C. The livers were forced through a Harvard tissue press into three volumes of 0.25 M sucrose and the resulting suspension was homogenized in a plain glass test tube by 3-4 strokes with a loose fitting motor-driven Teflon pestle. The homogenate was centrifuged 12 min at 750 X *g* and the resulting supernatant fraction for 12 min at 8500 X *g*. The supernatant fraction was then discarded and the mitochondrial fraction, resuspended in fresh 0.25 M sucrose, was again centrifuged 12 min at 8500 X *g*. The washed mitochondria were then resuspended in 0.25 M sucrose and protein content of the suspension was determined by the method of Lowry et al. (13).

Procedure for Assay for Inhibitory Action

The following commercial substances were used in the mitochondrial incubations without purification: ^{14}C-26-cholesterol, 18.6 mC/mmole; sodium octanoate, 13.0 mC/mmole; adenosine monophosphate (AMP); adenosine triphosphate (ATP); nicotinamide adenine dinucleotide (NAD); and glutathione (GSH), all from Calbiochem, Los Angeles; sodium lauryl sulfate (Fisher Scientific Company, Fair Lawn, N.J.); Triton X-100 (Arbor-Huron Laboratories, Ann Arbor, Mich.); and Tween 80 (Atlas Chemical Co., Wilmington, Del.). Radioactivity was estimated by liquid scintillation counting (Packard Tricarb) with a toluene scintillation fluid (Omnifluor, New England Nuclear Corp., Boston, 4 g/l of toluene). The radioactivity data are subject to a ± 5% counting error.

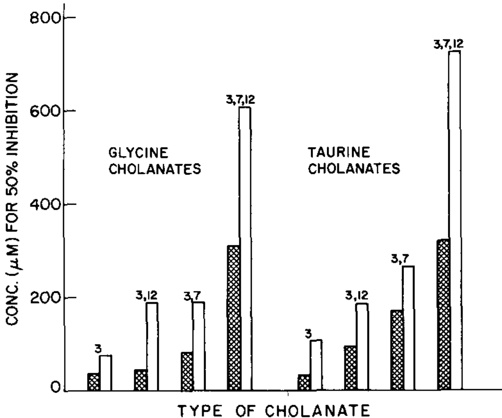

FIG. 1. Effect of length of alternate light-dark periods on susceptibility of mitochondrial oxidation to inhibition by different conjugated cholanates. Hepatic mitochondria were prepared from rats maintained eight days on alternate light-dark periods of 12 hr each, then killed after 3 hr of light (cross-hatched bars) and rats maintained eight days on alternate light-dark periods of 4 hr each, then killed after 1 hr of light (clear bars). Mitochondrial preparations were incubated with ^{14}C-26-cholesterol and four levels of the bile salt as described in Table I. Each bar represents the mean value from two or more incubations with different rat preparations. Numbers represent positions of α-hydroxyl groups.

Incubation mixtures contained 0.5 ml of the mitochondrial fraction (equivalent to 1.5-2.0 g of liver), 0.8 ml of 0.25 M Tris buffer (pH 8.5), 0.1 ml of ^{14}C-26-cholesterol (0.05 μC in 0.1% Tween 80), 8.0 mg ATP, 2.0 mg AMP, 1.5 mg NAD, 5.0 mg GSH, 0.2 mg sodium citrate, 3.0 mg $MgCl_2 \cdot 6\ H_2O$, the bile acid or its derivative in 0.1 ml of 0.1% Tween 80, and 1.0 ml of 0.125 M sucrose. After addition of the mitochondrial fraction, a shell vial containing a strip of filter paper was inserted, the flask was sealed and shaken for 4 hr at 37 C. Incubations were stopped by injection of 0.5 ml of 6 N sulfuric acid into the medium. Flasks were shaken an additional hour to collect the carbon dioxide in 0.5 ml Hyamine hydroxide which had been injected into the shell vial. On removal of the shell vial, the outer surface was rinsed with alcohol and wiped dry, the vial inverted into a flask which contained scintillation fluid and radioactivity was then determined.

RESULTS AND DISCUSSION

Inhibition of Oxidation of ^{14}C-26-Cholesterol

The mono- and di-α-hydroxy cholanic acids were the most potent inhibitors of mitochondrial oxidation of ^{14}C-26-cholesterol, and there was little difference between them except for the 3α,6α-compound which was clearly less active and more nearly resembled the 3β-compound in activity (Table I). The trihydroxy- and the trioxo-compounds were also distinctly less inhibitory. The methyl esters, in most cases, were slightly less inhibitory than the free acids. However, formation of the glycine derivative reduced inhibitory power much more than formation of the methyl ester. The free alcohol of the dihydroxy compound was also substantially less inhibitory than the free acid.

Oxidation of ^{14}C-1-Octanoate

To examine the specificity of inhibition of cholesterol oxidation by cholanates parallel studies were performed with ^{14}C-1-octanoate. The octanoate (ca 0.01 μmole, containing 2 x 10^5 counts per minute) in 0.1% Tween 80 was added to the incubation flask in place of the ^{14}C-26-cholesterol and incubated for 2 hr (ca 40-50% label collected as $^{14}CO_2$). Four hydroxycholanic acids (3α; 3α,7α; 3α,12α; and 3α,7α,12α) were tested as the free acid, the methyl ester, the glycine and taurine conjugates and the free alcohol. In the 20 comparisons of octanoate oxidation with and without cholanate addition a range of 93% to 104% as much oxidation occurred in the presence of the cholanate as in its absence. Inhibition of octanoate oxidation also was not detectable at a 10-fold higher level (1.0 mM). It was evident, therefore, that these hydroxy cholanates and their derivatives exerted no measurable inhibitory effect upon octanoate oxidation by hepatic mitochondrial preparations. The inhibitory effect of these compounds upon cholesterol oxidation, therefore, can be considered a specific one.

Influence of Light and Dark Periods on Inhibition

When animals were kept on alternating 12 hr light-dark periods and the livers removed at the third hour in a light period, 50% inhibition was produced with lower concentrations of cholanate conjugates than when animals were kept on alternating 4 hr light-dark periods and livers removed in the first hour of a light period (Fig. 1). These different light-dark periods did not alter measurably the inhibitory effect of free cholanic acids upon the mitochondrial oxidation of cholesterol. Thus, it appears that a diurnal variation exists in the cholanic acid levels of the liver and enterohepatic circulation. Perhaps hepatic mitochondria from animals in the postabsorptive state have a greater sensitivity to conjugated cholanates than those in the absorptive state. This could reflect a reduced level of endogenous cholanic acid conjugates during the absorptive period.

The studies of Lee and Whitehouse (3) suggested an inverse relationship between degree of hydroxylation and inhibitory power of cholanic acids upon mitochondrial oxidation of cholesterol. This relationship holds for the purified acids which we have tested. In addition, it holds for the methyl esters and the free alcohols and, in a geometric progression, for the glycine conjugates as well as for the taurine conjugates (Fig. 1). One concludes from these data that the hydroxylation pattern is the determining factor in inhibition of cholesterol oxidation. It appears that rat liver synthesizes those bile salts which have the lowest inhibitory effect upon oxidation of the cholesterol side chain. Whether this extends to all aspects of cholesterol oxidation will be of interest to observe.

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The Effect of Dietary Sterculic Acid on the Hepatic Lipids of Rainbow Trout[1,2]

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ABSTRACT

Groups of young rainbow trout (5 g) were fed a basal diet containing 9% salmon oil and 1% tristearin with 0, 100 or 200 ppm methyl sterculate. Liver lipids were separated into polar and non-polar fractions and the fatty acids quantitatively analyzed. Significant elevations of the stearic-oleic and the palmitic-palmitoleic ratios were found in liver fatty acid composition 10 days after the feeding trial began. Liver triglycerides of fish fed methyl sterculate for 87 days contained only 2-3% docosahexenoic acid as compared to 10.69% in control trout, suggesting an effect on the biosynthesis of long chain polyunsaturates. Dietary cyclopropenoid fatty acids (CPFA) suppressed growth rate during the first part of the 200 day feeding trial. After 90 days no differences in the rate of weight gain were observed between the control and CPFA groups. A seven day feeding trial with 0, 5, 20, 50 and 100 ppm CPFA resulted in a maximum change in the stearic-oleic ratio at 50 and 100 ppm levels. All levels of CPFA increased this ratio and caused marked alterations in the cellular morphology of the liver.

INTRODUCTION

Cyclopropenoid fatty acids (CPFA) occur naturally in the triglycerides of plants in the order Malvales (1). The most nutritionally and economically important of these lipids is cottonseed oil, which contains small quantities of both the 18 carbon cyclopropenoid malvalic acid, and the 19 carbon sterculic acid. These unique fatty acids have been shown to induce a variety of physiological disorders when fed to animals. Phelps et al. (2) have recently reviewed these disorders, which include abnormalities in the reproductive process and alterations of lipid metabolism. The latter effect has been studied by Reiser and Raju (3,4) and Johnson et al. (5), who determined that the stearic fatty acyl desaturase enzyme system was inhibited by sterculic acid. This explained the high levels of stearic acid and correspondingly lower levels of oleic acid generally observed in poultry and rats fed cyclopropenoids. This shift in fatty acid composition recently has been observed in rainbow trout (6) where it was found most pronounced in the liver lipids. Sinnhuber et al. (7,8) recently reported that sterculic and malvalic acids greatly increased the incidence and growth rate of aflatoxin-induced hepatoma in rainbow trout.

Many investigations have been made of the effect of CPFA in certain warm-blooded species but very little work has been done with fish. In a study designed to determine the influence of dietary fatty acids and water temperature on

TABLE I

Per Cent Composition of Fatty Acids in Trout Liver Lipids After Feeding CPFA for 10 Days[a]

	CPFA in diet (ppm)		
Fatty acid	0	100	200
Triglycerides			
14:0	6.7	3.5	4.6
16:0	15.4	16.8	16.3
16:1	9.6	6.0	5.9
18:0	4.9	20.7	23.3
18:1	39.8	36.9	35.6
18:2ω6	2.5	1.5	1.5
20:1	4.9	5.2	5.0
18:4ω3	1.4	1.2	---
22:1	1.6	1.8	1.1
20:5ω3	1.3	---	---
22:5ω6	1.1	---	---
22:6ω3	4.6	3.7	4.8
Phospholipids			
14:0	2.6	2.5	3.6
16:0	20.9	21.6	19.9
16:1	3.3	2.4	2.5
18:0	4.5	12.3	13.3
18:1	14.8	12.3	12.8
18:2ω6	1.3	0.8	1.5
20:1	2.0	1.8	2.1
18:4ω	1.0	---	---
20:4ω6	1.9	1.6	1.5
20:5ω3	3.5	2.6	2.5
22:5ω6	2.4	1.3	2.4
22:6ω3	36.8	39.2	32.7

[a]Trace components omitted for clarity. In all cases at least 94% of the total composition is shown.

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[1]Presented in part at the annual meeting of the Federation of American Societies for Experimental Biology, Atlantic City, New Jersey, April, 1968.

[2]Technical Paper No. 2565, Oregon Agricultural Experimental Station.

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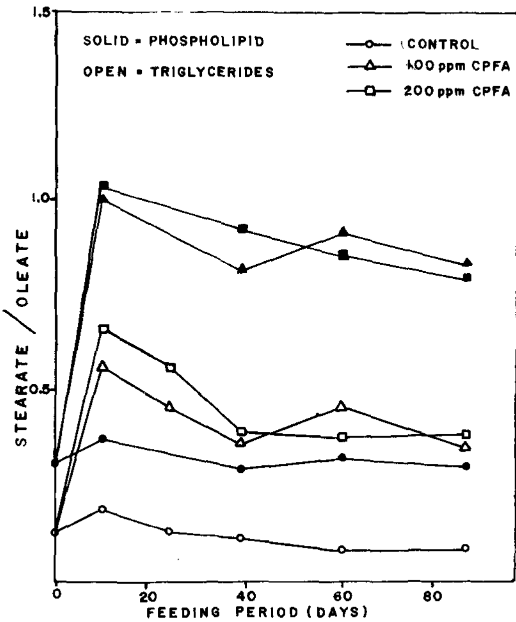


FIG. 1. Ratio of stearic to oleic acid in the liver lipids of trout fed methyl sterculate.

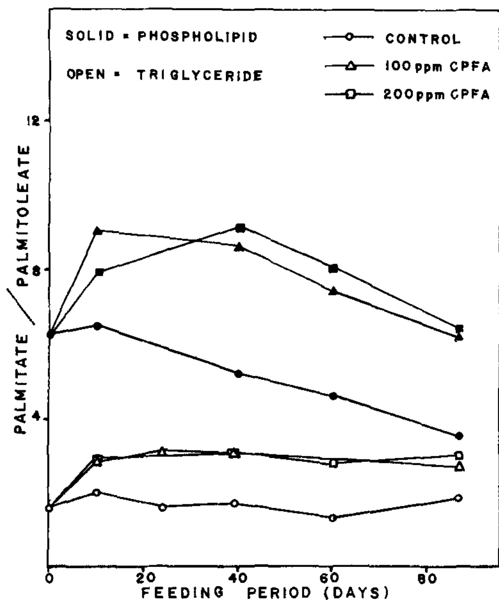


FIG. 2. Ratio of palmitic to palmitoleic acid in liver lipids of trout fed methyl sterculate.

the fatty acid composition of total body fat, Reiser et al. (9) noted that the body lipids from one fundulus (*Fundulus grandis*) fed a 30% cottonseed oil diet contained a high percentage of stearic acid. In contrast, seven mullet (*Mugil cephalis*) did not accumulate abnormal amounts of stearic acid in the body fat when fed the cottonseed oil. The cyclopropene content of the oil was not reported. Previous work at this laboratory with crude CPFA-containing oils indicated trout were very sensitive to dietary cyclopropenoids (6-8). This prompted the present study to determine the effect of purified sterculic acid methyl esters on the hepatic lipids of rainbow trout.

EXPERIMENTAL PROCEDURES

Experimental Animals

All fish used in this study were Mt. Shasta strain rainbow trout (*Salmo gairdneri*) that were spawned and reared at our laboratory. The fish were held in fiberglass tanks at a constant water temperature of 11.4 C and were fed the semi-purified diet described by Lee et al. (10) prior to the feeding trials. The only change being that 10% salmon oil was used instead of 5% corn oil and 5% salmon oil.

Feeding Studies

The stearic acid content of all the experimental diets, including the control, was increased to a level above that which is normally found in trout tissue by adding 1% tristearin to the dietary lipid. Methyl sterculate was prepared by the method described by Kircher (11). Lots of 150 fish each (3 months old 5 g fingerlings) were placed on diets containing 0, 100 or 200 ppm CPFA. This feeding trial was carried out for 200 days in order to determine the long term effects of dietary cyclopropenes. Fifty fish from the 200 ppm CPFA diet were placed on the control diet after 100 days in order to study their pattern of recovery. At various intervals samples of 10 fish were removed from each test group and their livers removed for lipid analysis.

In a second study, groups of 15 fish each (8 months old, 40-50 g) were fed 0, 5, 20, 50 and 100 ppm methyl sterculate for a seven-day period. The fish were then killed and the lipid composition of the livers determined. Histological examinations of the livers included hematoxylin and eosin stains as well as stains to demonstrate fat and glycogen content.

Lipid Analysis

Livers from the sampled fish were pooled and extracted by the method of Folch et al. (12) and the resulting oil was fractionated into polar and nonpolar lipids using silicic acid column chromatography. A glass column (14 mm i.d.) was packed with 5 g of washed silicic acid (100-200 mesh) mixed with 2.5 g Celite

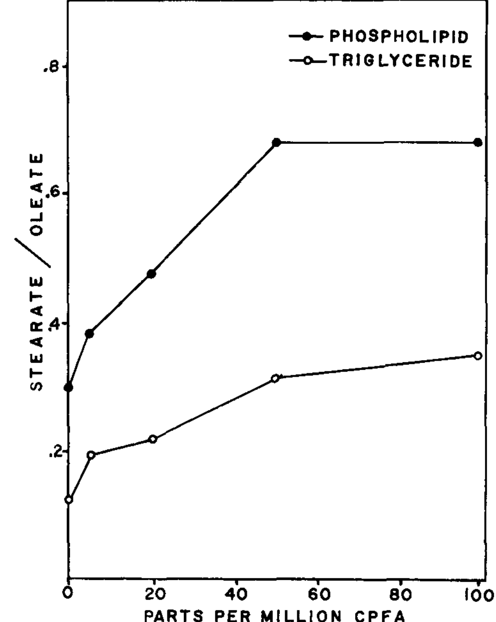


FIG. 3. Ratio of stearic to oleic acid in the liver lipids of trout fed various amounts of CPFA for seven days.

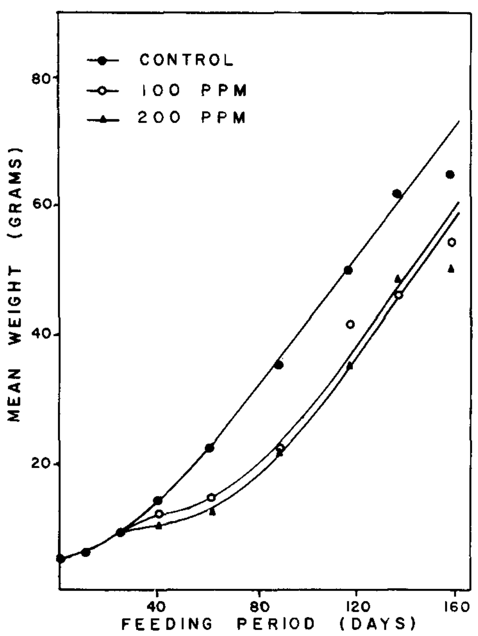


FIG. 4. Growth of trout fed methyl sterculate.

and charged with approximately 100 mg of oil. The nonpolar fraction which contained primarily triglycerides was eluted with 150 ml of chloroform and the remaining polar fraction which contained primarily phospholipids was eluted with 150 ml of methanol. Each lipid fraction was then converted to methyl esters using 14% boron trifluoride in methanol according to the procedure of Morrison and Smith (13).

The resulting fatty esters were analyzed using an Aerograph Model 600-B gas chromatograph equipped with a hydrogen flame detector. A 12 ft by 1/8 in. o.d. aluminum column packed with 10% DEGS on 120-140 mesh Celite was used. The Celite support was treated to remove impurities according to the procedure of Farquhar et al. (14). Column temperature was held at 195 C and the flow rate of nitrogen was maintained at 20 ml/min. Injection port and detector temperature were 245 C.

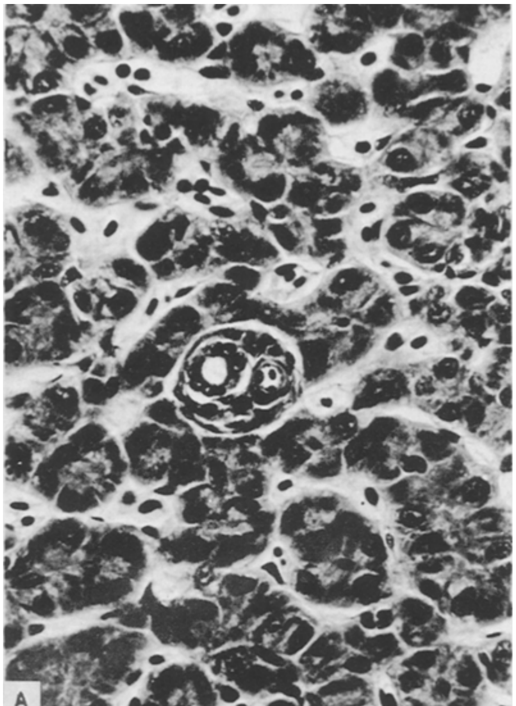
Qualitative analysis of the methyl esters was accomplished primarily through comparison with authentic standards. Conformation of identity was also carried out using preparative GLC in combination with silver ion chromatography according to the method of DeVries (15) and hydrogenation as described by Farquhar et al. (14).

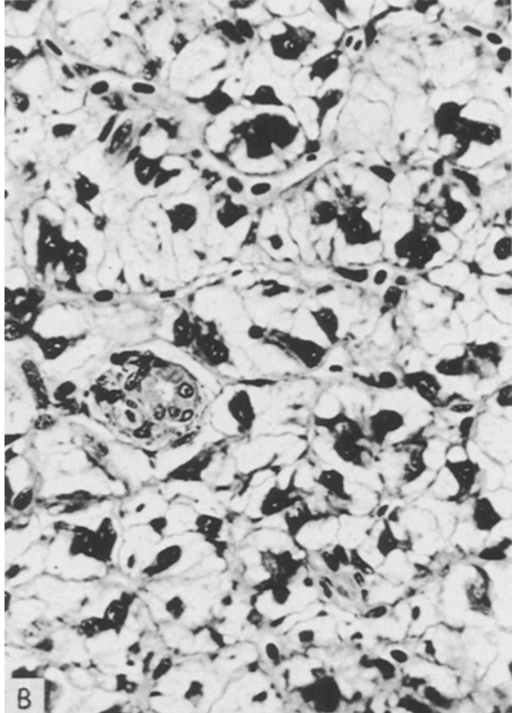
Quantitative analysis of the ester mixtures was expressed as area percent calculated by simple triangulation, planimetry or disc integration. Area per cent was compared with actual weight per cent using NIH quantitative standards and found to be within 3.1% relative error for all major components (greater than 10%) and to within 4.8% for all minor components (less than 10%).

RESULTS AND DISCUSSION

The inclusion of methyl sterculate in the diet of rainbow trout resulted in significant changes in fatty acid composition of the liver lipids (Table I). These changes were characterized by a very large increase in stearic acid and a small decrease in oleic and palmitoleic acids. The large increase in stearic acid content without a corresponding decrease in the levels of oleic was probably due to the biosynthesis of the monoene from acetate. Reiser and Raju (3) found this system in the rat was not inhibited by cyclopropenoid fatty acids. The results support the findings of Raju and Reiser (4) and Johnson et al. (5) which indicate that the stearic fatty acyl desaturase enzyme system is inhibited by cyclopropenes. For this reason, the ratios of stearic to oleic acid and palmitic to palmitoleic acid were selected as indices of biological change.

The maximum alteration in these ratios





occurred at or before the 10th day of feeding (Fig. 1,2). Continued feeding for a period of 87 days produced a slight reduction in this change but the CPFA-containing diets remained significantly higher than the control. The only other fatty acid which changed composition significantly over this feeding period was docosahexenoic acid (22:6). The concentration of this acid in the control group increased steadily in the triglycerides as the fish matured. This increase, however, was not observed in the fish fed methyl sterculate. At the end of the feeding period the respective concentration of 22:6 were 10.69% in the control, 1.68% in the 100 ppm diet, and 3.20% in the 200 ppm diet. These data indicate that cyclopropenoid fatty acids interfere with long chain fatty acid metabolism as well as stearic and palmitic dehydrogenation. It should be noted that all of the changes observed would produce a more saturated and therefore higher melting lipid. The calculated iodine value was 104, 62 and 65 for neutral liver lipids from fish fed 0, 100 and 200 ppm methyl sterculate for 87 days. This fact becomes increasingly important when one considers that the environmental temperature of these trout was 11.4 C. There was no significant difference observed between the two levels of cyclopropene fed. Apparently 100 ppm methyl sterculate in the diet was sufficient to

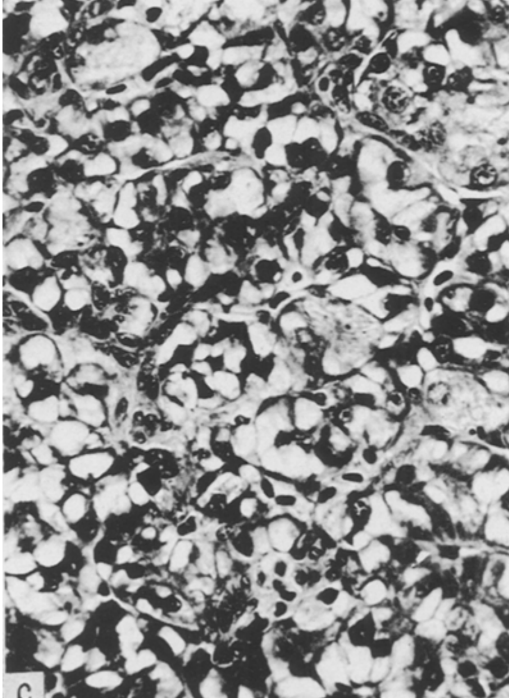


FIG. 5. Liver parenchymal cells of trout fed (A) CPFA-free control diet for eight months, (B) 20 ppm CPFA for seven days, (C) 100 ppm CPFA for eight months (H & E stain X 320, reduced approximately 15%).

induce a maximum in the changes observed, and doubling the dietary dose produced no additional change.

When more mature trout were fed dietary levels of 0, 5, 20, 50 and 100 ppm methyl sterculate for seven days, an increase in the stearic-oleic ratio was observed (Fig. 3). It should be noted, however, that an increase in stearic-oleic ratio was observed in all diets containing cyclopropenes. The observation that very low levels of dietary sterculate cause a shift in the stearic-oleic ratio supports the finding of Reiser and Raju (3) that this inhibition is of a non-competitive nature. Kircher (11) has shown that methyl sterculate reacts strongly with sulfhydryls and may be responsible for its physiological activity. The inhibition of desaturase enzymes in trout liver through a reaction of this type is certainly a possibility.

When fish which had been fed 200 ppm methyl sterculate for 100 days were placed on the control diet their hepatic lipids returned to normal within 30 days. This slow recovery in comparison to the time required to induce these changes is probably due to the movement of sterculic acid from other tissues to the liver.

Growth was also inhibited by feeding methyl sterculate as shown in Figure 4. The pattern of growth inhibition was similar to the change observed in liver fatty acid composition. The greatest suppression of growth occurred early in the feeding period, and there was no significant difference between the two levels of CPFA. Later in the feeding period the fish were gaining normally which suggests a recovery from the initial stress. This could possibly occur through an adaptation in the synthesis of unsaturated fatty acids.

Histological examination of the livers of these trout revealed profound morphological changes. The livers of fish fed CPFA were typically enlarged, very firm and pale in appearance. Microscopic examination of these livers showed them to have extremely large deposits of glycogen. Figure 5B shows typical liver parenchymal cells of trout fed 20 ppm methyl sterculate for seven days. Abnormal glycogen deposits were observed in all cells and the bile duct (center of Figure) is surrounded by heavy deposits as compared to that of normal liver (Fig. 5A). This is typical of the change observed in all trout fed CPFA for a short period. The liver parenchymal cells shown in Figure 5C are typical of those observed after continued feeding of CPFA. These livers, while still having large glycogen deposits, contain some areas of more normal parenchymal cells.

Conclusions

The lipid alterations observed in this study indicate that stearic fatty acyl desaturase activity is inhibited in rainbow trout in much the same manner as has been reported with warm blooded animals. A similar effect on the desaturation of palmitic to palmitoleic is indicated by the data shown in Table I. This phenomenon may have been more readily observed in trout tissue because their tissue lipids normally contain much higher quantities of palmitoleic acid than those of the other animals studied. Low levels of decosahexenoic acid found in the liver triglycerides of fish fed sterculate for 87 days indicate an additional effect on fatty acid metabolism. As would be expected in a rapidly metabolizing organ such as the liver, the influence of CPFA was apparent immediately in both the triglycerides and phospholipids.

Perhaps the most significant observation to be made in this study is the similarity in the time pattern of the various changes. For instance, the most extreme alteration in fatty acid composition, growth and liver histology occurred very early in the feeding period, and in all cases a slight recovery was observed as the experiment progressed.

ACKNOWLEDGMENTS

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Influence of Dietary *trans,trans*-Linoleate on Hematologic and Hemostatic Properties of Rat Blood

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ABSTRACT

Studies of the comparative effects of a semi-synthetic diet containing supplements of corn oil, no fat, linolenate or *trans,trans*-linoleate on blood coagulation parameters are reported. In spite of large differences in fatty acid composition of the tissue lipids of the different groups, the only diets that appeared to produce abnormal hematologic and hemostatic properties were those containing *trans,trans*-linoleate. These groups of animals showed significant differences from a control group of animals fed Purina rat chow in platelet and fibrinogen concentration, and values for hematocrit and prothrombin time. A positive fibrinolysin test was also obtained in about 50% of the animals fed *trans,trans*-linoleate.

INTRODUCTION

Although dietary fats have been implicated in the development of atherosclerosis and thrombosis, the role of lipids in these diseases has not been clearly defined. Atherogenic diets appear to have an effect on blood coagulation parameters and a body of evidence (1-6) indicates a relationship between saturated fatty acids and platelet adhesiveness. Platelets are rich in lipid, have high thromboplastic activity, and are involved at discrete stages in the chain of events that lead to blood coagulation (7-9). The influence of lipid on blood clotting has been shown to be due mainly to phospholipids (10-24). The clot-promoting activity of these compounds is believed to depend mainly on the surface charge of preformed lipid micelles (25-27). Daemen et al. (28,29) demonstrated that combinations of pure sub-classes of synthetic phospholipids containing certain fatty acids exhibited highly significant clot-promoting activity.

It is well known that fatty acid composition of tissue lipid is closely related to dietary fat composition, and changes in dietary fat are reflected by corresponding changes in tissue lipids (30,31). Accordingly, dietary fat might be expected to exert an influence on blood coagulation activity. Presented here are studies on the comparative effects of diets containing *trans,trans*-linoleate, corn oil, no fat or linolenate on the hematologic and hemostatic behavior of blood in rats.

MATERIALS AND METHODS

Diets

Listed below are experimental diets prepared by adding the fatty supplements to a basic semi-synthetic diet consisting of 30% vitamin test casein, 60% sucrose, 4% cellulose, 4% salt mixture and 2% casein containing known vitamins in the required amounts as described previously (32). Seven groups of animals of the Sprague-Dawley strain, obtained from Dan Rolfsmeyer Co., Madison, Wisconsin, were studied. One group was maintained on the basic fat-free diet from weaning to 10 months of age. A second group was fed the basic diet supplemented with 10% by weight of corn oil for the same period. Groups 3 and 4 were fed the basic diet supplemented with 7.5% of 99% all-*cis*-linolenate (The Hormel Institute), respectively, for the last two weeks of the 10 month feeding period. These groups of animals, No. 1-4, were raised at The Hormel Institute and shipped to the Department of Hematology of the University of Louisville for a study of blood coagulation parameters. Later, at the University of Louisville, three more groups (No. 5,6 and 7) of animals were fed the basic fat-free diet supplemented with a concentrate of *trans,trans*-linoleate to observe further the effects of *trans* acids on blood properties. The composition of this concentrate was 3.0% palmitate, 2.8% stearate, 2.1% elaidate, 7.2% oleate, 4.7% *cis,trans*-linoleate and 80.2% *trans,trans*-linoleate. The first of these groups, No. 5, was maintained on the basic fat-free diet for four months and then given a supplement of 15% of *trans,trans*-linoleate concentrate for 30 days. Groups 6 and 7 were fed 5% and 15% of the *trans,trans*-linoleate concentrate for 30 days after weaning.

In order to establish a normal range of values for the hematologic and hemostatic tests listed below, a colony of weanling Sprague-Dawley rats were also fed a Purina rat chow diet to four months of age.

Hematologic Tests

Hemaglobin (Hg), platelet count, hematocrit volume, white blood cell count (WBC) and total

TABLE I

Major Fatty Acids (Wt %)

Fatty supplement groups	Group 1, Fat-free (6 animals)	Group 2, Corn oil (5 animals)	Group 3, Linolenate (5 animals)	Group 4 *trans,trans* Linoleate (5 animals)
Livers[a]				
18:1	36.8	16.1	17.9	42.4
18:2	0.7	19.7	---	7.2
20:3	13.1	---	6.4	6.5
20:4	1.7	25.7	---	1.6
Hearts[a]				
18:1	29.0	11.9	13.7	30.2
18:2	1.7	27.0	0.8	13.4
20:3	25.9	---	7.0	16.3
20:4	8.0	26.3	3.2	6.7
Kidneys[a]				
18:1	27.4	13.4	18.4	31.6
18:2	1.6	13.7	---	9.3
20:3	17.6	---	2.2	9.1
20:4	10.5	27.8	5.7	9.1

[a]Livers, 18:3 = 8.5, 20:5 = 15.0, 22:6 = 6.7; Hearts, 18:3 = 12.6, 20:5 = 7.2, 22:6 = 7.1; Kidneys, 18:3 = 8.2, 20:5 = 24.9.

serum protein (33,34) were carried out from blood obtained from the tail amputated for the performance of the bleeding time.

Hemostatic Tests

Bleeding times were evaluated by the time necessary for bleeding to cease after amputation of the tail 1 in. from the end. The following tests were performed with arterial blood obtained from the abdominal aorta surgically exposed: prothrombin time, partial thromboplastin time, fibrinogen concentration and fribrinolysin (33,34).

After withdrawal of blood, the animals were placed in the anesthesia chamber and killed by ether vapors. Platelets were isolated via differential centrifugation as previously described (35) and examined by electron microscopy.

The lipids of pooled hearts, kidneys and livers of the animals in the first four groups of animals were extracted with chloroform-methanol (2:1) and fatty acid composition was determined by GLC on methyl esters prepared by interesterification with HCl-methanol (38). These analyses were made with an F & M Model 1609 flame ionization gas chromatograph equipped with a 6 ft x 1/4 in. column packed with 8% ethylene glycol succinate polyester on Gas Chrom P (EGSS-X, Applied Science Laboratory, State College, Pa.) at 185 C with a carrier gas flow (helium) of 85 ml/min. The instrument was calibrated with standard mixtures of methyl esters (The Hormel Institute) of NIH specifications (37).

TABLE II

Summary of Platelet Lipid Analysis

Fatty supplement	Group 4[a] *trans, trans* Linoleate, %	Group 2[a] Corn oil, %
Unknown R_f above cholesterol esters	4.5	2.9
Cholesterol esters	12.0	5.3
Triglycerides	9.1	5.1
Fatty acids	8.9	3.4
Cholesterol	10.4	12.9
Total neutral lipid	44.9	29.6
Total polar lipid[b]	55.1	70.4
Cardiolipin	7.3	5.6
Phosphatidyl-ethanolamine	4.7	13.7
Phosphatidyl-inositol	2.4	3.0
Phosphatidylserine	4.3	7.6
Phosphatidylcholine	14.8	21.7
Sphingomyelin	8.6	12.1
Unknown R_f zero[b]	13.0	6.7

[a]Five animals in each group.
[b]By difference.

Lipid class analysis was performed on the platelets of pool samples using quantitative thin layer chromatography and the charring-densitometry technique (36). Analyses were performed in triplicate with a relative error of approximately ±5% for major components and ±10% for minor components, < 5%, determined on standard mixtures.

RESULTS

The effect of diet on the fatty acid composition of the tissue lipids of the first four

TABLE III

Hematologic Tests (Mean and Standard Deviation) on Rats Fed Diets Containing *trans, trans*-Linoleate, No Fat, Corn Oil and Linolenate With Normal Rats

Group	No. of animals	Hemoglobin (g/100 ml)	Platelet (10^3/mm^3)	Hematocrit (% vol)	White blood cells (10^3/mm^3)	Serum protein (g/100 ml)
Normal	35	14.4	1059	50.3	10.8	7.0
		0.6	237	3.7	2.6	0.5
1	6	15.4	945	48.0	11.6	6.9
(Fat free)		0.9	124	3.2	2.5	0.9
		a	b	b	b	c
2	5	15.1	952	48.4	11.8	6.9
(Corn oil)		0.3	315	0.9	2.3	0.3
		a	b	b	b	b
3	5	14.7	948	47.0	10.8	6.4
(Linolenate)		0.8	272	3.0	4.8	0.3
		b	b	b	c	a
4, 5, 6, 7	27	14.9	860	47.3	16.6	6.2
(tt-linoleate)		0.9	310	3.5	6.2	0.8
		c	a	a	c	c

[a]Significant (difference in means from normal significant; difference variance not significant).

[b]Nonsignificant (difference in means nonsignificant; difference in variance not significant).

[c]Difference in variance significant.

groups of animals is shown in Table I. Fatty acid composition varied widely and characteristically in these groups. The tissue lipids of the corn oil group, No. 2, which consisted of normal, healthy animals contained large percentages of linoleic and arachidonic acids. In contrast, the fat-free group, No. 1, was virtually devoid of these acids and high in oleic and 5,8,11-eicosatrienoic acid; all the animals of this group exhibited advanced symptoms of an EFA deficiency. Lipids of the organs of the linolenate group, No. 3, were also low in 18:2 and arachidonic acid (20:4), but these animals had high values for linolenic (18:3), 20:5 and 22:6 acids; linolenic acid is readily converted to 20:5 and 22:6 by the rat. The animals of this group as well as Group 4 also exhibited dermal symptoms of an EFA deficiency. Group 4 animals which differed from the others by receiving a supplement of 7.5% *trans,trans*-linoleate in their diets resembled Group 1 to some extent, but differed in that they contained appreciable amounts of *trans,trans*-linoleate (18:2) in their tissue lipids. The animals in Group 5 also exhibited EFA deficiency symptoms. This group of animals, as well as the animals in Groups 6 and 7 also should contain appreciable amounts of *trans,trans*-18:2 in their tissues on the basis of previous nutritional studies with *trans* acids (38-40).

Lipid class composition of the platelets of the corn oil group, No. 2, and the *trans,trans*-linoleate Group 4 are compared in Table II. The major difference between these groups is the much higher percentage of neutral lipids, except for cholesterol, and the corresponding lower percentage of polar lipids, especially phosphatidylethanolamine, in the platelets of the animals of the *trans,trans*-linoleate group. In spite of the wide differences in lipid composition, electron micrographs showed no abnormalities in the platelets of the different groups.

Results of the coagulation tests are summarized in Table III. The normal ranges for the values of each test were established in the control group fed Purina chow for statistical comparison with the other groups and are in accord with values previously reported for normal rats (41). The only animals that showed possible defects in blood coagulation activity were the groups that received the *trans,trans*-linoleate. These groups taken together showed significant differences in the means from the normals at the 95% confidence level in platelet concentration, hematocrit values, prothrombin time and fibrinogen concentration. Differences in the means of the *trans,trans*-linoleate fed groups from the normals were also indicated in bleeding time, hemoglobin concentration, white blood cell concentration, and serum protein, but the scattering of the values (analysis of variance) was too great to give significance to the differences. The test for fibrinolysin was positive in approximately 50% of the animals in the *trans,trans* groups. The only other positive tests observed were in two animals of Group 3, the linolenate supplemented animals. The only tests that showed significant differences from

TABLE IV

Hemostatic Tests (Mean and Standard Deviation) on Rats Fed Diets Containing *trans,trans*-Linoleate, No Fat, Corn Oil and Linolenate With Normal Rats

Group	No. of animals	Bleeding time (min)	Prothrombin time (sec)	P. Thromboplastin time (sec)	Fibrinogen Conc. (mg/100 ml)
Normal	a	5.6 (43)	13.5 (34)	34 (34)	280 (34)
		2.1	0.9	9	75
1		6.9	12.3	30	252
(Fat free)	6	3.3	1.8	4	82
		b	b	b	c
2		9.1	13.9	35	306
(Corn oil)	5	4.9	0.7	13	154
		b	c	c	b
3		7.5	13.4	25	229
(Linolenate)	5	3.1	0.9	1	93
		c	c	b	c
4, 5, 6, 7		8.0 (27)	12.8 (14)	30 (15)	171 (12)
(tt-linoleate)	a	4.7	1.3	10	56
		b	d	c	d

[a]No. of animals tested shown in brackets.

[b]Difference in variance significant.

[c]Nonsignificant (difference in means nonsignificant; difference in variance not significant).

[d]Significant (difference in means from normal significant; difference in variance not significant).

the normals outside of that for the *trans,trans*-linoleate groups were values for hemoglobin concentration for Groups 1 and 2 and serum protein concentration for Group 3.

DISCUSSION

Although the results on all of the *trans,trans*-linoleate fed animals were grouped together for statistical analysis, there is sufficient evidence in the present study to indicate an abnormal effect of this compound on hematologic and hemostatic properties of blood in the rat. The effect of *trans,trans*-linoleate becomes more apparent, considering that a deficiency of essential fatty acids had no comparable effect with the possible exception of increasing slightly hemoglobin concentration which was also observed in the animals fed corn oil. Although positive fibrinolysis is sometimes associated with thrombocytopenia, an interpretation of the effect of *trans,trans*-linoleate on blood coagulation activity must await further studies with large groups of animals and should include studies on platelet adhesiveness. Phosphatides having different molecular and fatty acid compositions may produce marked effects in in vitro blood coagulation tests (28,29), but the wide variations in the lipid classes of the platelets and the fatty acid composition of the tissues, and presumably blood lipids produced in the present study, had little effect on properties of the blood except for the animals fed *trans,trans*-linoleate.

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The Polymorphism of Odd and Even Saturated Single Acid Triglycerides, C_8-C_{22}

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ABSTRACT

The polymorphism of single fatty acid odd triglycerides, C_{11} through C_{17} has been reinvestigated, with extension of the study to C_9, C_{19} and C_{21}. With study of the even glycerides C_8, C_{20} and C_{22} it has been possible to review the whole series (odd and even) C_8 through C_{22}. The odd glycerides resemble the even in showing three distinct melting levels. Lowest melting forms are α. Stable forms are β except for C_9 and C_{11} which show a different structure type. Intermediate melting β' forms of odd glycerides are substantially more stable than their even counterparts as well illustrated by differential thermal analysis. There are no vitreous forms. Alternation in mp (between odd and even) is confirmed for stable phases and nonalternation for α and (within experimental error) for β'. Both β' and β long spacings show alternation but not α. Alternation is evident in the short spacings of β' and β forms. While short spacings of β' forms of even triglycerides are much alike especially for C_{14} through C_{22}, those of odd glycerides show a fortuitous 4-carbon cycle. This appears to involve no significant structural variation as chain length increases but simply an approximate 4-carbon cycle of variation in diffraction details derived from the presumed unvarying $0\perp$ type subcell structure.

INTRODUCTION

No detailed diffraction study of the phase behavior of the series of single saturated acid triglycerides with odd chain lengths has been reported since Clarkson and Malkin (1) reviewed their investigation of C_{11}, C_{13}, C_{15} and C_{17}. They reported four forms with four melting levels for each glyceride, repeating their claim of a lowest melting vitreous form lacking long spacings. Since the vitreous form of the even saturated triglycerides has long since been abandoned by several investigators, and only a few recognize any sort of fourth form with fourth distinct melting level for these even triglycerides, the case of the odd triglycerides deserves review. Conclusions by Larsson (2) from a brief exploration require some modification.

We examined C_9, C_{19} and C_{21} compounds as well as C_{11}, C_{13}, C_{15} and C_{17} studied earlier (1). In addition, data on C_8, C_{20} and C_{22} and on previously unreported aspects of C_{10} afford the opportunity of summarizing the phase behavior of the whole series of compounds from C_8 through C_{22}.

EXPERIMENTAL PROCEDURES

Odd fatty acids, C_9 through C_{19}, were prepared via the nitriles from the requisite even bromides of 1-carbon less chain length; bromides obtained from Humphrey Wilkinson, Inc. By analysis, including gas chromatography of the methyl esters, the distilled odd acids give fractions of 99% purity or better.

The acids were converted to methyl esters by acid catalyzed esterification with an excess of methanol. The methyl esters in 10% excess were reacted with sodium glyceroxide to form the triglycerides which were purified in approximately 10 g quantities by column chromatography, as indicated later, and crystallized once from hexane to yield products of better than 99% purity by thin layer chromatography (TLC).

From 1 g of C_{21} methyl esters (Sigma Chemical Co., St. Louis, Mo.), 99% pure by gas chromatography, the triglyceride was prepared as above to yield 0.3 g of the chromatographed triglyceride, better than 99% pure by TLC.

By reaction of 99.7% C_8 methyl ester with sodium glyceroxide the triglyceride was prepared and purified by chromatography followed by two crystallizations at -35 C from 93% acetone-7% ethanol. By TLC the C_8 triglyceride was of more than 99% triglyceride content.

Two preparations of C_{10} triglyceride were studied. One was the same preparation as used by Jensen and Mabis (3) in their crystal structure studies and was prepared by acid catalyzed reaction of C_{10} acid with glycerol followed by four crystallizations from 50-50 Skelly F-ethanol at -5 C. The other sample was purchased from Applied Science Laboratories, Inc., State College, Pa. Both were judged by chemical analysis or by TLC to be 99% pure or better. No significant difference in behavior of the two preparations was noted.

TABLE I

Melting Points of Single Acid Triglycerides

Acid chain length	α: This laboratory: Present study	α: This laboratory: Previous study (6)	α: C&M[a]	α: Av[b]	α: Calc.	β: This laboratory: Present study	β: This laboratory: Previous study (6)	β: C&M[a]	β: Av[b]	β: Calc.	Stable: This laboratory: Present study	Stable: This laboratory: Previous study (6)	Stable: C&M[a]	Stable: Av[b]	Stable: Calc.
22	68.2			68.0	67.4	74			74	73.3	82.5			82.5	82.3
21	65.0			65.0	65.1	71.0			71.0	71.4	75.9			76.0	76.1
20	61.8			62.0	62.3	69			69.0	69.2	78.1			78.0	78.3
19[c]	58.2			59.0	59.0	64.5			65.5	66.6	71.4			71.0	70.7
18	54.7	54.9	54.5	54.7	55.1	63.2	64	64.5	64.0	63.5	73.5	73.1	71.5	73.3[d]	73.1
17	50.0		50	50.0	50.5	60.1		61.0	60.5	60.1	64.5		63.5	64.0	63.9
16		44.7	45	45.0	45.2		56.6	56.0	56.5	56.1		66.4	65.5	66.0	66.3
15	38.5		40	39.0	39.1	50.1		51.5	51.0	51.6	56.2		54.0	55.0	55.3
14	32.8		33	33.0	31.9	45.0		46.5	46.0	46.5	58.5		57.0	58.0	57.6
13	24.4		25	24.5	23.7	41.5		41.0	41.5	40.6	45.1		44.0	44.5	44.5
12	15.2		15	15.0	14.2	34		35.0	34.5	34.0	46.5		46.4	46.5	46.4
11	3.7		1.0	2.5	3.2	27.5		26.5	27.0	26.5	31.2		30.5	31.0	30.9
10	-9, -7		-15	-10.5	-9.6	15.5		18.0	17.0	18.0	31.9, 32.3		31.5	32.0	32.2
9	-26[e]			-26		44.0			4.0		10.7			10.5	
8	-51[e]			-51		-18.0			-18.0		9.9			10.0	

[a]Clarkson and Malin (1).

[b]To nearest 0.5°, except α and β C_{18}.

[c]Av. includes 60, 66.8, 70.5 for α, β', β (10).

[d]Av. this lab.

[e]DTA data.

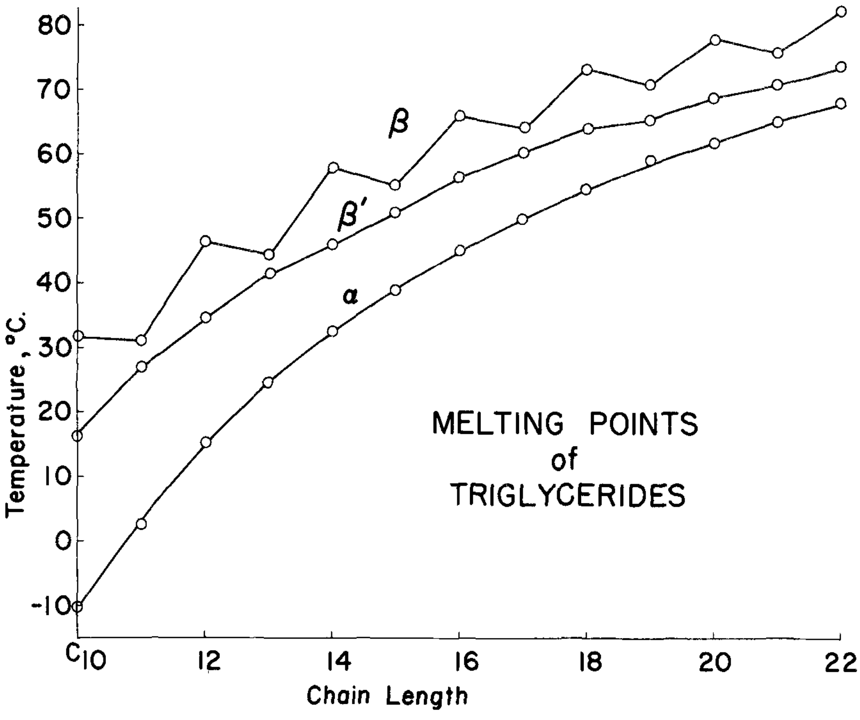

FIG. 1. Melting points of triglycerides vs. chain length.

The C_{20} triglyceride was prepared from 98.2% C_{20} acid by acid catalyzed esterification of fatty acid with glycerol. It was isolated by column chromatography (CG) on silica gel as indicated later and two crystallizations from hexane. The C_{22} triglyceride was prepared from 98+% C_{22} acid by acid catalyzed esterification with glycerol and five crystallizations from Skelly B and benzene-ethanol (50:50). Both C_{20} and C_{22} compounds were better than 99% triglyceride by TLC.

The C_8, C_9, C_{11} and C_{13} triglycerides were chromatographed on alumina columns after the convenient method of Jensen et al. (4). The C_{15}, C_{17}, C_{19}, C_{20} and C_{21} triglycerides were chromatographed on silica gel according to Quinlin and Weiser (5). Attempts to chromatograph C_{20} on alumina according to the Jensen procedure were not successful due to low solubility in the eluting solvents.

Examination of phase behavior was, in general, carried out as previously described by capillary melting point and diffraction methods (6) which require no further comment. Additional studies of thermal behavior were made by means of differential thermal analysis (DTA) on the DuPont 900 Analyzer.

We believe that we observed all possible phases (except low temperature sub-α states) of all glycerides, at least from C_{10} through C_{22}. Three phases of each glyceride were observed. Comment on individual glycerides is necessary in some cases but the following statements apply generally: the lowest melting metastable α form is obtained by chilling the melt to a temperature 20 C below the corresponding melting point. Diffraction patterns, which require 1-2 hr exposure, are obtained 10-20 C below the melting point. Stable forms (in most cases β) are generally obtained by solvent crystallization but normally readily obtained, also, by storage of solid samples slightly below the level of intermediate β' melting points. For intermediate forms in pure state, two principal techniques were employed, one preferred for odd triglycerides the other for even. For β' forms of odd triglycerides the sample is melted and then chilled below the α melting point and is then stored normally for 30 min at 2 C above the α melting point. However, C_{21} required 2 hr 1 C above the α melting point. For β' forms of even triglycerides the melt is normally crystallized at a temperature near the α melting point where it clouds in 5 min but not in 1 min. Crystallization time ranges from 15 min for C_{12} to 1 hr for C_{18}. For C_{10}, 5 min at -5 C is appropriate. For C_{20} 4 hr at 62.3 C was required. So slow was the even process for C_{22}

TABLE II

Long Spacings of Single Fatty Acid Triglycerides in A

Acid chain length	α					β					Stable				
	This laboratory		C&M[a]	Av	Calc.	This laboratory		C&M[a]	Av	Calc.	This laboratory		C&M[a]	Av	Calc.
	Present study	Previous study (6)				Present study	Previous study (6)				Present study	Previous study (6)			
22	61.5			61.5	61.1	56.0			56.0	56.0	54.0			54.0	54.2
21	58.5			58.5	58.6	53.2			53.2	53.0	52.7			52.7	52.3
20	55.8			55.8	56.0	50.7			50.7	51.3	49.5			49.5	49.6
19	53.1			53.1	53.4	48.1			48.1	48.3	48.2			48.2	48.2
18		50.6	50.6	50.6	50.8		46.8	47.2	47.0	46.7		45.1_5	45.1	45.1	45.0
17	48.4		48.5	48.5	48.3	43.9		43.7	43.8	43.7	43.4		43.5	43.5	43.5
16		46.0	45.6	45.8	45.7		42.3	42.6	42.5	42.0		40.9	40.6	40.8	40.4
15	42.9		42.9	42.9	43.1	39.3		39.1	39.2	39.0	39.4		38.9	39.2	38.9
14	40.3	41.4	41.2	41.0	40.5	36.7	37.7	37.6	37.3	37.4		35.5	35.8	35.7	35.8
13	37.9		37.7	37.8	38.0	34.2		34.2	34.2	34.4	33.8		34.1	34.0	34.2
12	35.5		35.6	35.6	35.4	32.9			32.9	32.7	31.2		31.2	31.2	31.2
11	32.3		33.0	32.7	32.8	30.0		29.5	29.8	29.7	29.5		29.6	29.6	26.6
10	30.2			30.2	30.3	27.7			27.7	28.1	26.3		26.8	26.5	26.6
9						25.3			25.3		24.9			24.9	
8											22.7			22.7	

[a]Clarkson and Malkin (1).

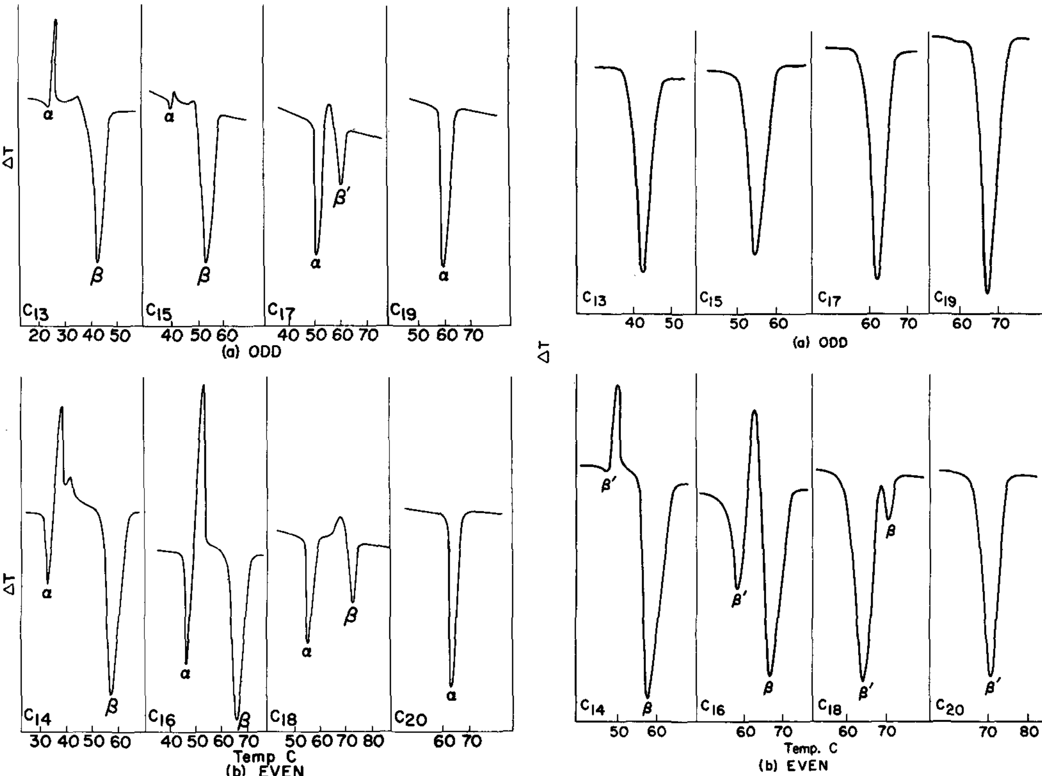

FIG. 2. DTA for odd vs. even triglycerides in α form.

FIG. 3. DTA for odd vs. even triglycerides in β' form.

that we resorted to the odd method; after chilling, the sample was held 16 hr at 69 C.

The melting points are presented in Table I and in Figure 1. Long spacing diffraction data appear in Table II.

We made two principal uses of DTA: (a) to confirm melting levels for the various forms and (b) to characterize and intercompare various members of the homologous series with respect to transformation tendencies. DTA curves were scrutinized for possibility of polymorphs other than stable, α, and β' forms. We found none, although artifacts occasionally appeared for C_{14} in Figure 2. These were eliminated from consideration because of inconstancy of temperature level with slightly altered conditions.

In general, 10 mg samples were tested against glass beads as reference material. Recommended heating rates of 10 or 20 C per minute were normally employed but, with the rapidly transforming metastable states of lower homologs, rates as high as 60 C were advantageous.

Stable form melting was confirmed on solvent crystallized samples except in the cases of the low melting C_8 and C_9 which readily transform to stable states in the course of the heating curve. Melting of the lowest melting form (usually α) was confirmed on chilled samples; with C_8 and C_9 the exotherms due to transformation were accepted. For intermediate β' forms, melting endotherms were readily obtainable on all odd homologs, but below C_{14}, among even homologs, very high heating rates were required to reveal melting before complete transformation had occurred.

The DTA plots characterize the individual homologs in their different states and distinguish the behavior of odd from even triglycerides. Illustrative cases appear in Figures 2 and 3. Figure 4 shows the behavior of C_{20} phases by DTA with a comparison of β' as prepared in pure state by the even method, but less pure state by the odd method.

RESULTS AND DISCUSSION

Nomenclature

In presenting the results, an initial discussion of nomenclature is in order, for while clear enough in the even case, it has not been clari-

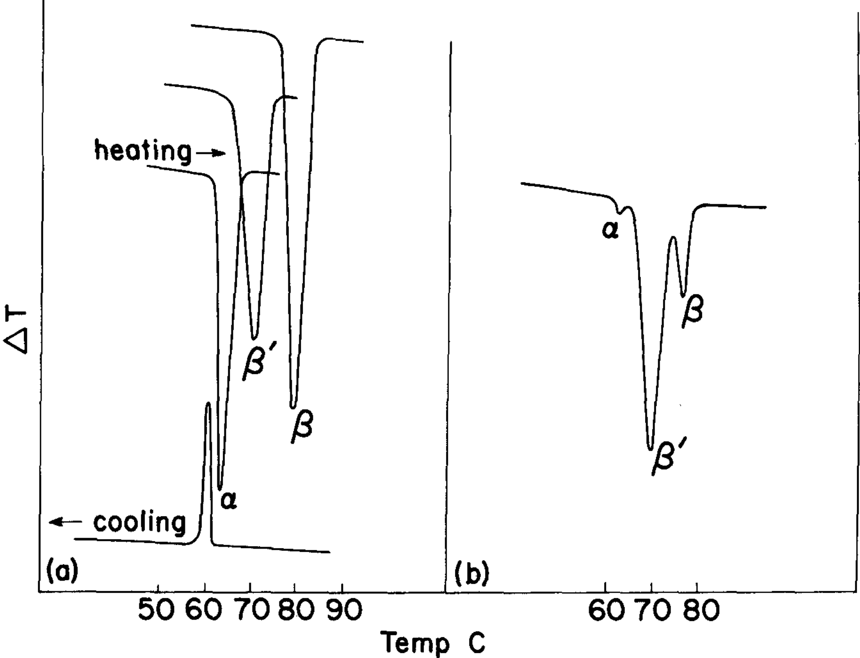

FIG. 4. DTA curves for triarachidin. (a) β from solvent crystals; β' by "even method"–melt, hold 4 hr 62.3 C; α by melting and chilling; cooling curve shows α from melt. (b), β' by odd method–melt and chill, hold 30 min 63.8 C.

fied for the series of odd compounds, among which a new complication arises. With two known exceptions (out of 45 cases) the phases encountered are α, β' and β, in increasing order of mp, or α_L, β'_L and β_L as suggested by Chapman (7). The basis for the nomenclature of the present paper is as follows:

α, single strong spacing at about 4.1 A, associated with a lowest melting form (no pattern obtained for C_8 or C_9).

β', strong spacing or spacings near 4.2 A and at about 3.8 A (variations discussed later); long spacings shorter than those of α, hence chains tilted with respect to end planes (must be distinguished from $I_{9,11}$. No β' pattern observed for C_8).

β, strong line at about 4.6 A, associated with a highest melting form (variations discussed later).

$I_{9,11}$ (an exceptional phase), standing for "stable form of C_9 and C_{11}," observed instead of β. The spacings are discussed in detail later. There would be reason to call this form by such a name as β'_2 or perhaps 0 ⊥ stable (or perhaps 0' ⊥ stable) since it seems to fall in the 0 ⊥ class as to sub cells, but perhaps the term $I_{9,11}$ applying only to C_9 and C_{11} contributes less confusion even if, for instance, the term may eventually prove to be applicable to C_7.

While further description of pattern types is possible, an excess of detail is to be avoided in characterization, since variations do appear with temperature and with chain length.

Accuracy and Variability of Data for Given Phase

In trying to clarify the polymorphism of triglycerides and to understand the aberrations that have crept into the subject, it is desirable to set down some points on the accuracy and reproducibility of measurement and the actual variations possible with a given phase.

Diffraction Data. With these triglycerides there is little problem of confusing one phase with another because of inaccuracies in diffraction data. The problems have arisen in the past from associating a given set of diffraction data with the wrong thermal behavior, or in regarding a set of data from mixed phases as due to a single phase.

Long spacings, of the order of 40 A, are accurate to about ± 0.3 A. They vary little with temperature but are apt to be much more diffuse with smaller crystal size as is obtained, for instance, with quick chilling or with higher molecular weight.

Short spacings,' of the order 4.0 A are obtainable to about ± 0.02 A. These do very with temperature, largely in accord with density variation. They also become more diffuse with smaller crystal size.

TABLE III

β' Short Spacings of Odd Triglycerides

C_{4n+1} Glycerides	Short spacings	C_{4n-1} Glycerides	Short spacings
C_9	4.48 VW,[a] 4.30 S, 4.05 W+, 3.83 M[a]	C_{11}	4.33 M, 4.11 S, 3.80 M
C_{13}	4.36 W,[a] 4.18 S, 3.99 W+, 3.79 M	C_{15}	4.30 S, 4.12 S, 3.84 M
C_{17}	4.30 VW, 4.15 S, 3.97 VW, 3.79 S[a]	C_{19}	4.23 S, 4.08 M, 3.78 M
C_{21}	4.25 W, 4.13 S, 4.03 W, 3.76 M		

[a]S, strong; M, medium; W, weak; VW, very weak.

Thermal Data. Thermal data are a different matter. While characterizing data can normally be obtained with great certainty, the true melting levels will definitely vary with change in phase preparation and treatment.

DTA peaks are typically precise to 0.5 or 1.0 C. Normally only with α states among triglycerides can values from heating (with concomitant melting) and cooling (with concomitant freezing) curves be compared. The heating curve values tend to exceed the cooling curve values by 2-3 C.

The familiar melting point method of raising a capillary sample at a rate of about 0.5 C per minute is properly applicable only to stable forms with a few additions of slowly transforming metastable states. Values are reproducible to ± 0.2 C for well stabilized samples, e.g., most solvent crystallized samples. But there is no doubt that manner of preparation of stable phase can affect melting level, lowering it by as much as 3 C in the case of rapidly prepared phase of small particle size.

Thrust-in melting points (6) are preferable, indeed essential, for most metastable phases. For α phases formed with minimum cloud, precision of ± 0.1 or even 0.05 C is possible, but such precision falls off as molecular weight decreases and speed of transformation increases. Alpha forms, like β forms, may show melting point variation, with increases as much as 1.0 C possible with stabilized α. Intermediate β' phases are the most difficult to characterize thermally. Reproducibility of ± 1.0 by preferred thrust-in techniques is as good as can be hoped for. Beta prime melting levels, also, definitely vary with sample preparation.

Thus the reasonable expectation in study of triglyceride polymorphism is an accuracy of ± 0.1 C for (minimum) α melting levels, ± 0.2 C for (maximum) stable form melting levels, and ± 1.0 C for β' melting levels (probably near their maximum). It is probably the very real variation of melting levels of β' and β phases which has given rise to many difficulties in the past, e.g., the seven tristearin polymorphs of Weygand and Gruntzig (8). This variation in melting level should be no surprise in view of the long experience of continuous variation in melting level of fats in analytical laboratories and the wide and continuous variation in melting level of the purest polymers (although with polymers, chain folding is a further factor in melting point variation).

Variation in α Spacings With Chain Length. The strong α short spacing varies from about 4.08 to 4.14 A with increasing molecular weight (and temperature). The α pattern for the very low melting lowest form of C_9 was not obtainable even at -35 C.

Variation in β Spacings With Chain Length. The even glycerides show much similarity in β short spacings which come close to 5.24 M, 4.61 VS, 3.84 S, 3.68 S. Patterns do alter continuously in character with change in chain length, but even at C_8 the spacings are recognizably similar to the familiar values - 5.28 M+, 4.55 S, 3.92 M, 3.80 S, 3.57 W+.

The odd glycerides, C_{13} through C_{21}, have a slightly different pattern type. Average figures are - 5.31 W, 4.61 S, 3.92 M+, 3.62 M. There is no sign of the 4-carbon cycle described later for β' forms of odd glycerides.

The Stable Form for C_9 and C_{11}. In the series of stable forms of odd triglycerides there is a break between C_{13} and C_{11} such as does not occur in the even series unless it be below C_8. The short spacings for C_{11} are 4.24 S, 4.10 M, 4.00 M, 3.87 S, and for C_9 are 4.29 M, 4.15 W+, 4.01 W, 3.89 S.
The spacings, obviously not of β type and, indeed, reminiscent of β', are of distinct character and pertain to some as yet unidentified structure type.

Variation in β' Spacings With Chain Length. The character of the β' short spacings is an intriguing matter. The even glycerides, especially C_{14} through C_{22}, have shown two main spacings close to 4.18 A (very strong but somewhat diffuse) and 3.78 A (strong). The even β' spacings are probably generally less sharp than odd β' spacings can be because the even β' forms are less stable, hence never well perfected. Shorter even chains than C_{14} exhibit minor β' variations. But the two distinct types of spacings observed for odd, $C4_{n+1}$ and $C4_{n-1}$

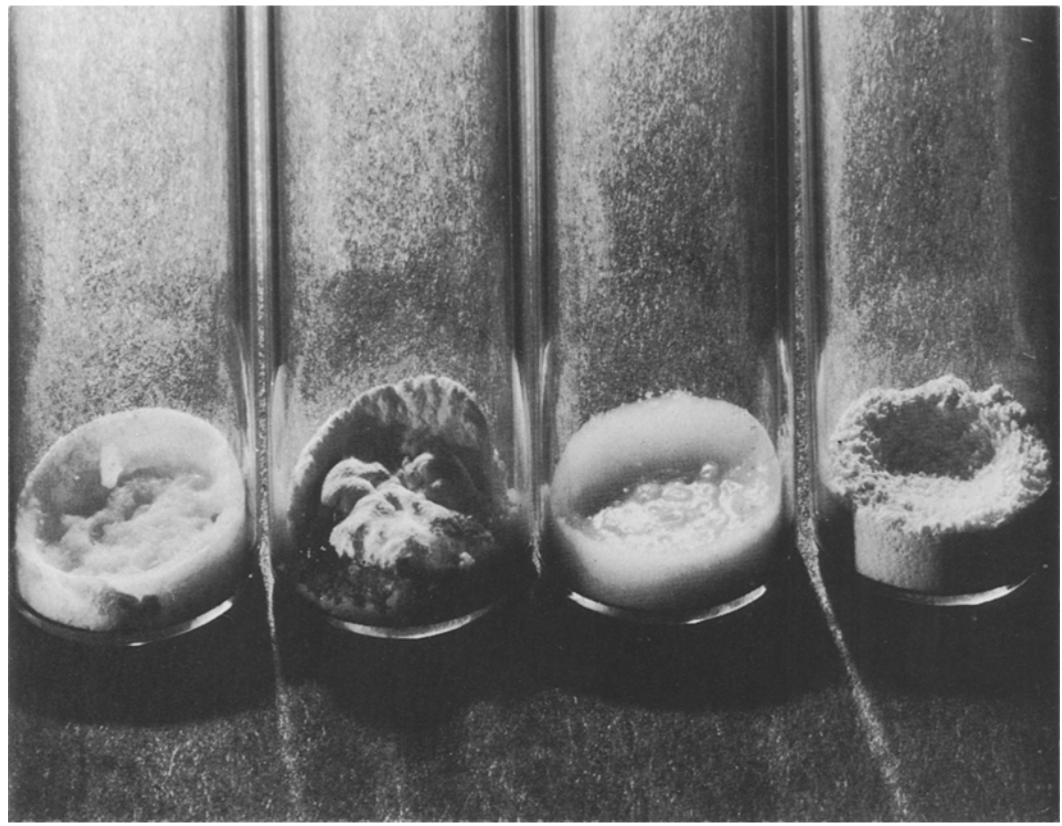

FIG. 5. Bloom or swelling of crystallized triglycerides. One gram crystallized 0.2 C below the α mp for 2 hr in air chamber. From left to right: C_{15}, C_{16}, C_{17} and C_{18}.

glycerides, e.g., C_{17} and C_{15} where n = 4, shown in Table III, are not observed with β' forms of even triglycerides.

The short spacing alternation seen for β' within the odd glyceride series at first suggests some significant structure variation involving a 4-carbon cycle. However, it has been possible to conclude, somewhat speculatively from analogy with β' stable $C_nC_{n+2}C_n$ glycerides, as briefly investigated by Webb (private communication) that the metastable odd β' phases are truly homologous structures probably of familiar O⊥ (or O⊥') subcell types (9). These β' phases in undergoing minor continuous structural change with chain lengthening exhibit diffraction changes in a fortuitous 4-carbon cycle such that C_{17} diffraction resembles that of C_{13} more than that of C_{15}.

Phase Stability From Comparison of DTA Curves. DTA curves are instructive for phase characterization, especially for tendency to transform. Alpha is more fleeting with lower molecular weight, but distinction between odd and even α phases can hardly be made on the basis of DTA curves in Figure 2. In Figure 3 it is plain that β' is decidedly more fleeting for even than odd. Complete melting without transformation is seen for C_{13} but not below C_{20} in the even case.

Beta is the stable phase (except for C_9 and C_{11}) for odd as well as even, however, as indicated by its appearing from solvent, its higher melting point and its development on standing of α or β' in every case except that of C_{21}. In that case the β' state did not convert to β in 1 month at 60 C. All even triglycerides, C_8 through C_{22}, can be readily prepared as β via melt without recourse to solvent, by judicious storage in the range of the α and β melting points.

Bloom or Swelling Phenomena. The tendency of familiar saturated triglycerides, e.g., tristearin or linseed hardstock to bloom or expand on crystallization is well known. This phenomenon, although not fully understood, is associated with the rapid development of β phase. It is readily seen in large samples but can be observed in samples as small as 1 g. In

present experience it is encountered with even but not odd triglycerides as shown in Figure 5.

Final Listing of Melting Points and Long Spacing. In the list of melting points in Table I and long spacings in Table II there appear (a) new data obtained in this work, (b) earlier data from this laboratory, (c) data of Clarkson and Malkin, (d) averages of the latter data with our data except as indicated, and (e) calculated data for glycerides C_{10} through C_{22}. In listing the data of Clarkson and Malkin their vitreous phase melting points were considered to be α and their α to be β'.

Calculated Melting Point and Long Spacing Values. In computing relations used for calculating melting points and long spacing values, data for C_8 and C_9 glycerides were not included, since for the most part, they were not obtained by comparable procedures and are probably not as precise. However, they are regarded as reliably extending an understanding of polymorphic behavior of the series. Data for C_{11} were included in calculations even for stable form melting points but not for stable form long spacings, since no β diffraction pattern was obtained.

Observations of alternation affect the procedure for calculating data for the series. Our observations of alternation or lack of alternation with chain length are readily interpretable according to the enlightening discussion of Larsson (9). Alternation for the stable phase very probably results from the difference between odd and even glycerides in methyl group packing that normally occurs with tilted chains. The lack of alternation with α indicates the similar methyl group packing in the absence of tilting. The special case of undetectable mp alternation with tilted β' probably indicates similarity of methyl group packing despite tilting. There is a β' long spacing alternation despite lack of melting point alternation.

Melting Points. The maximum melting point of polyethylene to which value all straight chain hydrocarbon series must extrapolate is about 140 C. Accordingly it was found that an equation of the following form well expressed melting point variation with chain length (from N = 10 to 22) for the various polymorphs:

$$\text{mp C} = 140 - e^{(a + bN + cN^2)}$$

or

$$\log(140 - mp) = a' + b'N + c'N^2$$

The following equations were obtained:

$$\log(140 - mp_{\alpha}) = 2.68776 - 0.0626939N + 0.00114085N^2$$

$$\log(140 - mp_{\beta'}) = 2.49726 - 0.0498375N + 0.00087426N^2$$

$$\log(140 - mp_{\beta,odd}) = 2.45691 - 0.0458687N + 0.00070647N^2$$

$$\log(140 - mp_{\beta,even}) = 2.43894 - 0.0488017N + 0.00081758N^2$$

For the β cases, the maximum deviation (Table I) is 0.4 C, for experimental values expressed only to the nearest 0.5 C, except for the more accurate tristearin value. For α and β' cases, maximum deviation is 1.1 C, again for values expressed only to the nearest 0.5 C and possibly, at the lower end of the scale, less precise than ± 0.5 C.

Long Spacings. The long spacing data were averaged and tabulated in manner similar to those for melting points. Both β and β' phases show alternation. It is interesting that long spacings for even glycerides are relatively shorter than those for odd in the β case but longer in the β' case. Equations best fit for linear relationships, long spacings vs. chain length N (10 to 22), were:

$$LS_{\alpha} = 4.523 + 2.4731N$$
$$LS_{\beta'} \text{ odd} = 4.058 + 2.3329N$$
$$LS_{\beta'} \text{ even} = 4.814 + 2.3250N$$
$$LS_{\beta} \text{ odd} = 4.080 + 2.3200N$$
$$LS_{\beta} \text{ even} = 3.686 + 2.2946N$$

The 39 averaged long spacing values are within 0.3 A of the calculated values (Table III) in all but six cases of which three show deviation of 0.4 and the maximum shows 0.6; all six cases are for even glycerides for which data were obtained at various times.

Commentary on the Discussion of Polymorphism by Hoerr and Paulicka

Hoerr and Paulicka (11) have recently contributed interesting and constructive discussion to the field of triglyceride polymorphism but do not always precisely state the case. Thus, in their Table I, their dotted line should refer to the next to highest melting level of Malkin, for it is this melting level which we claim and have claimed to be superfluous in Malkin's scheme. Also, the principal difficulties concerning triglyceride polymorphism were resolved, not "by the introduction of infrared spectroscopy", per se, but by acceptance of the true correlation between melting level and other characterizing features, diffraction pattern, infrared spectrum, dielectric constant, crystal density, etc. Chapman's (7) employment of infrared spectroscopy helped to promote the acceptance of the true correlation. Finally, while we did not investigate low temperature states in this study and the term sub-α may not have been a happy

choice in terminology for such states, we do not agree that "the term sub-α (or some equivalent term) should be eliminated in the classification. . . ." for this term (or some equivalent) represents a low temperature structural state different from α, whether or not reversibly transformable to α and whether or not related to α through one or more second order transformations.

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The Lipids of Thermophilic Fungi: Lipid Composition Comparisons Between Thermophilic and Mesophilic Fungi

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ABSTRACT

The lipid composition of nine thermophilic and nine mesophilic species of seven genera of fungi were compared. The total lipids varied between 8.0% and 54.1% with most fungi possessing between 8.0% and 18.3% lipids. The predominant fatty acids were found to be palmitic, oleic and linolenic. Lesser amounts of arachidic, linolenic, palmitoleic, pentadecanoic, myristic and lauric acids were found. The mesophiles varied between 0% and 18.5% linolenic acid, while the thermophiles did not contain any appreciable linolenic acid (< 0.5%). The mesophile, *Mucor globosus*, and the thermophile, *Mucor pusillus*, contain γ linolenic acid. The fatty acids of the thermophilic fungi were more saturated than the corresponding mesophilic species.

INTRODUCTION

Several hypotheses have been proposed to explain the ability of microorganisms to grow at high temperatures. These include the dynamic and the stable protein hypotheses (1). Several suggestions have also been made to explain the failure of thermophiles to grow at ordinary temperatures (1). Most of these hypotheses have focused attention on the enzymes of the thermophilic bacteria (2,3). Similar studies with thermophilic fungi have not been made, nor have the lipids been investigated (4). It was considered possible that a comparison of total lipids and the composition of the lipids of some representative thermophilic and mesophilic fungi might be enlightening as concerns the unique character of thermophily. Consequently, 18 fungi were examined for their lipid composition. Nine fungi were thermophiles of seven different genera and nine fungi were mesophiles of the same genera. The mesophiles were selected for their morphological similarity to that of the thermophiles.

EXPERIMENTAL PROCEDURES

Discs of mycelium and agar were cut from 4-day-old cultures, using a sterile cork borer, and one disc was placed in each Erlenmeyer flask containing 50 ml of medium (10 g glucose, 2 g peptone, 1 g yeast extract, 1 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, and 1 ml microelements per liter) (5). The flasks were incubated in stationary culture for four days at 45 C (thermophiles) or 25 C (mesophiles), except that *Malbranchea pulchella*, a slow growing organism, was incubated for 12 days. The cultures were placed at 2 C until analyzed. Uninoculated liquid medium and liquid medium containing a disc of uninoculated agar were included as controls.

The contents of three culture flasks of each species were selected for apparent uniformity in development and combined. The combined mycelium was then filtered into a previously tared fiberglass mat and washed with 150 ml of distilled water. The wet mycelium was transferred to a 125 ml Erlenmeyer flask, including the fiberglass mat, and diluted 20-fold (v/w) with chloroform-methanol (2:1 v/v). After standing at room temperature for 24 hr the extract was filtered. The residue was again extracted for 10 min with boiling chloroform-methanol and filtered. The filtrates were combined and evaporated to dryness under nitrogen. The lipid residues were dissolved in 20 ml of chloroform-methanol and washed according to Folch et al. (6). The mycelial residue, including the fiberglass mat, was dried at 105 C for 24 hr.

Methyl esters were prepared by transesterification with 12.5% methanolic borontrifluoride (7). The esters were analyzed by gas chromatography as previously described (8). The fatty esters were identified by comparison of retention times with methyl ester standards (Supelco, Bellefonte, Pa.), by bromination, by hydrogenation, and the γ linolenic acid was also confirmed by mass spectrometry and ozonolysis (9).

RESULTS AND DISCUSSION

The weight of the dried extracted mycelia, the weight of the lipids and the per cent lipids, based upon the total weight of the dried extracted mycelia and lipids are given in Table I. The fungi contained from 8.0% to 18.3% lipids with four exceptions, *Malbranchea pulchella* (24.8% and 26.5%), *Stilbella thermophila* (38.1%) and *Chaetomium globosum* (54.1%).

The high percentage lipids found in these fungi may be due to an accompanying pigment, which was extracted by the lipid solvent.

The relative percentage of fatty acid found in the 18 species of fungi are presented in Table II. The predominant fatty acids are palmitic, oleic, and linoleic with lesser amounts of arachidic, linolenic, palmitoleic, pentadecanoic, myristic and lauric. The thermophiles contained only traces of linolenic acid and longer chain fatty acids ($< 0.5\%$). Most mesophiles possessed significant quantities of linolenic acid (0 to 18.5%).

Oleic acid was the most abundant fatty acid in the thermophiles while linoleic acid was most common in the mesophiles. There was no significant difference between the per cent unsaturation of the fatty acids of the thermophiles, 37.9% to 73.8%, when considered as a group, versus the mesophiles, 57.4% to 83.3% (Table III). However, if one compares the per cent unsaturation of the fatty acids within a genus, the thermophiles were usually more saturated.

A truer measure of the degree of unsaturation is the number of double bonds per mole of fatty acid (Table III) (8,10). This index places a greater weight on the polyunsaturated fatty acids. All the thermophiles examined possessed 0.56 to 1.01 double bonds per mole fatty acid and the mesophiles 0.96 to 1.60. Therefore, the thermophilic fungi as a group, were more saturated than the mesophiles and when individual genera were compared the thermophiles were always more saturated than the corresponding mesophiles. No unusually long chain fatty acids, that might impart thermostability, were found in the thermophiles.

The degree of unsaturation of the component fatty acids was of special interest because it has been reported that the fatty acids of organisms tend to be more saturated the higher the culture temperature (8,10-13). However, many exceptions to this generalization are known (15).

In most temperature studies the fatty acid composition has been compared within the same organism when it is grown at different temperatures. The thermophylic fungi will usually not grow at 25 C. Consequently, nine mesophilic fungi, which belong to the same genera as the thermophilic fungi and which are morphologically very similar to the corresponding thermophiles, were selected for comparison purposes and cultured at 25 C. In each case the thermophilic fungi were more saturated than the corresponding mesophilic fungi, however, variation does occur with individual species.

TABLE I

Percentage Lipid in Fungi

Thermophile	Mycelium[a] mg	Lipid mg	Per cent[b] lipid	Mesophile	Mycelium[a] mg	Lipid mg	Per cent[b] lipid
Chaetomium thermophile	428.5	44.6	9.4	*Chaetomium globosum*	296.7	349.6	54.1
Humicola grisea var. *thermoidea*	255.9	38.4	13.0	*Humicola grisea*	338.5	40.8	10.8
Humicola insolens	378.5	62.5	14.2	*Humicola nigrescens*	267.0	23.2	8.0
Humicola lanuginosa	58.6	12.2	17.2	*Humicola brevis*	159.9	27.3	14.6
Malbranchea pulchella var. *sulfurea*	132.5	43.8	24.8	*Malbranchea pulchella*	113.2	40.9	26.5
Mucor pusillus	378.0	85.2	18.3	*Mucor globosus*	134.4	26.9	16.7
Penicillium duponti	175.4	30.4	14.8	*Penicillium chrysogenum*	444.2	48.2	9.8
Sporotrichum thermophile	235.6	43.1	15.5	*Sporotrichum exile*	200.2	21.1	9.5
Stilbella thermophila	66.8	41.3	38.1	*Stilbella* sp.	472.0	96.7	17.0

[a]Weight of oven dry (100 C) mycelial debris after extraction.

[b]Calculated from the total weight of dried mycelial debris and lipids. The lipid content of the growth medium was negligible.

TABLE II

Percentage Fatty Acids in Fungi

Organism	12:0	14:0	15:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0
Thermophiles										
Chaetomium thermophile	---	---	---	57.8	3.1	4.4	8.0	26.8	---	---
Humicola grisea var. *thermoidea*	---	---	---	28.8	---	2.2	40.4	28.5	---	---
Humicola insolens	---	---	---	29.9	---	1.1	37.3	31.8	---	---
Humicola lanuginosa	---	---	---	21.4	---	4.5	65.2	8.6	---	---
Malbranchea pulchella var. *sulfurea*	---	---	---	26.2	---	7.5	35.0	31.3	---	---
Mucor pusillus	---	---	---	23.5	1.2	2.9	59.4	11.2	1.1[a]	0.8
Penicillium duponti	---	---	---	25.2	---	10.8	42.2	21.8	---	---
Sporotrichum thermophile	---	---	---	28.4	---	6.8	2.7	35.1	---	---
Stilbella thermophila	---	2.1	---	42.5	1.9	13.7	25.4	14.3	---	---
Mesophiles										
Chaetomium globosum	---	1.4	1.0	30.6	10.8	9.6	9.7	35.6	1.3	---
Humicola grisea	---	---	---	15.3	---	1.5	30.9	33.9	18.5	---
Humicola brevis	---	---	---	28.8	1.9	3.7	20.4	41.3	4.0	---
Humicola nigrescens	---	---	---	20.5	---	3.6	29.2	34.3	12.2	---
Malbranchea pulchella	---	---	---	11.3	---	11.4	26.6	50.7	---	---
Mucor globosus	2.1	7.6	---	26.1	7.7	6.9	25.8	8.3	15.6[b]	---
Penicillium chrysogenum	---	---	---	12.2	---	5.5	10.9	65.4	6.0	---
Sporotrichum exile	1.4	1.9	---	17.0	2.1	8.8	8.3	58.4	---	2.1
Stilbella sp.	---	---	---	19.5	1.4	2.3	13.4	58.3	5.0	---

[a]Contains γ linolenic acid. Percentage was estimated from results of hydrogenation of trapped gas chromatograph peak consisting of arachidic and γ linolenic acids.

[b]Contains γ linolenic acid.

Ideally the fatty acid composition comparisons should be made between cultures of fungi of similar physiological age, not necessarily with days of culture. The total lipid composition is known to vary greatly with age; however, the relative percentage fatty acid usually does not change appreciably with days of culture (8,15). Also, the medium composition can significantly affect the degree of unsaturation, particularly if it is deficient in nitrogen; under our culture conditions this was not a problem.

Only *Mucor globosus* and *M. pusillus* were found to contain γ linolenic acid. This acid has been found in fungi only of the class Phycomycetes (13,14). Thus it appears that evolution of thermophily has not affected the fungi's ability to synthesize this acid, although its relative concentration was very small. It would be worthwhile to examine other thermophilic Phycomy-

TABLE III

Degree of Unsaturation in Fungi

Thermophiles	Per cent unsaturation	Double bonds per mole	Mesophiles	Per cent unsaturation	Double bonds per mole
Chaetomium thermophile	37.9	0.65	*Chaetomium globosum*	57.4	0.96
Humicola grisea var. *thermoidea*	68.9	0.97	*Humicola grisea*	83.3	1.54
Humicola insolens	69.1	1.01	*Humicola nigrescens*	75.7	1.34
Humicola lanuginosa	73.8	0.82	*Humicola brevis*	67.6	1.17
Malbranchea pulchella var. *sulfurea*	66.3	0.98	*Malbranchea pulchella*	77.3	1.27
Mucor pusillus	71.8	0.83	*Mucor globosus*	57.4	0.96
Penicillium duponti	64.0	0.86	*Penicillium chrysogenum*	82.3	1.60
Sporotrichum thermophile	64.8	1.00	*Sporotrichum exile*	68.8	1.27
Stilbella thermophila	41.6	0.56	*Stilbella sp.*	78.1	1.47

cetes to determine whether they also possess the ability to synthesize γ linolenic acid. Zygospores have never been found for *Mucor globosus*, and only infrequently for *M. pusillus* (heterothallic). Therefore, the synthesis of this acid by these two fungi, plus the morphology of their hyphae and asexual structures, confirms their placement in the class Phycomycetes. The fatty acid composition of *Chaetomium globosum* and *Penicillium chrysogenum*, found in our study, varies somewhat from previous reports (13,14).

The differences in fatty acid composition between the thermophiles and mesophiles were not great, and would at first glance, appear not to aid in explaining thermophily. However, if these differences reside in the membrane lipids they could be highly significant and this will be investigated further.

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Mevalonate Biosynthesis in Rat Liver

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ABSTRACT

Mevalonate synthesis in rat liver can occur not only from HMG-CoA utilizing particle-bound HMG-CoA reductase, but from malonyl-CoA utilizing soluble enzymes. From a strictly quantitative standpoint, synthesis from malonyl-CoA appears to be of minor importance. The possible regulatory significance of two parallel routes to mevalonate is discussed.

INTRODUCTION

The reactions leading from acetyl-CoA via HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) to mevalonate, originally proposed for mammalian liver (1) have been substantiated both for this tissue (2-5) and for yeast (6-9). A second pathway for mevalonate biosynthesis occurs in soluble extracts of pigeon liver extensively purified as a fatty acid synthesizing system (10). Malonyl-CoA is an obligatory intermediate, and enzyme-bound acetoacetate and HMG (rather than their CoA thioesters) are intermediates. A ^{14}C-malonyl-S-enzyme complex has been isolated and shown to incorporate isotope both into fatty acids (in the presence of NADPH) and into HMG (in the absence of NADPH) (11). We wished to know whether malonyl-CoA can serve as a precursor of mevalonate in mammalian liver. We report that rat liver can form HMG and mevalonate directly from malonyl-CoA without prior decarboxylation to a 2-carbon precursor. As in pigeon liver, the reactions are catalyzed by soluble enzymes. Unlike pigeon liver, rat liver appears to synthesize most of its mevalonate from HMG-CoA utilizing microsomal HMG-CoA reductase.

MATERIALS AND METHODS

Radioactive Counting

Polar and nonpolar compounds were counted in 10 ml of dioxane (12) or toluene scintillation fluor [4.0 g 2,5-diphenyloxazole, 0.10 g 1,4-bis-2-(phenyloxazolyl)benzene plus toluene to 1 liter], respectively. Samples were counted three times for 10 min in a Packard Tri-Carb scintillation spectrometer, then recounted after addition of an internal standard to correct for quenching.

Chemicals

Chemicals obtained commercially included: HMG, DL-mevalonic acid, NADP, CoA, glucose-1-phosphate and ATP (Sigma Chemical Co.); glycylglycine (Calbiochem); β-*p*-dibromoacetophenone (Eastman Corp.); Celite 535 (Johns Manville Co.); 2,5-diphenyloxazole and 1,4-bis-2-(phenyloxazolyl)benzene (Packard Corp.); 2-^{14}C-acetic acid (sodium salt) (Volk); 1-^{14}C-acetic anhydride and 2-^{14}C-malonic acid (New England Nuclear Corp.); and 2-^{14}C-mevalonic acid (Nuclear Chicago Corp.).

Synthesis of Acyl-CoA Thioesters

Acetyl-CoA prepared from acetic anhydride and CoA (13) and malonyl-CoA prepared via the monothiophenyl ester (14) were purified before use by paper chromatography (15). Yield and purity of CoA thioesters was determined by absorption at 260 mμ, by hydroxamate formation and, where applicable, by measurement of radioactivity. Hydroxamates were quantitated by the procedure of Rudney (18). The molar absorbancy at 540 mμ for acetohydroxamate and malonohydroxamate was 1050.

Protein Determination. Protein was determined by a biuret method (16) standardized against serum albumin.

Quantitation of Mevalonolactone. Mevalonolactone was determined as its hydroxamate (17) using a modified ferric chloride reagent (8) which gives a more stable color complex.

Isolation of Mevalonate. Mevalonate was separated from other ether-soluble acids by chromatography on Celite columns (8,18). Ten grams of Celite 535 thoroughly mixed with 5.0 ml of 0.2 N H_2SO_4 (19) was used to prepare a firmly packed 2 cm diameter column. Incubation mixtures (see Tables), which contained 10 μmoles of carrier mevalonate, were deproteinized by heating at 80 C for 3 min and centrifuged. The protein pellet was washed with 1.0 ml of water and centrifuged. Mevalonate in the combined supernatant liquid and washing was converted to mevalonolactone, extracted into ether (17), and evaporated to dryness. The residues were dissolved in 0.3 ml of 12 N H_2SO_4 and 0.3 ml of water were then added. This solution was absorbed onto a portion of Celite (18), applied to the column, and the

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TABLE I

Mevalonate Synthesis by the 10,000 x *g* Supernatant Fraction

Substrate	Incorporation into mevalonate[b]	
	Trial 1	Trial 2
2-^{14}C-Acetate	68	149
1-^{14}C-Acetyl-CoA		5.3
2-^{14}C-Malonyl-CoA	4.1	1.8
2-^{14}C-Malonyl-CoA + 6 μmoles acetate	9.6	
2-^{14}C-Malonyl-CoA + 1.5 μmoles acetyl-CoA		5.7

[a]Tubes contained, in 3.25 ml: 160 μmoles glycyl-glycine (pH 7.5), 44 μmoles $MgCl_2$; 50 μmoles potassium citrate (pH 7.5), 30 μmoles potassium ATP, 1.2 μmoles NADP, 10 μmoles carrier mevalonate, 67 mg (Trial I) or 75 mg (Trial II) of 10,000 x *g* supernatant fraction protein, and either 6.0 μmoles 2-^{14}C-acetate, 0.3 μmoles 1-^{14}C-acetyl-CoA, or 0.3 μmoles (Trial I) or 0.5 μmoles (Trial II) 2-^{14}C-malonyl-CoA. The ATP of Trial I had been stored frozen several months. Fresh ATP was used in Trial II. Incubation was for 2 hr at 38 C.

[b]Results are expressed as μmoles substrate incorporated per gram protein per hour.

TABLE II

Mevalonate and Total HMG Synthesis[a]

Substrates	Incorporation into	
	Mevalonate[b]	Total HMG[b]
2-^{14}C-Acetate	195	410
1-^{14}C-Acetyl-CoA	6.3	76
2-^{14}C-Malonyl-CoA	1.9	59
2-^{14}C-Malonyl-CoA + 1.5 μmoles acetyl-CoA	2.5	36
2-^{14}C-Malonyl-CoA + 1.5 μmoles CoA		96

[a]Paired incubation mixtures were utilized for determination of mevalonate and of total HMG synthesis. Each contained, in 3.1 ml: 310 μmoles potassium phosphate (pH 7.0), 60 mg of 5,000 x *g* supernatant fraction protein, and either 6.0 μmoles 2-^{14}C-acetate, 0.3 μmoles ^{14}C-acetyl-CoA, or 0.3 μmoles 2-^{14}C-malonyl-CoA. Incubations for mevalonate synthesis contained, in addition, 15 μmoles glucose-1-phosphate, 1.4 μmoles NADP and 10 μmoles carrier mevalonate. Preliminary experiments showed addition of other cofactors failed to stimulate mevalonate synthesis in this crude, undialyzed preparation. Incubation was for 2 hr at 38 C.

[b]Results are expressed as μmoles substrate incorporated per gram protein per hour.

column was eluted with chloroform saturated with 0.2 N H_2SO_4. Mevalonolactone emerged in the 75-125 ml fraction. Paper chromatography in ethanol:15 N NH_4OH:H_2O::8:1:1 (v/v) revealed only one acid spot on visualization with ninhydrin (20). This had an R_f, 0.7, identical to that of mevalonolactone. Chloroform was removed in vacuo at 45 C and the residue dissolved in 1.0 ml of water. Duplicate 0.2 ml aliquots of this solution were used for counting or to determine mevalonolactone present. The sulfate present in chloroform extracts did not affect quantitative estimation of mevalonate (21).

Quantitation of Total HMG. Total HMG (free HMG plus HMG derivatives) was quantitated as the di-*p*-bromophenylacyl ester of the free acid (10). Incubation mixtures were brought to pH 11 with KOH and saponified for 1 hr at 38 C. Carrier HMG (100 mg) was added and the pH adjusted to 6.2 ± 0.2. Precipitated protein was removed by centrifugation, washed with 1 ml of water, and the combined supernatant liquid and washings used for preparation of di-*p*-bromphenylacyl esters (10). Di-*p*-bromophenylacyl esters isolated from incubation mixtures required two precipitations before a clearly crystalline product was obtained. From five to six total recrystallizations were required to obtain a final product (colorless plates, mp 131-132 C uncorr.) of constant specific activity.

Enzymatic

Liver extracts were prepared in two ways. The soluble pigeon liver system catalyzing mevalonate synthesis from malonyl-CoA had been extensively purified for fatty acid synthesis. A rat liver 10,000 x *g* supernatant fraction actively catalyzing fatty acid synthesis was employed [Doering (1964) reports that this system converts acetate to fatty acids about 100 times as effectively as to sterols]. The second preparation yields a 5,000 x *g* supernatant fraction from lightly perfused livers (3). This catalyzes cholesterol synthesis from acetate, the rate limiting reactions being those leading from acetate to mevalonate. Crude systems were intentionally selected to permit comparison of the relative importance of acetate and malonyl-CoA in mevalonate synthesis.

Preparation of the 10,000 x g Supernatant Fraction. The preparation is that of Doering (22). Livers were chilled in cold potassium phosphate buffer, blotted and weighed. Subsequent procedures were at 0 to 4 C. Livers were blended in a Monel metal blender for 15 sec with 3 vol of 0.1 M potassium phosphate buffer, pH 7.5. The resulting brei was homogenized at low speed with 10 strokes of a loose-fitting (0.5 mm clearance), Teflon-pestle Potter-Elvehjem homogenizer. After centrifugation at 10,000 x *g* for 25 min, the supernatant fraction was removed using a syringe with large bore needle to avoid disturbing either

TABLE III

Utilization of Preformed Total HMG for Subsequent Synthesis of Mevalonate[a]

Total incubation time, hr	NADP and glucose-1-phosphate added, hr	Mevalonate formed[b]
1	0	8.8
2	0	18
2	1	27

[a]Tubes containing, in 2.6 ml: 260 μmoles potassium phosphate (pH 7.0), 0.5 μmoles 2-^{14}C-malonyl-CoA, 10 μmoles carrier mevalonate, and 40 mg 5,000 x *g* supernatant fraction protein were incubated at 38 C for 1 or 2 hr; 12 μmoles glucose-1-phosphate and 1.0 μmole NADP were added when indicated.

[b]Expressed as μmoles malonyl-CoA incorporated into mevalonate per gram protein.

TABLE V

Effect of Avidin and Biotin on Mevalonate Synthesis From Acetate[a]

Additions	Incorporation into mevalonate[b]
None	116
5 μg Avidin	120
5 μg Avidin + 5 μg biotin	117
5 μg Biotin	109

[a]Tubes contained, in 2.6 ml: 260 μmoles potassium phosphate (pH 7.0), 12.5 μmoles glucose-1-phosphate, 1.1 μmoles NADP, 10 μmoles carrier mevalonate, 6.0 μmoles 2-^{14}C-acetate, and 46 mg 5,000 x *g* supernatant fraction protein. Incubation was for 2 hr at 38 C.

[b]Results are expressed as μmoles of acetate incorporated into mevalonate per gram protein per hour.

the lipid layer or the sediment. Two tenths of a milliliter of this 10,000 x *g* supernatant fraction were used in incubations.

Preparation of the 5,000 x g Supernatant Fraction. The preparation is essentially that of Bucher and McGarrahan (3). Livers perfused lightly with 8 ml of ice-cold phosphate-nicotinamide buffer were excised, chilled in buffer, blotted and weighed. Subsequent operations were at 0 to 4 C. Livers in 2 vol of buffer were homogenized by hand using six strokes of the above homogenizer. The buffer used for perfusion, rinsing and homogenization was 0.1 M in potassium phosphate (pH 7.4), 0.004 M in $MgCl_2$, 0.03 M in nicotinamide and 0.125 M in sucrose. The homogenate was centrifuged at 700 x *g* for 10 min to remove cell debris, and the resulting supernatant solution centrifuged at 5,000 x *g* for 15 min to obtain the 5,000 x *g* supernatant fraction. This was removed by syringe as described above. Incubations were started by addition of 0.2 ml of this fraction to tubes containing all other reactants.

TABLE IV

Mevalonate Synthesis in Air, Under Nitrogen and in the Presence of Added Bicarbonate[a]

	Mevalonate formed[b] from	
Gas phase	Acetate	Acetyl-CoA
N_2	323	6.0
Air	252	4.8
N_2 + $KHCO_3$	224	5.4

[a]Thunberg tube sidearms contained either 6.0 μmoles 2-^{14}C-acetate or 0.24 μmoles 1-^{14}C-acetyl-CoA. The body contained 7.0 μmoles glutathione, 0.2 μmoles CoA, 3.0 μmoles potassium ATP, 0.7 μmoles NADP, 9.0 μmoles glucose-1-phosphate, 8.0 μmoles $MgCl_2$, 200 μmoles potassium phosphate (pH 7.0), 10 μmoles carrier mevalonate and 98 mg 5,000 x *g* supernatant fraction protein. Tubes were evacuated and flushed five times either with air or with prepurified N_2 (less than 8 ppm O_2). Tubes to receive bicarbonate were then opened and 80 μmoles $KHCO_3$ added. Incubation volume after mixing was 3.0 ml. Incubation was for 2 hr at 38 C.

[b]Results are expressed as μmoles of acetate or of acetyl-CoA incorporated into mevalonate per gram protein per hour.

Fractionation of Liver Homogenates. To assess the ability of subcellular fractions to synthesize mevalonate from acetate, acetyl-CoA and malonyl-CoA, a liver homogenate prepared by the procedure of Bucher and McGarrahan (3) was fractionated by centrifugation at 0-5 C (Table VI). The 105,000 x *g* pellet was suspended in nicotinamide-phosphate buffer to a volume one tenth that of the 105,000 x *g* supernatant fraction.

Radioactive Substrates. The specific activities of substrates used in all incubations were: 2-^{14}C-acetate (4.32 x 10^5 cpm/μmole), 1-^{14}C-acetyl-CoA (2.02 x 10^6 cpm/μmole), and malonyl-CoA (4.42 x 10^5 cpm/μmole).

RESULTS

Mevalonate Synthesis From Malonyl-CoA

Both the 10,000 x *g* (Table I) and 5,000 x *g* (Table II) supernatant fractions of rat liver catalyze incorporation of isotope from 2-^{14}C-malonyl-CoA into mevalonate. Although total incorporation was low, synthesis was of the same order of magnitude for malonyl-CoA as for acetyl-CoA. Addition of a 5 to 10-fold molar excess of carrier acetyl-CoA or acetate to 2-^{14}C-malonyl-CoA incubations stimulated rather than depressed isotope incorporation into mevalonate. This suggests that malonyl-CoA can form mevalonate without prior decarboxylation and equilibration with a two-carbon pool. It furthermore is consistent with

TABLE VI

Mevalonate Synthesis by Subcellular Fractions[a]

Cell fraction assayed	Mevalonate formed[b] from		
	Malonyl-CoA	Acetyl-CoA	Acetate
600 x *g* supernatant	0.26	0.70	19
10,000 x *g* supernatant	0.55	0.65	22
105,000 x *g* supernatant	1.72	0.24	10
105,000 x *g* supernatant + 105,000 x *g* pellet (1X)	0.44	0.51	16
105,000 x *g* supernatant + 105,000 x *g* pellet (2X)	0.32		22

[a]Tubes contained, in 3.0 ml: 7.0 μmoles glutathione, 0.2 μmoles CoA, 3.0 μmoles potassium ATP, 9.0 μmoles glucose-1-phosphate, 8.0 μmoles $MgCl_2$, 0.7 μmoles NADP, 200 μmoles potassium phosphate (pH 7.0), the indicated radioactive substrate, and various cellular fractions as a source of enzymes. Substrates were either 0.5 μmole 2-^{14}C-malonyl-CoA, 0.5 μmoles 1-^{14}C-acetyl-CoA, or 6.0 μmoles 2-^{14}C-acetate. The quantity of each supernatant fraction was that calculated to be derived from 0.7 g of intact liver. The 105,000 x *g* pellet was resuspended in phosphate-nicotinamide buffer in one tenth the volume of the homogenate from which it derived, then added at the calculated original concentration (1X) or at twice this concentration (2X).

[b]Results are expressed as μmoles of substrate converted to mevalonate per gram of original liver protein per hour. The values are thus not directly comparable to those of other Tables where results are in terms of grams of protein.

formation of mevalonate from malonyl-CoA via reactions analogous to those shown for pigeon liver (10).

Activated forms of HMG (HMG-CoA or HMG-enzyme) are involved in mevalonate synthesis both from acetyl-CoA and malonyl-CoA. Rat liver preparations catalyzing mevalonate formation from malonyl-CoA ought therefore to form activated HMG. In the absence of NADPH, isotope from 2-^{14}C-malonyl-CoA was incorporated into total-HMG (HMG plus its alkali-labile derivatives) even more readily than into mevalonate (Table II). This observation parallels that of Brodie et al. (10) with pigeon liver. Isotope incorporation is stimulated by addition of CoA, but, unlike incorporation into mevalonate, is decreased by addition of carrier two-carbon precursors.

Utilization of Preformed Total HMG for Subsequent Synthesis of Mevalonate

The enhanced synthesis of Total HMG over mevalonate raised the question whether the Total HMG is subsequently available for mevalonate formation. Mevalonate formed after 1 or 2 hr was compared to that obtained after 2 hr when NADPH was withheld for the first hour (Table III). Preincubation in the absence of NADPH stimulated subsequent mevalonate synthesis. This is consistent with formation from malonyl-CoA of an HMG-derivative which subsequently is converted to mevalonate. The stimulation was far less than anticipated were all the accumulated Total HMG (Table II) subsequently converted to mevalonate. This is consistent with the observation (3) that the rate-limiting in rat liver occurs immediately prior to mevalonate synthesis.

Mevalonate Synthesis Under Conditions Designed to Prelude Malonyl-CoA Formation

Mevalonate synthesis via malonyl-CoA was proposed by Brodie et al. (10) as the major pathway for mevalonate synthesis by pigeon liver. To attempt to assess the relative contribution of synthesis via malonyl-CoA to total mevalonate synthesis we next studied mevalonate synthesis from two-carbon precursors under conditions designed to preclude malonyl-CoA formation. Mevalonate synthesis from acetyl-CoA and from acetate was studied in the presence and absence of bicarbonate, avidin and biotin. Mevalonate synthesis was unaffected by removal of CO_2 and was depressed rather than stimulated by addition of bicarbonate (Table IV). Addition either of avidin or of biotin were also without significant effect on mevalonate synthesis (Table V). While rat liver can utilize malonyl-CoA for mevalonate synthesis, its overall ability to form mevalonate is thus quantitatively unimpaired by blocking malonyl-CoA formation.

Mevalonate Synthesis by Subcellular Fractions

Since mevalonate synthesis from malonyl-CoA is known to involve soluble enzymes in pigeon liver, we asked whether this was true also for rat liver. When mevalonate synthesis was studied in homogenate fractions obtained by differential centrifugation, comparable

results were obtained with either acetate or acetyl-CoA in all fractions (Table VI). Incorporation of isotope from two-carbon precursors was depressed by the removal of the microsomal fraction and stimulated by its addition. By contrast, mevalonate synthesis from malonyl-CoA was most readily catalyzed by a particle-free supernatant fraction. In further contrast to mevalonate synthesis from two-carbon precursors, mevalonate synthesis from malonyl-CoA was inhibited by addition of a microsomal fraction. It thus appears that, like pigeon liver, rat liver utilizes soluble enzymes for conversion of malonyl-CoA to mevalonate.

DISCUSSION

Rat liver homogenates convert carbon 2 of malonyl-CoA both to Total HMG and to mevalonate. This conversion is direct, i.e., without prior decarboxylation to two-carbon compounds. For Total HMG synthesis, malonyl-CoA compares favorably with acetyl-CoA. For mevalonate synthesis, the two-carbon precursors are far more efficiently utilized. Mevalonate synthesis from malonyl-CoA is more readily observed using a 105,000 x *g* supernatant fraction as enzyme. Here malonyl-CoA serves as a relatively efficient precursor of mevalonate.

Although malonyl-CoA may serve as a major precursor of certain metabolities (i.e., ketone bodies) derived from Total HMG, it appears to be quantitatively of minor importance for mevalonate synthesis. Consideration of quantitative factors alone might suggest that the malonyl-CoA pathway is of minor physiological significance in rat liver. We do not consider this to be the case. What does seem clear is that rat liver can synthesize mevalonate in at least two ways: from acetyl-CoA via a particulate HMG-CoA reductase and from malonyl-CoA via soluble enzymes. The data of Siperstein and Fagan (23) suggest that cholesterol synthesis involves only the particulate pathway. These investigators showed that the soluble fraction of rat liver formed small quantities of mevalonate from acetate, and that this synthesis was insensitive to dietary cholesterol. We suggest that the soluble activity they report may represent synthesis via malonyl-CoA. The metabolic fate of mevalonate is not limited to steroid biosynthesis. Coenzyme Q and N^6-(Δ^2-isopentenyl)adenosine of transfer RNA (25) represent two key products of mevalonate in animals. Compartmentalization of two pathways for mevalonate synthesis could permit unrestricted synthesis of other key metabolites under conditions (such as cholesterol feeding) where steroid synthesis is curtailed. The physiological function of the soluble, malonyl-CoA pathway may thus ultimately relate to synthesis of isoprenoids other than sterols.

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Cholesterol Metabolism and Vitamin B_6: II. Intestinal Cholesterogenesis in Vitamin B_6 Deficient Rats

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ABSTRACT

The in vitro incorporation of 1-^{14}C-acetate into cholesterol by vitamin B_6 deficient and pair-fed rat intestinal tissue was assayed. It was found that vitamin B_6 deficiency increases, by a twofold factor, the incorporation of 1-^{14}C-acetate into the cholesterol biosynthetic chain, when using a segment of the ileum, and on the other hand no increase was observed when using a segment from the jejunum. The production of $^{14}CO_2$ from 1-^{14}C-acetate was not found altered by a vitamin B_6 deficiency when using segments from the jejunum or the ileum. The effect of sodium cholate and sodium chenodeoxycholate on the incorporation of radioactivity into cholesterol and on the production of radioactive CO_2, with 1-^{14}C-acetate as a substrate, was assayed with the two groups of animals. The significance of these findings and the possible relationships between these factors are discussed.

INTRODUCTION

Srere et al. have demonstrated that virtually all mammalian tissues are capable of synthesizing cholesterol, with the exception of the mature brain (1). The hepatic tissue has until only recently been considered the most important source of circulating cholesterol (2). It has now been shown (3,4) that in the dog and in the rat, extrahepatic cholesterogenesis contribute between 10% and 15% to the cholesterol circulating pool when hepatic cholesterol synthesis is suppressed by cholesterol feeding. More recently, Wilson, in a similar experiment with the squirrel monkey, demonstrated that 40% of the cholesterol circulating pool could be accounted for by extrahepatic cholesterol synthesis, and gives evidence for a contribution by the intestinal wall (5). Results by other investigators suggests that in man this situation may well exist, since cholesterol feeding does not suppress cholesterogenesis (4,6,7).

This laboratory has been interested for some time on the effects of vitamin B_6 deficiency upon cholesterol synthesis (8-12). It has been shown (12,13) that in vivo and in vitro, the incorporation of 1-^{14}C-acetate into liver cholesterol is increased by a 10-fold factor in the vitamin B_6 deficient rat. It was therefore of interest to assay intestinal cholesterogenesis in vitamin B_6 deficient rats, especially in the light of the postulate that the pathways of rat intestinal cholesterol synthesis are similar to those of rat liver (14).

MATERIALS AND METHODS

Sodium cholate and sodium-chenodeoxycholate (Steraloids Inc., N.Y.) were checked for purity by thin layer chromatography (TLC), using a solvent system of Ganshirt et al. (15), consisting of butanol-acetic acid-water (10:1:1).

Diets

Two groups of animals were used: a pyridoxine deficient group which had free access to tap water and to a semi-synthetic diet free of pyridoxine (Nutritional Biochemical Corp., Cleveland, Ohio); a second group (pair-fed animals) which received the same diet supplemented with pyridoxine hydrochloride (4.5 mg/kg) and which was allowed the same amount of food as that consumed by the deficient group. After eight weeks on such a diet the first group presented all the major signs of vitamin B_6 deficiency, such as loss of weight, swelling and necrosis of the paws, etc. The deficiency state was further confirmed by the determination of the taurine levels in the urine, according to the method of Bergeret and Chatagner (16).

Preparation of the Intestinal Tissue

Immediately after decapitation of the animals, the small intestine was excised, and the proximal and distal ends were identified and placed in an ice cold solution of Krebs-Ringer bicarbonate buffer (pH 7.4). A section of the jejunum (central 10 cm) and a section of the ileum (distal 10 cm) were cut away and flushed with a solution of Krebs-Ringer bicarbonate buffer containing 2 mg/ml of glucose.

Incubation Medium

Incubations were carried out in Warburg flasks containing 200 mg (wet weight) of either a central or distal segment of the small bowel, 4.0 cc of Krebs-Ringer bicarbonate buffer and 6 μc (2 mg) of 1-^{14}C-acetate (New England Nuclear Corp.). Whenever bile salt inhibition

TABLE I

^{14}C-1-Acetate Incorporation Rates Into Cholesterol and $^{14}CO_2$ Production of Pair-Fed and Vitamin B_6 Deficient Rat Intestinal Segments

	Pair-fed group				Deficient group			
Segment	No. animals	Cholesterol X ± SE DPM/g tissue	No. animals	$^{14}CO_2$ X ± SE DPM/g tissue	No. animals	Cholesterol X ± SE DPM/g tissue	No. animals	$^{14}CO_2$ X ± SE DPM/g tissue
Central	5	3230 ± 2916	3	$6.9 \times 10^5 \pm 1.8 \times 10^5$	5	4758 ± 1212	3	$5.2 \times 10^5 \pm 1.4 \times 10^5$
Distal	4	18,546 ± 2914	3	$13 \times 10^5 \pm 1.4 \times 10^5$	5	31,631 ± 470	3	$12.8 \times 10^5 \pm 4 \times 10^5$

was assayed, sodium cholate or sodium chenodeoxycholate at a final concentration of 2.5×10^{-6}M was added. Concentrated sulfuric acid (0.25 ml) was pipetted into the side-arm of the flasks and a piece of multi folded Watman No. 1 filter paper which had been moistened with hyamine hydroxide 10 X, was introduced in the center well. The reaction vessels were then gassed with 95% O_2/5% CO_2 and closed with a glass stopper, and the mixture was incubated in a Dubnoff metabolic shaker for 2 hr (37 C).

Determination of $^{14}CO_2$

The determination of radioactive CO_2 was carried out essentially as described previously (17). Briefly, the technique consists of stopping the reaction by the addition of the sulfuric acid contained in the side-arm of the flask; the flasks are then shaken for 15 min at 37 C to assure the total recovery of the $^{14}CO_2$, and each filter paper is introduced into a counting vial containing 15 ml of a PPO-POPOP solution in toluene (42 ml of liquifluor + 1000 ml of toluene).

Determination of 1-^{14}C-Acetate Into Cholesterol (Digitonine Precipitable Sterols)

The contents of the Warburg flasks were transferred to a 50 ml Erlenmeyer equipped with a condenser tube; the flasks were rinsed twice with 5 ml of distilled water. Fifteen pellets of KOH, ethanol (15 ml) and carrier cholesterol (4 mg in 1 ml) were added, the suspension heated at 80 C overnight and the neutral lipids were extracted with petroleum ether (bp 30 C-50 C) as described by Lupien and Migicovsky (18). The neutral sterols were precipitated as their digitonides and, after solubilization with 1 ml of hyamine hydroxide 10 X in a counting scintillator vial, the volume made up to 15 ml with a PPO-POPOP solution in toluene.

RESULTS

Daily Urinary Excretion of Taurine

The daily urinary excretion of taurine by the B_6 deficient rats was significantly lower ($P < 0.01$) than that of the pair-fed animals, as was expected. While the pair-fed animals excreted 14.2 ± 0.37 mg of taurine/24 hr, the urinary excretion of taurine by the deficient animals was found to be 2.2 ± 0.59 mg/24 hr.

1-^{14}C-Acetate Incorporation Into Cholesterol and Production of $^{14}CO_2$

As shown in Table I, the central intestinal segments from vitamin B_6 deficient rats showed no increased capacity of incorporation of 1-^{14}C-acetate into cholesterol when compared

to the pair-fed group. However, vitamin B_6 deficiency did appreciably affect the incorporation of 1-^{14}C-acetate into cholesterol when distal segments of the intestine were incubated; a twofold increase in incorporation rates were consistently obtained with this tissue ($P < 0.01$).

The $^{14}CO_2$ production from 1-^{14}C-acetate with central and distal segments from both groups of animals was identical.

Effects of Bile Salts on 1-^{14}C-Acetate Incorporation Into Cholesterol and $^{14}CO_2$ Production

The two bile salts assayed, sodium cholate and sodium chenodeoxycholate, had essentially the same effects at the concentrations used when added to the incubation media containing small bowel segments of either group of animals (Tables II and III).

Addition of sodium cholate and sodium chenodeoxycholate showed less inhibition ($P < 0.05$) on the incorporation of 1-^{14}C-acetate into cholesterol with the central segments; since the untreated distal segments had a higher specific activity relative to the untreated central segments, the effect of bile salts added to the distal segments was much more pronounced, although the specific activities of the treated distal and central segments showed no difference. Obviously the bile salt concentrations used (2.5×10^{-6} M) were sufficient to overcome the higher specific activities of the untreated distal segments. The inhibition by sodium chenodeoxycholate on both segments, jejunum and the ileum of the two groups of animals, was significantly greater ($P < 0.05$) than the inhibition by sodium cholate.

On the other hand, these two bile salts also inhibited the $^{14}CO_2$ production from 1-^{14}C-acetate in all intestinal segments of both groups of animals; in this case, however, sodium cholate proved to be a more potent inhibitor of $^{14}CO_2$ production than sodium chenodeoxycholate inversely to the results obtained for the incorporation of 1-^{14}C-acetate into cholesterol (Tables II and III). Again the results obtained show no significant differences between the central and distal segments of both groups of animals when expressed as their specific activities. It is noteworthy to mention that in both groups of animals, sodium chenodeoxycholate did not significantly inhibit the production of $^{14}CO_2$ from 1-^{14}C-acetate when the central segments were used.

DISCUSSION

Cholesterol Biosynthesis and CO_2 Production

It appears from these results that the distal intestinal segments of vitamin B_6 deficient ani-

TABLE II

Bile Salts Inhibition on ^{14}C-1-acetate Incorporation Into Cholesterol and $^{14}CO_2$ Production With Central and Distal Segments of Pair-Fed Rats. *Bile Salts Added at Final Concentration of 2.5×10^{-6}M. Pair-Fed Group*

Segment	Addition	Cholesterol			$^{14}CO_2$		
		No. animals	X ± SE DPM/g tissue	% Inhibition	No. animals	X ± SE DPM/g tissue	% Inhibition
Central	None	4	3230 ± 636	0	3	$6.9 \times 10^5 \pm 1.8 \times 10^5$	0
	Cholate	2	306 ± 84	90.6	4	$9.3 \times 10^4 \pm 1.8 \times 10^4$	86.5
	Chenodeoxycholate	3	87 ± 15	97.3	4	$4.04 \times 10^5 \pm 0.95 \times 10^4$	non Signif.
Distal	None	3	18,540 ± 2914	0	3	$1.3 \times 10^6 \pm 1.8 \times 10^4$	0
	Cholate	3	240 ± 52	98.7	3	$1.1 \times 10^5 \pm 1.5 \times 10^4$	91.2
	Chenodeoxycholate	4	115 ± 30	99.4	3	$3.05 \times 10^5 \pm 5.7 \times 10^4$	77

TABLE III

Bile Salts Inhibition on ^{14}C-1-acetate Incorporation and $^{14}CO_2$ Production With Central and Distal Segments of Vitamin B_6 Deficient Rats. Bile Salts Added at Final Concentration of 2.5×10^{-6}M. Deficient Group

Segment	Addition	Cholesterol			$^{14}CO_2$		
		No. animals	X ± SE DPM/g tissue	% Inhibition	No. animals	X ± SE DPM/g tissue	% Inhibition
Central	None	5	4758 ± 1212	0	3	$5.2 \times 10^5 \pm 1.4 \times 10^5$	0
	Cholate	4	381 ± 77	92	3	$9.3 \times 10^4 \pm 1.1 \times 10^4$	82.7
	Chenodeoxycholate	4	200 ± 19.7	95.8	4	$3.2 \times 10^5 \pm 7.0 \times 10^4$	non Signif.
Distal	None	5	31,636 ± 470	0	3	$12.8 \times 10^5 \pm 4.0 \times 10^5$	0
	Cholate	3	608 ± 172	98.1	4	$1.7 \times 10^5 \pm 3.0 \times 10^4$	86.7
	Chenodeoxycholate	4	172.5 ± 19.3	99.4	4	$2.8 \times 10^5 \pm 3.5 \times 10^5$	78

mals have a greater capacity of incorporation of 1-^{14}C-acetate into cholesterol (digitonine precipitable sterols) than those from pair-fed animals. Previous in vitro and in vivo studies in this laboratory have shown this to be also true for hepatic tissue (12).

Intestinal cholesterogenesis has been shown to contribute significantly to the serum cholesterol pool in most laboratory animals studied and in man (3-7). One would therefore expect serum cholesterol levels to rise in the vitamin B_6 deficient rat. The fact that this is not the case, as shown by Shah et al. (13), probably means that either the acetate pool is diminished or that cholesterol conversion to bile acids and the excretion of the latter are increased in vitamin B_6 deficient animals. Preliminary results from this laboratory suggest that some steps in the bile acid synthesis may be affected by vitamin B_6 deficiency. It therefore can be postulated that in vitamin B_6 deficient rats, the overall turnover of cholesterol may be increased.

The presence of sufficiently high concentrations of bile salts in the central segments of intestines has been shown (19) to be related to the low cholesterol synthesis in that region. These findings could explain the results obtained with the central segments, where no difference was observed in the rate of 1-^{14}C-acetate incorporation into cholesterol between the two groups of animals. Further evidence of this are the results obtained after the addition of bile salts in vitro. The fact that the bile salts assayed in these experiments caused the same rate of inhibition in tissues from both groups of animals seems of importance, especially in view of the recent suggestion that bile salts may be the primary regulatory mechanism of intestinal cholesterol synthesis. It can be concluded that this regulatory mechanism is not perturbed in vitamin B_6 deficient animals. However, it is recognized that other digitonine precipitable sterols might be implicated in the above phenomena, since changes in the composition of the unsaponifiable fraction could account in part or in whole for some of the effects observed.

Furthermore, the rate of $^{14}CO_2$ production, which grossly estimates the rate of oxydation of 1-^{14}C-acetate through the Krebs cycle, was found identical in tissues from both groups of animals, indicating again that the energy production mechanisms of the intestinal cells are not altered in the deficiency state.

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The Incorporation of Glycerol into the Glyceride-Glycerol of Fat Cells Isolated from Chronically Cold-Exposed Rats[1]

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ABSTRACT

The rate of 2-^{14}C-glycerol incorporation into the glyceride-glycerol moiety was measured in isolated fat cells from control rats maintained at 25 C and from animals chronically exposed to cold (5 C) for six to eight weeks. The rate of glycerol conversion (μM/μg DNA/30 min) was less in the cold-exposed rats, than in comparable controls (1.96 ± 0.14 vs. 2.68 ± 0.17). Very little glycerol was oxidized to CO_2 or converted to fatty acids. In both groups of animals, the glyceride-glycerol formation was apparently stimulated by noradrenaline in the presence of glucose, but this incorporation does not significantly bias the estimation of lipolysis based upon glycerol release.

INTRODUCTION

By use of the classical enzymological approach, Wieland and Suyter (1) were unable to find any significant glycerokinase activity in adipose tissue although they had purified and described the enzyme from rat liver. Other reports have apparently confirmed this result (2-5). On the other hand, Cahill et al. (6) demonstrated that glycerol was incorporated into glyceride-glycerol at a rate equivalent to 15% of glucose incorporation. In addition, Lochaya et al. (7) found that the glycerokinase activity in the adipose tissue of obese hyperglycaemic mice was greater than that of the nonobese controls and could be further increased over the controls by either insulin or epinephrine. Despite this evidence to the contrary, it became acceptable dogma that adipose tissue lacked any significant glycerokinase activity and that glycerol release could be used for accurately measuring triglyceride breakdown (8). Recently, however, Robinson and Newsholme (9) have rediscovered glycerokinase activity in adipose tissue and on the basis of their investigations have questioned the accuracy of estimating the rate of lipolysis based upon glycerol release. This work was carried out in adipose tissue. It is possible that glycerokinase could exist in either a much greater or lesser concentration in the adipocytes themselves. For this reason, we have measured the incorporation of radioactive glycerol into glyceride-glycerol in isolated fat cells and have determined whether conditions that markedly stimulate lipolysis such as chronic cold exposure and noradrenaline treatment (10) would likewise stimulate glycerokinase activity.

MATERIALS AND METHODS

Animals

Groups of 26 male Sprague-Dawley rats weighing between 50-100 g were obtained from Charles River Breeding Laboratories. These animals were divided into two groups and maintained in individual cages at either 25±2 or 5±1 C for six to eight weeks before being killed. In order to keep both the age and body weights of the control and cold-exposed rats similar, the heavier animals were placed into the 5 C chambers. Each group of rats was weighed once a day for the first five days of the experiment and then twice a week thereafter. Any animal whose weight deviated from the mean of previously established growth curves (11) by more than two standard deviations was not used. All rats were fed a Purina Lab Chow diet and water ad lib. The rats weighed approximately 350 g each at the end of the experimental period.

Preparation of Isolated Fat Cells

Isolated fat cells were prepared by a modification of the procedure of Rodbell (12). Rats were killed by decapitation, and the epididymal adipose tissue rapidly excised, blotted and weighed. Each gram of tissue was added to a 25 ml polyethylene vial containing 6 ml of 4% bovine serum albumin (Lot 55, Pentex, Inc.) Krebs-Ringer phosphate buffer without calcium, pH 7.4, plus 3 mg/ml of bacterial collagenase (Worthington Biochemical Co.). The buffer solutions containing either albumin or albumin plus collagenase were serially filtered through 0.65, 0.45 and 0.22 membrane filters (Millipore Corp.) prior to adding the tissue. The tissue was incubated at 37 C in a gyrorotary shaking water bath with a 1/2 in. circular orbit (New Brunswick Scientific Co.,

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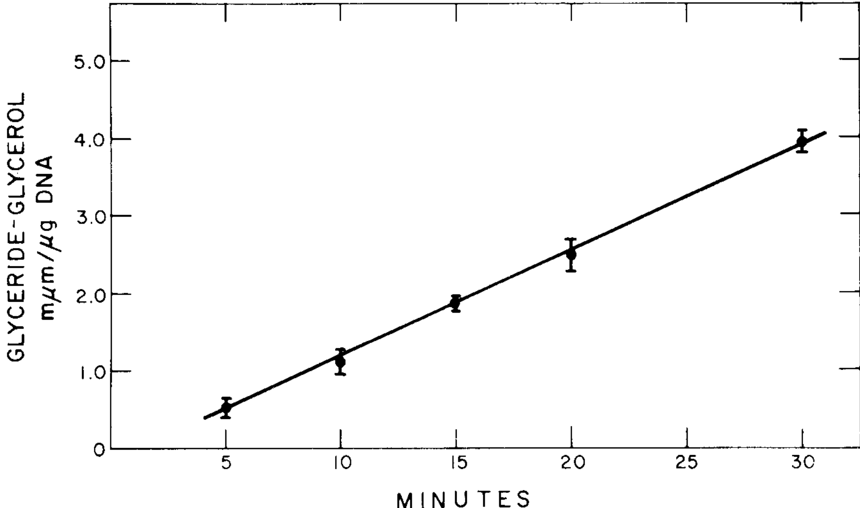

FIG. 1. Glyceride-glycerol formation in isolated fat cells incubated in the presence of 2-^{14}C-glycerol. Fat cells were prepared from the pooled epididymal fat pads of 12 rats maintained at 25 C. Each point represents the mean ± S.D. of three replications. Wherever possible, the analyses were performed in triplicate. The mean adipocyte content per vial for all samples was 19.67 ± 0.76 μg DNA (Mean ± S.D., n=15). Fat cells were suspended in 4 ml of Krebs-Ringer phosphate buffer containing per ml: albumin, 40 mg; 2-^{14}C-glycerol, 1.25 μC and 108.5 μmoles; and noradrenaline, 3 nmoles. Other conditions reported in Materials and Methods.

Inc.) at 200 revolutions per minute for 75 min. At this point, the fat cell preparation was strained through plastic mesh into a clean polyethylene vial and incubated an additional 15 min to insure uniformity in cellular disaggregation. The suspension was transferred to polyethylene tubes (16 x 114 mm) and centrifuged for 30 sec at 600 rpm in an International Clinical Centrifuge, Model CL. The fat cells floated to the surface. The sediment and infranatant solution were removed by aspiration and the cells washed with 4% albumin-phosphate buffer.

Histological examination of the cells after staining with methylene blue revealed the presence of mainly unilocular fat cells. The typical recovery of tissue triglyceride as washed fat cell triglyceride was 85% for the control and 65% for the cold-exposed rats.

DNA Determination

A portion of the washed, packed, isolated fat cells was transferred to 15 ml glass-stoppered conical centrifuge tubes. Lipid was extracted three separate times with 10 ml chloroform-methanol (2:1), followed by a final wash with 10 ml methanol. Centrifugation was carried out after each extraction, and the clear supernatant collected by aspiration. The lipid extracts were combined, the solvent evaporated to dryness, and the residue dissolved in 10 ml chloroform. The chloroform solution was washed with distilled water to remove nonlipid contaminants, and aliquots of the washed solution were used for gravimetric determination of lipid. The insoluble pellet remaining after lipid extraction was used for DNA analysis. The pellet was heated at 90 C for 45 min in 2 ml of 2% perchloric acid, and DNA was determined by the method of Ceriotti (13).

Incubation of Fat Cells

The remaining packed cells were suspended in an appropriate volume of albumin-phosphate buffer pre-equilibrated at 37 C. The reaction was started by adding 3.7 ml of the fat cell suspension by means of a large bore plastic pipette, to 0.3 ml of substrate in polyethylene vials. Incubation was carried out at 37 C in the gyrorotary water bath at 200 rpm. Appropriate zero time controls as well as experimental tubes containing everything but adipocytes were run concurrently. At the end of the incubation period the reaction was stopped by the addition of 5 ml Dole's (14) acid extraction medium (hexane as solvent). The extraction medium was added forcefully using an automatic pipette (International Applied Science Laboratory). After adding an additional 3 ml of hexane the vials were capped and shaken mechanically for 15 min. At least 15 min were required after shaking for separation of the 4.5 ml hexane and 7.5 ml aqueous phases.

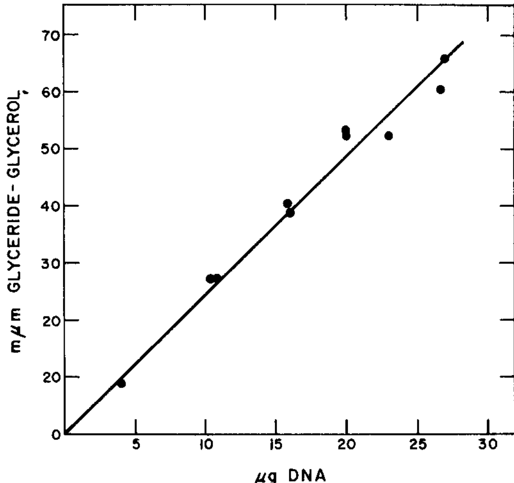

FIG. 2. Effect of fat cell concentration on the incorporation of 2-^{14}C-glycerol into glyceride-glycerol. Fat cells were prepared from the pooled epididymal fat pads of 12 rats maintained at 25 C. Each point represents the mean ± S.D. of three replications. Conditions are the same as in Figure 1, except that no hormone was added. Reaction stopped at 30 min.

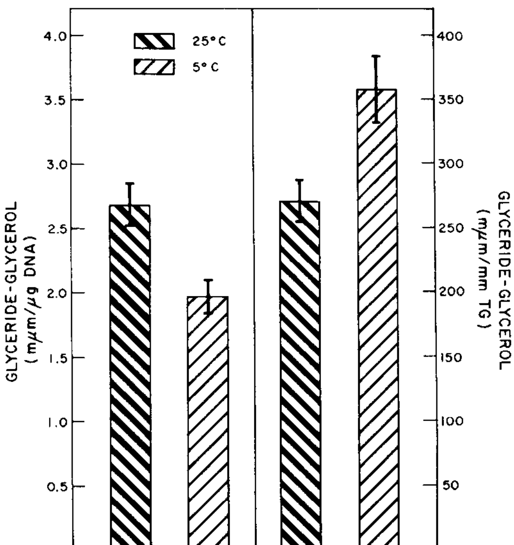

FIG. 3. The incorporation of 2-^{14}C-glycerol into adipocyte glyceride-glycerol based upon fat cell triglyceride and DNA. The conversion of 2-^{14}C-glycerol to glyceride-glycerol is shown as a function of adipocyte triglyceride content (right) and DNA (left). Fat cells were isolated from the epididymal fat pads of 6-12 rats per experiment. The results represent the mean ± S.E.M. of four separate experiments in the cold-exposed group (5 C) and five separate experiments in the controls (25 C). There were three replicate vials of cells in each experiment and analyses were performed in triplicate wherever possible. Incubation conditions as in Figure 2.

Glyceride Fatty Acid Determination

A 3.0 ml portion of the upper hexane phase was transferred into glass test tubes having screw caps fitted with teflon liners. The free fatty acids (FFA) were washed from the hexane phase using 3.0 ml of alkali extracting reagent (15), 2.0 ml hexane, 2.5 ml isopropanol and 4.0 ml of water. The tubes were capped, shaken 15 min and allowed to separate into a 5.0 ml hexane and 9.5 ml aqueous phase. A 1 ml aliquot of the hexane phase was taken for triglyceride radioactivity. To determine glyceride fatty acids, 1 ml of the hexane phase was evaporated and the lipid saponified at 75 C for 1 hr with 2 ml of ethanolic KOH (1 ml of saturated KOH per 20 ml of 95% ethanol, freshly prepared). After 2.0 ml of isopropanol and 4.0 ml of water were added, the sample was neutralized with 6N H_2SO_4 to a Nile Blue A end point and the fatty acids were extracted with 4 ml of hexane by shaking for 15 min. After phase separation, a 0.1 ml aliquot of the hexane phase was titrated by the procedure of Dole and Meinertz (14) using a motorized titrator (Menisco-Matic Buret, American Instrument Co.). The accuracy of the titrator was improved by the addition of a fine glass capillary tube recurved at the tip and attached to the NaOH reservoir. The radioactivity in the glyceride-fatty acids was measured by evaporating an additional 3 ml aliquot of the hexane phase in polyethylene counting vials and counted in a Packard Tri Carb scintillation spectrometer in 15 ml of scintillation fluid (16).

Free Fatty Acid Determination

In order to determine the amount of FFA produced during the incubation of fat cells, an 8 ml aliquot was taken from the 9.5 ml of aqueous phase produced by alkali washing the original lipid extract. This aqueous phase was neutralized to a Nile Blue A end point and extracted with 4 ml of hexane with shaking. A 1 ml aliquot of the hexane phase was evaporated in counting vials for the determination of FFA radioactivity. Another hexane aliquot (2 ml) was taken for FFA titration. The total fatty acid content of each vial of fat cells was the sum of free and glyceride fatty acids determined by titration. Since the lipids of adipose tissue are primarily triglycerides (17), the difference in radioactivity found in glyceride fatty acids and that in the intact triglyceride represents radioactivity in the glycerol moiety and is referred to as glyceride-glycerol.

Calculation of Adipocyte Concentration

The adipocyte concentration was calculated

by multiplying the triglyceride content by the adipocyte DNA to triglyceride ratio measured on aliquots of the initial packed cell suspension

Conversion of Glycerol to CO_2

The conversion of added 2-^{14}C-glycerol to $^{14}CO_2$ was measured in polyethylene vials containing the incubation mixture fitted with double rubber septums and polypropylene center wells (Kontes Glass Co.) containing 10% KOH on fluted filter paper as the CO_2 trapping agent (18). The reaction was terminated by injecting the acid extraction medium through the rubber stopper into the incubation mixture. The vials were allowed to stand at room temperature overnight to absorb the CO_2. At the end of this period the polypropylene center wells were transferred to 15 ml of scintillation fluid (16). The counting efficiencies of all radioactivity determinations were made using an internal ^{14}C-toluene standard.

Materials

2-^{14}C-Glycerol (New England Nuclear Corp.) was purified before use on a Polystyrene alkyl quaternary amine (Rexyn AG-1) ion exchange resin (19). The Rexyn AG-1 was washed successively with large volumes of 2 N NaOH, water, 2 N HCl, water and finally treated with 2 N NaOH followed by flushing with large volumes of water. The resin was then filtered to dryness and stored in a brown glass bottle. Glass columns (11 mm i.d.) were packed to a height of 11 cm with approximately 9 g of Rexyn AG-1. The sample of radioactive glycerol was applied to the column in 5 ml of water. The glycerol was quantitatively eluted from the resin with 25 ml water. Any polar contaminants remained on the column and could be eluted with 30 ml of 2 N HCl. (The degree of contamination by an unknown polar constituent was 14%.)

A solution of albumin bound fatty acids was prepared by combining 1.68 m moles oleic acid (Fisher, U.S.P.) with 50 ml of 8% bovine serum albumin (Pentex, Lot 55) in Krebs-Ringer phosphate buffer, pH 10.5. The mixture was stirred for 1 hr in a 37 C oven, then adjusted to pH 7.4 with HCl and brought to 100 ml with Krebs-Ringer phosphate buffer. The molar ratio of oleic acid to albumin was 27:1.

L-Arteronal bitartrate (noradrenaline) USA grade was purchased from Sigma Chemical Company.

Salmon testes DNA was obtained from Worthington Biochemical Corporation.

RESULTS

The rate of glycerol conversion to glyceride-glycerol by isolated fat cells was constant for at least 30 min (Fig. 1). On the other hand, the incorporation of 2-^{14}C-glycerol into CO_2 was low and nonlinear and there was no detectable incorporation into either free fatty acids or glyceride fatty acids. Similar results were obtained with fat cells isolated from rats exposed to 5 C for eight weeks. The incorporation of 2-^{14}C-glycerol into glyceride-glycerol of cold-exposed rats was also linear for 30 min; however, the rate was much less (2.21 ± 0.00 vs. 3.97 ± 0.14 nmoles/μg DNA).

The conversion of 2-^{14}C-glycerol to glyceride-glycerol by fat cells from control rats was proportional to the cell concentration up to 30 μg adipocyte DNA per 4 ml of medium (Fig. 2).

With isolated fat cells from rats of equivalent body weights (350 g), the ratio of microgram DNA per gram triglyceride was 114 ± 12 for controls and 207 ± 10 for cold-exposed rats. Thus, a mmole of triglyceride represents many more adipocytes from cold-exposed than control rats. The effect of this phenomenon on metabolic comparisons is demonstrated by Figure 3. On the basis of cell numbers (DNA), there is less incorporation of 2-^{14}C-glycerol into glyceride-glycerol in cells from cold-exposed rats (left side of Figure). However, when the activity is calculated on the basis of triglyceride content of the cells, the opposite is seen; there is a greater conversion of glycerol to glyceride-glycerol in cells from cold-exposed rats.

Figure 4 illustrates the effects of norepinephrine and glucose on the incorporation of 2-^{14}C-glycerol into the glyceride-glycerol of fat cells from rats maintained at 25 C and 5 C. A total of 47 control rats weighing 342 g and 27 cold-exposed rats weighing 334 g were used. The pooled epididymal fat pads from at least five rats were taken for each experiment. The left hand side of the Figure represents results obtained with fat cells from the 25 C rats. The bars are the mean ± SEM for five separate experiments without added glucose and mean ± S.D. of three replications in one experiment with added glucose. The right hand side of the Figure represents results obtained with fat cells from the 5 C rats. Here the bars represent the mean ± S.E.M. of four separate experiments without added glucose and the mean ± S.E.M. of three separate experiments with added glucose. Under all conditions there was significantly less 2-^{14}C-glycerol incorporated into glyceride-glycerol in fat cells from cold-exposed than control rats. The addition of glucose (GLUC) to the medium depressed the conversion of 2-^{14}C-glycerol into glyceride-glycerol. Even though norepinephrine had little

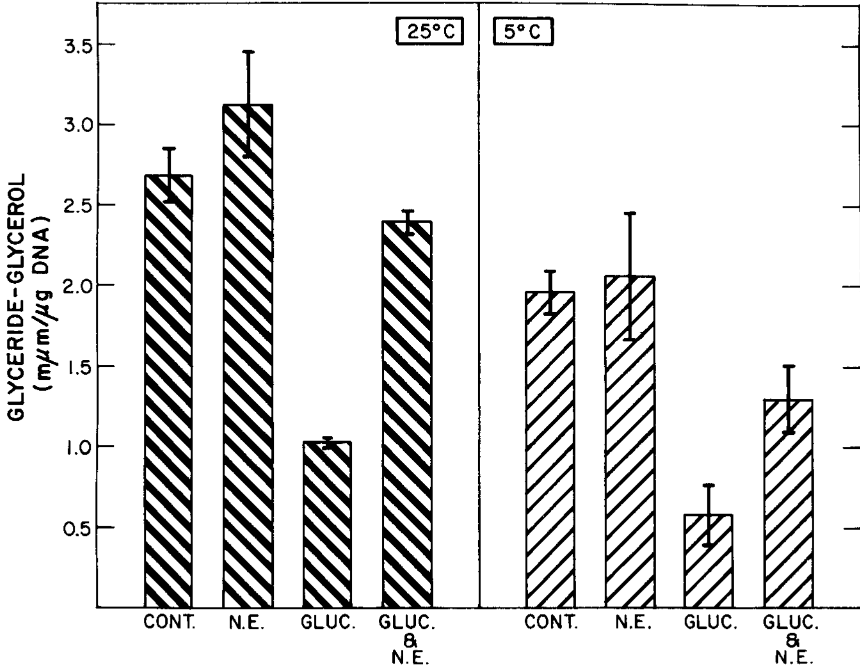

FIG. 4. Effects of noradrenaline (norepinephrine) and glucose upon the incorporation of 2-^{14}C-glycerol into the glyceride-glycerol of fat cells from rats maintained at 25 C and 5 C. Norepinephrine (N.E.) and glucose (GLUC), where present, were 3 nmoles and 1 mg/ml, respectively. There were three replicate vials of cells in each experiment and the concentration of cells per vial for all experiments (mean ± S.E.M.) was 22.1 ± 5.0 μg DNA per four ml total volume. Wherever possible, analyses were performed in triplicate. Incubation was for 30 min. Other conditions as in Figure 1.

effect on the incorporation of 2-^{14}C-glycerol into glyceride-glycerol in the absence of added glucose, it significantly stimulated the conversion in the presence of glucose. In each of three experiments on control rats, three replicate vials of cells were incubated in the presence of albumin bound oleic acid (4.5 mM). The incorporation of 2-^{14}C-glycerol (mean ± S.E.M.) was equivalent to 2.53 ± 0.5 μmoles glyceride-glycerol per μg DNA and was not significantly different from controls carried out in the absence of oleic acid.

DISCUSSION

In most studies with adipose tissue or the fat cells isolated from the tissue, metabolic activity has been expressed per unit weight of the tissue or cells. Recent work on the lipid composition and metabolism of adipose tissue (20-22) indicates that this may not be the basis of choice for making comparisons of metabolic function. As is evident from the preceding results, when a comparison is to be made between two groups of animals which have been subjected to different conditions such a consideration is of prime importance. Since the adipocyte fat content of rats exposed to a cold environment is considerably less than that of rats kept at normal ambient temperature (10,11,21) adipose tissue from rats exposed to cold would have more cells than an equivalent amount of tissue from rats maintained at 25 C. It is imperative under these conditions that the activity be expressed in some unit related to cell number rather than tissue mass. This is evident when we see, as is shown in Figure 3, the complete reversal of results obtained when adipocyte DNA is replaced by triglyceride content as the basis for measuring the incorporation of glycerol into glyceride-glycerol.

An analysis of other reports would indicate that measurements of ^{14}C glycerol incorporation into glyceride-glycerol can underestimate the true rate of enzyme activity due to the dilution effects of glycerol produced by fasting (2) or catecholamine administration (3). In this study glycerol was added to the fat cells in high concentrations in order that no significant change in its specific activity would occur through release of endogenous glycerol due to lipolysis. Since in 30 min norepinephrine (3

nmoles/ml) stimulates the release of 200 μmoles glycerol/μg adipocyte DNA (10), the use of 30 μg of adipocyte DNA results in less than 2% dilution of 2-^{14}C-glycerol. The experiments were carried out in the absence of glucose, in order to avoid as much as possible dilution of the α-GP pool by α-GP produced by glycolysis. Because of the low glycogen content in adipose tissue (23) and the length of time required for preparing the cells, it is unlikely that there were significant levels of endogenous glucose.

Results show that norepinephrine stimulates the conversion of glycerol to glyceride-glycerol, especially in the presence of added glucose. These results agree with the findings of Lochaya et al. (7) on the adipose tissue of mice but seem to be at variance with those reported by Lynn et al. (3) which indicate that epinephrine inhibits the conversion of ^{14}C-glycerol to glyceride-glycerol in rats. It should be pointed out, however, that the latter workers used very small amounts of carrier glycerol and ^{14}C-glycerol in their studies. The increased lipolysis due to the presence of epinephrine results in an increased release of unlabeled glycerol into the medium which would significantly dilute the ^{14}C-glycerol. It is suspected that the decreased incorporation of ^{14}C-glycerol into glyceride-glycerol is due to the dilution of label rather than a direct effect of epinephrine. The addition of glucose to adipose tissue is known to result in an increased production of α-GP (5). It is not surprising, then, that the presence of glucose inhibits the conversion of ^{14}C-glycerol to glyceride-glycerol, since the α-GP arising from glucose would dilute the ^{14}C-labeled α-GP formed from 2-^{14}C-glycerol. Norepinephrine also stimulates glucose transport and causes an increased conversion of glucose to α-GP (3,24-26). If norepinephrine had no direct effect on the conversion of glycerol to glyceride-glycerol we would expect a decreased conversion of 2-^{14}C-glycerol to glyceride-glycerol due to a further dilution of the ^{14}C α-GP. This is not the case. In the presence of glucose, the addition of norepinephrine results in a pronounced increase in 2-^{14}C-glycerol conversion to glyceride-glycerol. The question which cannot be answered at this time is whether this increased conversion is due to the stimulation of the glycerol $\rightarrow$ α-GP reaction or to the acceleration of reactions responsible for the conversion of α-GP to triglycerides. The apparent inhibition by added glucose would indicate that glycerol incorporation into glyceride-glycerol proceeds via α-GP rather than by direct esterification of glycerol with fatty acids. This is further supported by the lack of effect of added fatty acids. The lack of effect of exogenous FFA also indicates the incorporation does not proceed through either pyrophosphatase or glycerophosphatase activity (5).

The demonstration of glycerokinase activity in fat cells confirms previous findings by Robinson and Newsholme (9), who demonstrated the presence of glycerokinase activity in adipose tissue homogenates. However the activity found in isolated fat cells is at least twice as great as the maximum activity reported by Robinson and Newsholme. It should be concluded, therefore, that the glycerokinase activity is located primarily in the adipocytes rather than in other cells that make up adipose tissue. In order to compare our results with those of Robinson and Newsholme we assumed an equivalent amount of DNA in their preparation and converted the activity based on DNA.

The conclusion by Robinson and Newsholme that the glycerolkinase activity in adipose tissue may affect the accuracy of estimating the rates of lipolysis based on glycerol release by 15% may be valid when such measurements are made on intact fat pads. The specific activity of triglyceride lipase is low in fat pads, and the amount of released glycerol reincorporated into glyceride-glycerol could be significant. However, the specific activity of the triglyceride lipase in isolated fat cells is much higher than that of fat pads (200 μmoles/μg DNA/30 min). Considering that the incorporation of glycerol into glyceride-glycerol is only 3 μmoles/μg DNA/30 min when stimulated by norepinephrine, the error introduced by glycerol kinase in isolated fat cells would be no more than 1-2%.

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Lipid Composition of Beef and Human Pituitary Glands

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ABSTRACT

The lipid composition of beef and human pituitary was determined by chromatographic and spectrophotometric methods. Beef pituitary lipid contained about 25% nonpolar lipids and 75% phospholipids whereas nonpolar lipids made up approximately 60% of the total in human pituitaries. The main nonpolar (i.e., low polarity) lipids in human pituitary were triglycerides, cholesterol, free fatty acids and an unidentified component in the triglyceride fraction. Cholesterol was the major nonpolar lipid component in freshly collected beef anterior and posterior pituitary, but the amount of free fatty acids appeared to increase during storage. Preliminary investigation of the unknown nonpolar lipid in human pituitaries suggested that it was an unsaturated hydroxy compound with no carbonyl functions. Thin layer chromatography indicated that it was also present in smaller amounts in freshly collected beef pituitaries. The main phospholipids of beef anterior, posterior and human pituitary were phosphatidyl ethanolamine, phosphatidyl choline, phosphatidyl inositol, phosphatidyl serine and sphingomyelin. The fatty acid composition of total nonpolar lipids, free fatty acids, total phospholipids, phosphatidyl ethanolamine and phosphatidyl choline of beef anterior and posterior pituitary was determined by gas liquid chromatography. Mixtures of saturated and unsaturated fatty acids ranging from C_{12} to C_{22} were present; the main fatty acids were palmitic, stearic, oleic, linoleic and arachidonic.

INTRODUCTION

Although the pituitary gland is an important organ and has been the subject of numerous investigations, little information is available on its lipid composition. A comprehensive study of the lipids of beef anterior pituitary was carried out by Denstedt (1) using classical methods of analysis, but to our knowledge, pituitary lipids have not been analyzed by modern chromatographic methods.

The present studies were carried out initially with beef pituitaries because of their ready availability and larger size in comparison with pituitaries of common laboratory animals. The same methods were then applied to an analysis of human pituitary lipids. Anterior and posterior parts of the beef pituitaries were analyzed separately, but were found to be quite similar in lipid composition, and the human pituitaries were therefore analyzed as a whole. Substantial amounts of an unusual component were found in the nonpolar lipids of human pituitaries and this component was isolated chromatographically and a preliminary investigation of its structure was carried out. Thin layer chromatography (TLC) indicated that it was also present in smaller amounts in freshly collected beef pituitaries.

MATERIALS AND METHODS

Phospholipid standards were obtained from Mann Research Laboratories, New York; 1-monopalmitin and 1,3-distearin from F. H. Mattson, Procter and Gamble Laboratories, Miami Valley Labs., Cincinnati, Ohio; standard fatty acid methyl esters (NIH standards) from Applied Science Laboratories, State College, Pa., and the Hormel Institute, Austin, Minn; perchloric acid (Analar) from B.D.H., Toronto, Ontario; ascorbic acid from Fisher Scientific Co., Toronto, Ontario, and ammonium molybdate from J. T. Baker, Phillipsburg, N.J. Reagent grade chloroform, methanol and Skellysolve B (bp 67-69 C, dried over sodium) were redistilled from glass. Ether, acetone and acetic acid were of A.C.S. specifications. Silica gel was obtained from Brinkmann Instruments, New York and Florisil from the Floridin Company, Hancock, W.V. Beef pituitaries were obtained from L. J. Rubin and P. Ziegler, Research Laboratories, Canada Packers Ltd., Toronto, Ontario, and were kept frozen until extracted. Human pituitaries were obtained from D. M. Mills and A. C. Wallace, St. Joseph's Hospital and Westminister Hospital, London, Ontario. They were obtained at autopsy from both male and female individuals ranging in age from 11 to 78 years. Autopsies were carried out from 3 to 15 hr after death and the pituitaries were stored in the frozen state.

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TABLE I

Lipid Composition of Pituitary Gland

Tissue	Total lipid[a], %	Nonpolar lipid[b], % of total lipid	Phospholipid[b], % of total lipid
Beef anterior pituitary	3.2	25 - 27	73 - 75
Beef posterior pituitary	3.5	27 - 30	70 - 73
Human pituitary	3.8	57 - 60	40 - 43

[a]Averages for three determinations, calculated on the basis of weight of wet tissue.
[b]Range of values for three experiments in each case.

Extraction of Lipids

The beef pituitaries were freed of adipose and connective tissue and separated into anterior and posterior parts. Most of the pituitaries weighed between 1 and 1.5 g, and the anterior lobe made up 80% to 90% of the total. The separated portions from 10 to 15 pituitaries were pooled, ground with sand in a mortar and extracted with chloroform-methanol (2:1). For 1 g of tissue, 20 ml of solvent was used for the first extraction, and 10 ml each for two subsequent extractions. Each extraction was carried out for 24 hr at room temperature. The lipid extract so obtained was freed of nonlipid impurities by the procedure of Folch et al. (2), concentrated in a rotary evaporator at room temperature, made up in chloroform and stored at 4 C. Measured aliquots of this stock solution were evaporated under nitrogen, dried over KOH in a vacuum desiccator and weighed before chromatography.

Whole human pituitaries (average weight 0.5 to 0.6 g) were pooled in lots of 4 to 10 for extraction. In the earlier experiments extraction was carried out as described above. Later the pituitaries were homogenized in 20 vol of chloroform-methanol (2:1) with a VirTis homogenizer. The mixture was filtered and the residue re-extracted for 15 to 20 min and then overnight with 10 vol of the same solvent mixture in each case. Similar results were obtained with both methods.

Chromatographic Separations

The lipids were applied in chloroform solution to a 5 g column (1.2 x 8 cm) of acid-treated Florisil (3). Nonpolar (i.e., low polarity) lipids were eluted with chloroform (100 ml) and phospholipids with methanol (150 ml). The nonpolar lipids (20 to 100 mg) were analyzed further by chromatography on 12 g columns (1.2 x 20 cm) of Florisil deactivated with 6% (w/v) of water (4), and the fractions were monitored by TLC on plates of Silica Gel H, activated for 30 min at 120 C and stored at room temperature. Chromatography was performed in chromatographic chambers completely lined on all four sides with Whatmann 3 MM filter paper, saturated with solvent 30 min before insertion of the plate. The nonpolar lipids were separated with the solvent systems Skellysolve B-ether-acetic acid (70:30:1) or (60:40:1). The spots were developed either with iodine or by spraying with 0.6% potassium dichromate in sulfuric acid (55% by weight) and charring as described by Rouser et al. (5). Plates of Silica Gel H impregnated with silver nitrate were used for some separations. These were prepared by first running the plates in methanol-water (95:5) containing 15% $AgNO_3$ and then drying in air for 30 min. The phospholipids were separated on 20 x 20 cm plates by two-dimensional TLC (6) and were quantitated by the procedure of Rouser et al. (7), with the following modification. Instead of aspirating the charred spots into Kjeldahl flasks for digestion and later transferring to centrifuge tubes, it was found convenient to carry out the digestion with perchloric acid in the centrifuge tube itself. Optical densities for phosphorus determinations were measured with a Coleman Junior II Spectrophotometer 6/20.

Phosphatidyl ethanolamine and phosphatidyl choline for fatty acid analyses were isolated by preparative TLC, using the solvent system chloroform-methanol-water (65:25:4) and locating the lipid bands by spraying the plates with water (8). These preparations were shown to be homogeneous on TLC in two solvent systems as described above. The unknown component in nonpolar lipids was eluted from Florisil columns with the triglyceride fraction and was isolated for further study by preparative TLC with the solvent system, Skellysolve B-ether-acetic acid (70:30:1). The lipid was located by spraying with water and was recovered by scraping the appropriate portion of the plate and eluting with chloroform.

For analysis by GLC, methyl esters of fatty acids were prepared by refluxing the lipids with HCl in methanol. The reagent was prepared as described previously (9). The phosphatidyl ethanolamine fraction contained aldehydes as plasmalogens and the dimethyl acetals formed

TABLE II

Nonpolar Lipid Composition by Florisil Chromatography[a]

No.	Fraction	Stored beef pituitaries Anterior, %	Stored beef pituitaries Posterior, %	Human pituitary, %	Fresh beef pituitaries Anterior, %	Fresh beef pituitaries Posterior, %
1	Hydrocarbons	0.3	1.8	0.8	3.8	3.2
2	Cholesteryl esters	7.2	3.0	4.6	7.5	6.3
3	Triglycerides	3.0	3.2	55.6[b]	11.7	8.8
4	Cholesterol and diglycerides	34.2	58.7	18.7	65.3	70.0
5	Monoglycerides and pigments	1.7	3.0	1.7	6.0	10.1
6	Fatty acids	53.3	29.9	13.5	5.8	1.3

[a]Typical figures for one experiment are given in each case, but three separate analyses were carried out on the stored beef anterior pituitaries, two on the posterior and at least six on human pituitaries.

[b]Contains another major unidentified component (Fig. 1).

from these during transesterification were separated from methyl esters by TLC (10), for analysis by GLC. The analyses were carried out on a polar column (6 ft x 1/4 in. o.d. of 6% ethylene glycol succinate on Diataport S, 80-100 mesh at 170 C), using an F and M No. 402 gas chromatograph with flame ionization detector, and on a nonpolar column (6 ft x 3/16 in. of 3% SE-30 on siliconised Chromosorb W at 190 C) using a Barber-Colman Model 10 gas chromatograph with radium ionization detector. Fatty acid methyl esters were identified by comparison with pure standards and by a plot of log retention versus carbon numbers. In cases where standards were not available for unsaturated acids, equivalent chain length was employed (11-13). The relative amounts of fatty acids were estimated by the product of peak height and retention time as a measure of peak area (14). Our experience has shown that this method gives satisfactory results with standard mixtures, and this has also been confirmed recently by Brockerhoff and Ackman (15). The dimethyl acetals were identified on the basis of their retention times relative to those of fatty acid methyl esters.

RESULTS AND DISCUSSION

The total lipid and percentages of nonpolar and phospholipids of beef anterior, posterior and human pituitary are shown in Table I. Phospholipids constituted about three quarters of the total in beef anterior and posterior pituitary, while nonpolar lipids made up more than half of the human pituitary lipids.

Nonpolar Lipids

The composition of nonpolar lipids as determined gravimetrically after column chromatography on deactivated Florisil is shown in Table II. The TLC patterns of various fractions obtained from the column are shown in Figure 1. The main constituents of the nonpolar lipids of stored beef anterior and posterior pituitary were free fatty acids and cholesterol, which together made up 85% to 90% of the total fraction. The remainder consisted of cholesteryl esters and triglyceride, with some other minor components.

The nonpolar lipids of human pituitary constituted a much larger proportion of the total and showed some interesting differences in comparison to those of beef pituitary. In this case triglyceride and another unknown compound eluted with the triglyceride fraction (Fig. 1) were major components in addition to cholesterol and free fatty acids. As with beef

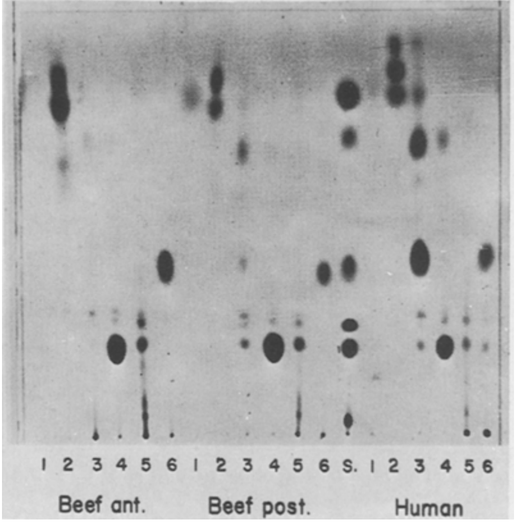
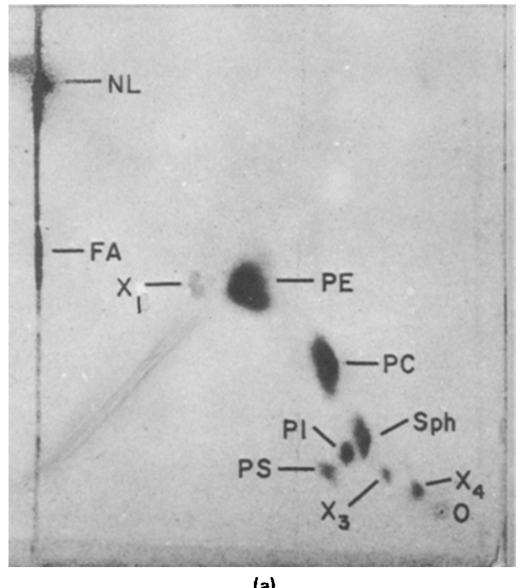
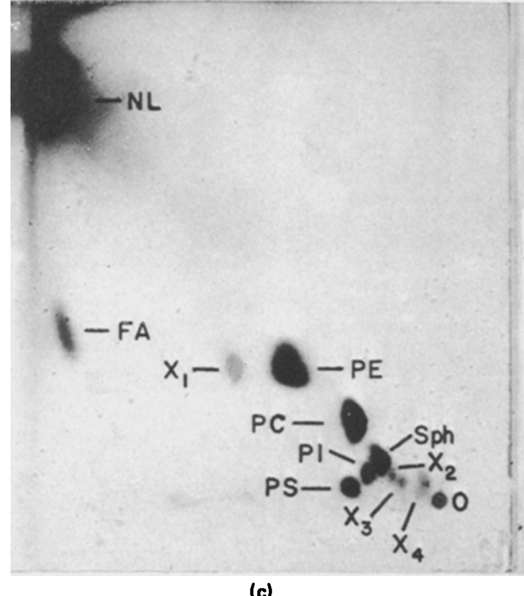


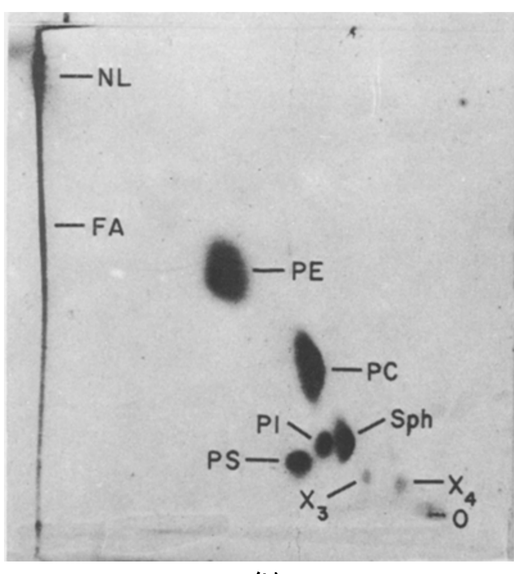
FIG. 1. Thin layer chromatogram of column fractions (1 to 6) of nonpolar lipids of beef anterior, beef posterior and human pituitary on Silica Gel H with the solvent system Skellysolve B-ether-acetic acid (60:40:1). Fraction S is a standard mixture of (from top to bottom) cholesteryl ester, triglyceride, fatty acid, diglyceride, cholesterol and monoglyceride.



(a)



(c)



(b)

FIG. 2. Two-dimensional TLC of lipids from (a) beef anterior (b) beef posterior and (c) human pituitary, showing the phospholipid components. The chromatograms were developed in the vertical direction with chloroform-methanol-28% by weight aqueous ammonia (65:35:5), followed by drying for 10 min and development from right to left with chloroform-acetone-methanol-acetic acid-water (5:2:1:1:0.5). Abbreviations: PE, phosphatidyl ethanolamine; PC, phosphatidyl choline; Sph, Sphingomyelin; PI, phosphatidyl inositol; PS, phosphatidyl serine; X_1, X_2, X_3 and X_4, uncharacterized components; FA, fatty acids; NL, neutral lipids; O, origin.

pituitary, the nonpolar lipids of human pituitary contained small amounts of cholesteryl esters and other unidentified components. Thin layer chromatograms showed three distinct spots in the cholesteryl ester fraction of human pituitary and only two in the corresponding fraction of beef anterior and posterior pituitary (Fig. 1). Minor spots corresponding to diglycerides are seen in the cholesterol fraction and similar spots are visible in the triglyceride and monoglyceride fractions. Some overlap of cholesterol into adjacent fractions is also apparent from the thin layer chromatograms, but this was not enough to significantly affect the results given in Table II. The above results illustrate the usefulness of combining thin layer and column chromatography for detection of minor components in naturally-occurring lipids and for demonstrating the presence of unknown lipids which overlap with other major components in one or other of the two chromatographic systems.

In order to obtain more information about the unknown component in the triglyceride fraction of human pituitaries, a small amount was isolated by preparative TLC. The purified material was a waxy semi-solid which showed no characteristic UV absorption. It stained readily with iodine vapor on thin layer chromatograms and could not be stored dry without alteration, presumably because of autoxidation. Hydrogenation with PtO_2/charcoal as catalyst gave a product which could readily be separated from the original compound by TLC. On Silica Gel H with the solvent system, petroleum

TABLE III

Phospholipid Composition of Pituitary Gland[a,b]

Phospholipid	Beef anterior	Beef posterior	Human pituitary
Phosphatidyl ethanolamine	32.5	25.3	25.0
Phosphatidyl choline	37.1	35.6	37.7
Sphingomyelin	8.8	11.2	11.4
Phosphatidyl inositol	5.9	6.7	4.2
Phosphatidyl serine	2.0	7.3	5.7
x_1	0.2	---	2.3
x_2	---	---	2.8
x_3	1.8	2.1	1.1
x_4	---	---	0.5
Origin	1.1	---	0.5

[a]The values are averages of six determinations for beef anterior, eight for beef posterior and four for human pituitary.

[b]Expressed as percentage of total phosphorus.

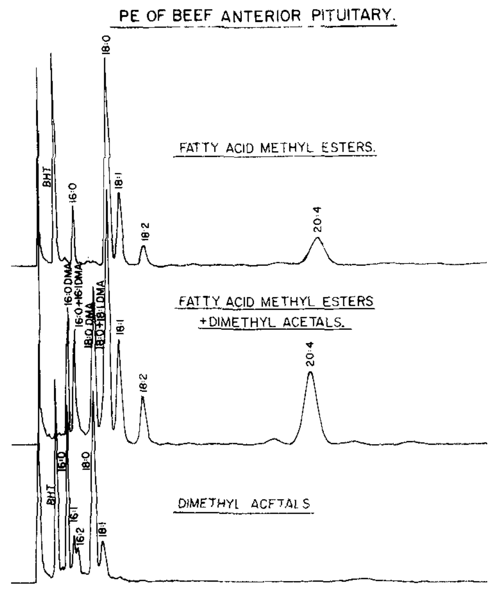


FIG. 3. GLC tracing of the fatty acid methyl esters and dimethyl acetals of phosphatidyl ethanolamine (PE) of beef anterior pituitary. Top and bottom tracings show the fatty acid methyl esters and dimethyl acetals after separation by preparative TLC. The anti-oxidant BHT (2,6-Di-tert-butyl-p-cresol) was added to the developing solvent during TLC.

ether-ether-acetic acid (70:30:1), the hydrogenated compound had a slightly greater R_f than the original, and on Silica Gel H, impregnated with silver nitrate, it had about the same R_f while the parent compound remained at the origin. Both compounds reacted with acetic anhydride-pyridine at room temperature to give acetyl derivatives which moved with the solvent front on Silica Gel H developed with the above solvent system.

The unknown lipid had an R_f value on TLC similar to that of free fatty acids, but the latter are not normally eluted from Florisil with triglycerides and the infrared spectrum of the unknown showed no absorption in the carbonyl region. Substances with similar chromatographic properties have been reported in tissue lipid extracts on previous occasions but have been found to be contaminants from various laboratory sources, including plastics (16,17). In an effort to eliminate this possibility, care was taken to collect the pituitaries in glass containers and to avoid any contact with plastics during extraction and chromatography. Arrangements were also made to collect pituitaries from two different hospitals to check on possible contamination at the source, but the unknown compound was present in all cases.

The lipids of beef pituitary, by contrast, did not appear to contain significant amounts of the unknown and, as noted earlier, the proportion of free fatty acids was considerably higher than in human pituitary lipids. Although the beef pituitaries were extracted soon after they were received in our laboratory, it was later learned that they might have been stored frozen for some time before shipment. It therefore seemed possible that the unknown compound, if present, could have been altered during storage and also that gradual hydrolysis of lipids might have given rise to the larger amounts of free fatty acids.

To check these points, a small lot of freshly collected beef pituitaries was obtained from Canada Packers in the frozen state and these were thawed, dissected and extracted immediately on arrival. The lipid extracts of anterior and posterior lobes contained 22% and 23% respectively of nonpolar lipids as compared to 25% to 30% in the lipids of stored pituitaries (Table I), and the proportion of free fatty acids was much smaller (Table II). Analysis of the triglyceride fraction by TLC also showed the presence of significant amounts of a component corresponding to the unknown in human pituitary lipids. It therefore seems probable that freshly-collected beef pituitaries can be used as an alternative source of this material for further structural analysis. The lipids of the anterior pituitary contained a larger proportion of the unknown than those of the posterior pituitary and a similar result was obtained in one experiment with human pituitaries in which anterior and posterior lobes were extracted separately.

TABLE IV

Fatty Acid Composition of Beef Pituitary Lipids[a]

	Beef anterior pituitary				Beef posterior pituitary			
Fatty acid[b]	Total nonpolar lipids	Total phospholipids	PE	PC	Total nonpolar lipids	Total phospholipids	PE	PC
14:0	0.5	0.5	0.2	0.9	1.4	0.5	0.6	0.8
15:0 + 16:0 DMA[c]	---	2.2	4.5	1.4	1.6	2.2	5.8	0.9
16:0 + 16:1 DMA	15.6	21.8	5.5	34.1	19.9	18.5	5.1	27.5
16:1	1.6	1.1	---	1.5	2.5	1.2	---	1.6
16:2	0.9	1.0	---	1.1	1.6	0.9	---	1.3
18:0 DMA + 17:0	0.7	3.8	9.5	1.3	1.8	2.5	8.9	---
18:0 + 18:1 DMA	16.1	15.3	18.9	14.4	15.8	16.8	14.8	18.1
18:1	18.2	15.2	9.3	20.5	22.7	16.6	7.7	23.9
18:2	17.1	7.6	5.4	9.5	9.7	6.2	2.4	7.4
20:0	---	0.6	0.3	---	0.9	0.7	---	---
20:1	2.4	0.7	0.4	0.7	1.2	0.8	---	1.7
20:2	3.1	1.0	0.5	1.2	0.5	0.9	---	4.2
22:0	2.0	1.8	1.7	1.4	1.6	1.6	4.3	2.3
20:4	20.3	14.8	20.7	8.5	13.7	12.5	21.3	9.4
20:5	---	0.9	1.1	---	---	---	---	---
22:4	---	2.7	4.1	---	1.8	7.9	14.2	---
22:5	---	7.0	14.2	---	---	4.7	7.9	---
22:6	---	2.5	2.5	---	---	4.1	6.2	---

[a]Average of two determinations.

[b]In the notation used, the first two digits give the number of carbon atoms and the third digit the number of double bonds.

[c]DMA, dimethyl acetal derivatives of the aldehydes. These were found mainly in the PE fraction.

Phospholipids

The phospholipid composition determined by two-dimensional TLC, followed by phosphorus analysis of the separated lipids, is given in Table III. Two-dimensional TLC patterns of the phospholipids of beef anterior, posterior and human pituitary are shown in Figure 2.

The main phosphatides of beef anterior, beef posterior and human pituitary were phosphatidyl ethanolamine and phosphatidyl choline, which together made up 60% to 70% of the total. Beef anterior pituitary contained more phosphatidyl ethanolamine and less sphingomyelin and phosphatidyl serine than either beef posterior or human pituitary. A few additional components, x_1 to x_4, were also seen on the two-dimensional chromatograms. Compound x_1 is probably cardiolipin and the others may be lysophosphatides.

The overall phospholipid composition was thus rather similar in each case and the same pattern was also seen on two-dimensional thin layer chromatograms of extracts from freshly-collected beef pituitaries. It is interesting to note that beef anterior and posterior pituitary show such close resemblance in both nonpolar lipid and phospholipid composition, since they represent quite different types of tissue.

Fatty Acid Composition

The fatty acid composition of total nonpolar lipids, free fatty acids, total phospholipids, phosphatidyl ethanolamine and phosphatidyl choline of beef anterior and posterior pituitary was determined. The identifications and percentages of the different fatty acids are given in Table IV, and GLC tracings are shown in Figure 3.

In general, the fatty acid composition of corresponding fractions was essentially the same in both anterior and posterior pituitary but fatty acid analyses of phosphatidyl ethanolamine and phosphatidyl choline showed characteristic patterns with significant differences. The patterns resemble those reported for the corresponding lipid classes in other tissues of various animal species (18-20). The major saturated fatty acid in beef anterior phosphatidyl ethanolamine was stearic, while palmitic predominated in phosphatidyl choline. The main unsaturated fatty acids in phosphatidyl ethanolamine were arachidonic, followed by oleic. In phosphatidyl choline the main unsaturated fatty acids were oleic, linoleic and arachidonic. Further, phosphatidyl choline had more saturated and monounsaturated acids while phosphatidyl ethanolamine had more polyunsaturated acids. It is significant that phosphatidyl ethanolamine contained C_{16} and C_{18} aldehydes and some other minor aldehydes while phosphatidyl choline contained very little of these. This is in general agreement with results

from other laboratories (21,22).

The fatty acid composition of the nonpolar lipids was similar in many respects to that of the total phospholipids. These analyses were carried out on lipid extracts from stored pituitaries in which most of the fatty acids of the nonpolar lipid fraction were present as free fatty acids, and analysis of the free fatty acid fraction alone gave rather similar results as well. These results might be interpreted as further evidence that the free fatty acids were derived from hydrolysis of phospholipids during storage.

We have recently become aware of a paper by J. Clement, G. Clement and M. Fontaine, C. R. Soc. Biol. 157:1716-1721 (1963), in which modern methods were used for analysis of the pituitary lipids of normal rats and of rats from which various other endocrine glands had been removed.

ACKNOWLEDGMENTS

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Long-Term Responses of Rats to Heat-Treated Dietary Fats: IV. Weight Gains, Food and Energy Efficiencies, Longevity and Histopathology

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ABSTRACT

Representative cottonseed salad oils, corn oils, lards and hydrogenated vegetable shortenings, and portions of the same fats heated at 182 C for 120 hr were fed as 20% of nutritionally adequate diets to weanling albino rats in longevity studies. Differences in the responses of rats fed diets containing the unheated and heated fats were generally small with respect to rates of gain, 12th week and adult weights, efficiencies of utilization of absorbed energy, incidences of grossly detectable diseases and longevities. There were no indications that the feeding of the heated fats had shortened survival times in comparison with the comparable unheated fat. Animals fed hydrogenated vegetable shortening, heated or unheated, survived the longest. However, gains were slightly slower with the heated cottonseed oil diets, and food efficiencies were slightly lower with the heated cottonseed oil and heated lard diets because of decreased digestibilities of these fats. The usual disabilities of old age such as nephritis, respiratory disease and periarteritis were present in all groups. The incidence of mammary tumors was high but did not differ significantly with the kind of fat, heated or unheated. Tumor incidence other than mammary was similar in both sexes and there was no significant difference between fresh and heated fats. Absence of adverse effects attributable to the heated fats during the life span of the rats is further evidence of the safety of these fats of the quality customarily consumed by the human population.

INTRODUCTION

Edible fats undergo chemical and physical changes when exposed to heat/or oxygen or both and, if the treatment is severe enough, some of the products may be harmful to experimental animals when fed under appropriate conditions. The nature and extent of the changes have been investigated extensively during the last few years, yet because of the complexity of the mixtures, there is still much to be learned regarding the composition and the biological effects of heated or oxidized fats. Three general types of chemical changes occur: oxidation, cleavage to smaller compounds and polymerization.

There is considerable evidence that the treatments to which edible fats may be subjected during practical home or institutional use do not cause the formation of deleterious substances in amounts sufficient to impair health (1-15). On the other hand, several teams of scientists have observed adverse biological effects when fats which had been severaly heated (2,11-13,16-25) or extensively oxidized (12,23-42) were fed or injected. Toxic substances can be concentrated in certain fractions of the treated fats by distillation or urea adduction procedures (12,16-21,28,30-37). Fats containing relatively large proportions of unsaturated fatty acids, particularly polyenoic acids, yield more of these toxic substances than the saturated fats (20,21,30). Factors in addition to unsaturation appear to be involved since in our experience different lots of a single type of oil yield reproduceably differing amounts of urea non-adducting fraction (NAF). In general, the biological effects of heated or oxidized fats are in proportion to the NAF content (25,27,31), but the effects differ depending on the nature of the fraction. Concentrates that are predominately monomeric tend to be absorbed more readily and are more toxic than dimers or longer chain polymers (12,20,31).

The small amounts of lipid-derived products developed in fats during cooking ordinarily cannot be detected biologically without extraction and concentration of the NAF. Furthermore, substantial amounts of severely heated fat can be included in the diet without adversely affecting growth or well-being of rats, particularly if associated with some unchanged fat (5,16,35,36).

Nevertheless, the presence in foods of even small amounts of possible deleterious substances, such as NAF, is cause for concern until there is sufficient evidence that these amounts are harmless. As mentioned above, existing data support the belief that fats used in foods or in cooking of foods are safe in this respect, even

though other evidence makes it quite clear that abuse of fats by excessive exposure to heat or oxygen will result in formation of toxic products. For the most part previous biological tests have been of short duration and may not have detected substances that would elicit a response on prolonged administration or in massive doses. It was to study the cumulative and chronic effects of heated fats, if such effects exist, that the present experimental work was designed and conducted. The findings, as reported, confirm the conclusions of the short-term tests, i.e., heat treatments more severe than those used in cooking foods yet in a practical range do not result in formation of sufficient quantities of fat-derived products to cause serious adverse biological effects. They are in substantial agreement with the observations of Nolen et al. (15) who conducted longevity studies with rats fed fats heated in the presence of foods.

EXPERIMENTAL PROCEDURES

In order to obtain the information desired, uniform groups of rats were fed diets which contained either a typical commercial fat or portions of this fat which had been subjected to a relatively severe heat treatment. Responses of the animals were compared with respect to: (a) rate of growth, (b) ability to digest the fats, (c) efficiency of food and energy utilization, (d) incidence of various abnormalities during life, (e) longevity, (f) gross changes observed at autopsy, and (g) organ weights. Tissues were taken at autopsy and preserved for subsequent histopathological study in the Human Nutrition Research Division, ARS, USDA.

Four widely used dietary fats were selected: cottonseed salad oil (CS), corn oil (CO), lard (L) and hydrogenated vegetable oil shortening (S). Three prominent brands of each were purchased during the course of the study in the necessary quantities in regular packages (38-50 lb) from commercial suppliers. Equal aliquots of the three brands of a specific fat were blended together for use in the diets and for heating prior to use. Both heated and unheated samples were stored in filled, sealed glass jars at 3 C until used.

Heat Treatment

Fats were heated at 182 C continuously for 120 hr in 46 lb quantities in a commercial, electrically heated deep fat fryer equipped with a chromed steel container and a stainless steel clad heating element. When used in this quantity there were 6.5 sq in. of surface exposed to air for each pound of fat heated, a ratio typical for this type of equipment. During heating the fats were stirred gently to avoid local differences in temperature. The stirring was not vigorous enough to cause entrapment of air bubbles, but it did provide a continuous change of the surface exposed to air and to the heating element. Fats treated in this manner are designated as HCS, HCO, HL and HS to differentiate them from the unheated CS, CO, L and S.

This relatively long and severe heat treatment was considered to be a compromise between short-term, single use of fats which would result in only minimal changes that might not be detectable biologically and more drastic treatments that have no practical counterpart. It was known from previous work (12,25,31) that such a treatment would be severe enough to decrease the digestibility of CS, the fat which had been most extensively studied. On the other hand, rats fed diets containing fats heated under similar conditions were known to grow well and to be apparently healthy for periods up to 12 weeks (11). It was also known that CS treated in this manner would be beyond practical commercial usage, as it would foam violently if attempts were made to introduce foods such as potatoes (12,14,42,43).

Certain analytical characteristics of the fats are shown in Figure 1. The general increases in viscosity and in the content of the urea non-adducting fraction, and the decreases in linoleic acid content and iodine values are greater than is typical for such fats used in practical food frying operations. Peroxide values were low and changed little by the heating procedure.

Preparation and Composition of Diets

Nutritionally complete, synthetic-type diets including 20% of the various test fats were prepared by adding appropriate quantities of the fats and 1% of wheat germ oil to aliquots of a uniform supply of a basal diet mix having the following composition: purified casein, 20.1; purified lactalbumin, 10.0; Jones and Foster salt mixture, 4.0; cellulose (Cellu Flour), 2.0; sucrose, 35.2; primary grown yeast, 5.0; d-α-tocopherol dry mix (Myvamix, 44 I.U./g), 1.0; choline chloride dry mix (25%), 0.4; vitamin A dry mix (Stabmix A, 10,000 I.U./g), 0.2; vitamin D_2 dry mix (Daves Sterol D-2, 1500 I.U./g) 0.2; and vitamin premix, 1.0. The vitamin premix contained, in mg/g, riboflavin, 0.45; niacin, 1.5; d calcium pantothenate, 3.5; pryidoxine hydrochloride, 0.65; biotin, 0.033; 2 methyl naphthoquinone, 1.0; *p*-aminobenzoic acid, 10.0; vitamin B_{12}, 0.001; and sucrose sufficient to bring the total to 1.000 g. The first five com-

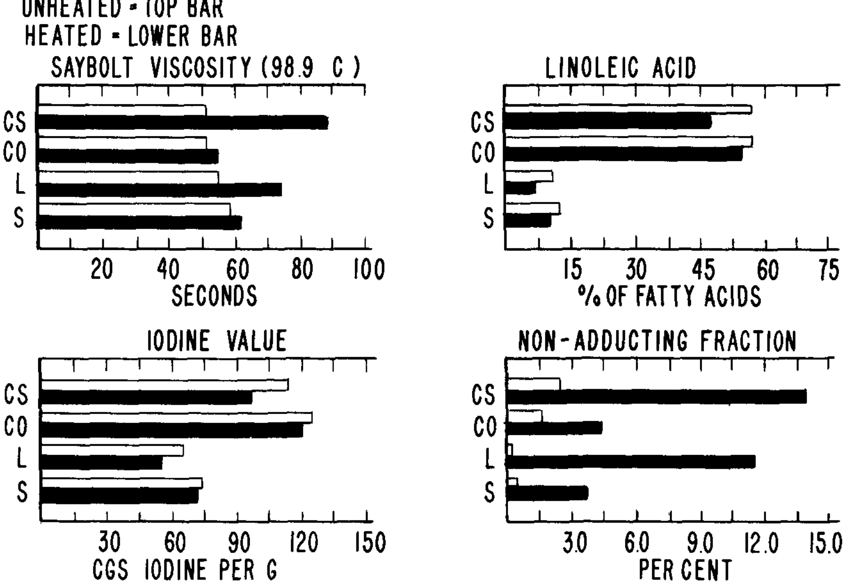

FIG. 1. Analytical values of fats before and after heating.

ponents of the basal diet were premixed in advance (premix 1 stored at room temperature), and the remaining six were likewise premixed (premix 2 stored in brown bottles at 3 C).

The diets were made by first mixing the proper aliquots of premix 1 and premix 2, then adding, during mixing, the appropriate melted fat and, finally, the wheat germ oil. Analyses of the diets gave average values as follows (in per cent): moisture, 3.3; protein, 27.2; fat, 21.9 and ash, 3.6. Fresh batches of each diet were prepared at intervals of about two weeks. Weighed quantities of fresh food in 3 or 6 oz widemouth glass jars were supplied three times weekly to the animals during the growth period and at least weekly thereafter. Food and water were supplied ad libitum. Uneaten food was weighed to determine food usage.

Care of Animals

Fifteen replications, each comprising one male and one female weanling rat (Holtzman albinos) for each unheated fat diet group and two of each sex for each heated fat diet group, were started weekly on the test diets for 15 weeks. Rats were caged individually in drawer-type cages with wire mesh fronts and bottoms. The rats were randomly distributed in cages suspended on racks in an air conditioned room with temperature controlled at 25-26 C and humidity at 45-50%.

Animals were weighed at about the same time each Wednesday. At that time uneaten food was removed for weighing, and each animal was inspected carefully for disease or physical abnormalities. A few rats with rales or suspected middle ear infections were isolated, but no treatment of any kind was administered to the animals except for trimming a few elongated incisors when necessary. Animals which showed severe or consistent losses of weight or other conditions that indicated ill health were observed more frequently. Those which became obviously moribund were decapitated and autopsied immediately to assure tissues suitable for histopathological evaluation. Animals which were found dead were autopsied as soon after death as possible.

During the 2nd, 12th and 52nd week of feeding, complete collections of feces were made for 10 randomly selected rats in each of the dietary groups. Lipid was extracted with ethyl ether from the pooled, acid hydrolyzed sample for each group for each period and weighed following solvent removal. Fat intakes for the same rats were calculated from the food intake and food composition records. The difference between the total dietary fat intake and the total fecal fat output represents gross fat absorption (uncorrected for endogenous fat). The weights of fat absorbed divided by weights of fat consumed gives practical digestibility values.

When the animals were 22 weeks of age, two males and two females which had been pre-

TABLE I

Average Weights of Rats Fed Diets Containing Unheated or Heated Fats for 12, 52 or 78 Weeks

Fat in diet	Average weights of rats in grams					
	Males			Females		
	12 wk	52 wk	78 wk	12 wk	52 wk	78 wk
CS[a]	451	655	681	268	385	458
HCS[b]	427[c]	634	674	253[c]	380	455
(CS/HCS) x 100	106	104	101	106	101	101
CO[a]	432	666	697	260	391	469
HCO[b]	431	653	723	265	394	464
(CO/HCO) x 100	100	102	96	98	99	101
L[a]	436	613	632	257	367	428
HL[b]	430	638	722	256	365	440
(L/HL) x 100	102	96	88	100	101	97
S[a]	440	643	680	262	358	442
HS[b]	428	615	648	254	361	425
(S/HS) x 100	103	105	105	103	99	104
All unheated	440	644	672	262	375	449
All heated	429	635	692	257	375	446
(Unheated/heated) x 100	103	101	97	102	100	101

[a]Fifteen animals started in each group.

[b]Thirty animals started in each group.

[c]Significantly less ($P \leq 0.05$) than for rats fed the unheated oil. Data for the 78 week periods were not statistically analyzed because of the small and irregular group sizes.

selected randomly for each group were killed for gross and histopathological examination. This procedure was repeated with other pairs of animals at ages of 36 and 50 weeks.

Except for the six randomly selected animals in each group killed for autopsy and tissue samples, as just described, all animals were maintained on their respective diets until they died or became moribund, or until a preselected termination point based on reduction of the colony to about 10% of the starting number was reached. At this time (928 days of age), all survivors were killed for termination gross and histopathological observations. At the time of autopsy, observations regarding the gross appearance of the animal and its organs and tissues were recorded; designated organs (liver, kidneys, adrenals) were removed, weighed and preserved in 10% neutral formalin for possible future histopathological study. All autopsies for this experiment were coded, i.e., done without knowing the diet group to which the animal belonged.

RESULTS AND DISCUSSION

Two general types of observations were made with respect to the influence of heating on the wholesomeness of fats, those relating to the effectiveness and efficiencies of the several fats tested as measured by growth and food utilization, and those relating to the health and longevity of the animals. Neither of these criteria gave any indication that fats heated for 120 hr at 182 C adversely affected the general health or well-being of rats to which they had been fed for their full life span. There were some minor differences between groups when heated-unheated comparisons were made, but these tended to balance out, some being in favor of the heated, others in favor of the unheated.

Growth

Animals fed heated fats grew well and at about the same rates as their counterparts fed unheated fats and maintained their weights over the long-term, as shown by the data in Table I. The only effect noted was a slightly slower rate of gain for each sex in the HCS diet group ($p \leq 0.05$ at 12 weeks, not significant thereafter). The slightly slower rate of growth for HCS fed rats is consistent with previous observations (21,25,31). There was generally more variation

TABLE II

Food and Calorie Efficiencies of Rats Fed Unheated or Heated Fats for 12 Weeks and Digestibility of Fats Fed

1	2	3	4	5	6	7	8	9
Sex and diet type	Average 12 wk gain, g	Average total 12 week food disappearance, g[a]	Grams food consumed per gram gain	Calories absorbed per gram gain[b]	Digestibilities[c] of fats in the diet, %			Digestible energy per gram diet, calories[d]
					2nd wk	12th wk	52nd wk	
Males								
CS	398	1219	3.06	14.4	91.6	92.3	93.7	4.70
HCS	376	1231	3.28	14.3	80.0	76.3	65.9	4.37
CO	381	1175	3.08	14.6	93.6	92.8	93.3	4.71
HCO	380	1192	3.14	14.6	91.1	90.2	89.8	4.66
L	385	1186	3.08	14.6	91.0	92.0	93.7	4.73
HL	378	1265	3.34	15.0	76.1	79.9	77.4	4.48
S	385	1244	3.23	14.6	86.0	83.7	89.3	4.52
HS	376	1216	3.23	14.6	86.7	84.1	76.8	4.53
All unheated	387	1206	3.11	14.5	---	---	---	4.66
All heated	378	1226	3.25	14.6	---	---	---	4.51
Females								
CS	217	874	4.02	19.1	93.7	94.2	95.0	4.74
HCS	203	922	4.55	20.0	79.7	78.1	72.6	4.41
CO	210	869	4.14	19.6	95.4	94.8	94.2	4.75
HCO	214	900	4.21	19.7	92.2	91.9	91.7	4.69
L	206	864	4.19	20.0	92.6	94.4	94.6	4.78
HL	206	915	4.44	20.1	75.9	82.3	79.1	4.53
S	211	906	4.30	20.0	88.6	91.0	79.0	4.67
HS	204	883	4.33	20.0	86.4	88.6	82.2	4.62
All unheated	211	878	4.16	19.7	---	---	---	4.73
All heated	207	905	4.38	19.9	---	---	---	4.56

[a]Food disappearance corresponds closely with food consumption, as only a few crumbs were scattered and not weighed.

[b]Calculations based on digestible energies shown in the last column in this table.

[c]See text.

[d]Calories were calculated using average diet composition and factors of 3.95 for sucrose, 4.27 for protein, 9.30 for vegetable oil and 9.50 for lard, according to USDA Handbook 8, (44). The 9.30 and 9.50 combustible energy values were used for fat, because digestibility was based on data for 12 week fat digestibilities listed in Column 7.

between the types of unheated fats fed than there was between the heated vs. unheated variants of each type. The long-term weight differences between the four types of unheated fats fed were never more than 9% and were not significant statistically at any age.

Food Efficiency and Digestibility

At the end of 12 weeks on diet the rats fed HCS or HL had consumed more food per gram gain than animals on diets containing unheated fats of the same types, but those fed HCO or HS had not consumed more than their counterparts (Table II, Column 4). The increased food consumptions were statistically significant ($P \leq 0.01$) for the males and females fed HL and for the females fed HCS. However, when the comparisons were made on a per calorie absorbed basis rather than on a per gram of food consumed basis, the differences between the heated and unheated fats disappear (Table II, Column 5). It seems probable that the decreased food efficiencies of animals fed diets containing HCS or HL are due to nondigestible substances in the fats, presumably polymers originating during the heating of the fats (19,20,31). The presence of nondigestible lipids is demonstrated by the digestibility values for each of the three time periods (Table II, Columns 6, 7 and 8). Substantial decreases in digestibility for HCS vs. CS and HL vs. L were observed for all periods. The digestibility values are similar for the 2nd, 12th and 52nd week for each unheated fat, however, there is some indication that the heated fats were progressively less well digested as the animals aged. The statistical significance of the differences observed could not be determined because it was necessary to pool feces for rats in each group to provide sufficient sample for analysis.

The equal utilizations of energy other than that of the nondigestible portions of the fats is evidence that digestibilities of the other components of the diets were not affected. These observations differ from those of Friedman et al. (31) who, using cottonseed oil stirred for 190 hr at 225 C, found that, "This difference in nutritive value (i.e., energy) cannot be accounted for by the decreased digestibility of the heated cottonseed oil." However, their fat, having been much more severely treated, was not comparable to those used in our tests.

Incidence of Diseases or Abnormalities

In general the animals remained healthy and lived long, relatively disease-free lives but, as expected with a group of aging animals, a variety of physical abnormalities was noted. These observations were recorded in detail and tabulated according to type and treatment of the fats and sex of the animal. The abnormalities were typical of those occurring in the rat and there were no substantial differences in frequencies between types of fat or treatments.

TABLE III

Survival of Rats Fed Diets Containing Unheated or Heated Fats

Dietary fats	Average survival in rat days		
	M	F	M & F
CS	635	738	687
HCS	650	706	678
Both	646	715	680
CO	656	683	669
HCO	657	706	681
Both	656	700	678
L	551	808	679
HL	662	696	679
Both	632	727	679
S	667	767	717
HS	691	765	728
Both	684	766	725
All unheated	627	749	688
All heated	665	718	692
Entire experimental colony	646	734	691

Longevity

The overall survival of the rats in this experiment was good, an average of 646 days for the males and 734 days for the females, and there were no indications that animals fed heated fats had shortened lifespans. In fact, in five of the eight heated-unheated comparisons, the average survival was greatest in the groups fed heated fats although the differences were not statistically significant (Table III). When data for both sexes and all fats are combined the average survival periods for the heated and unheated fat-fed animals are almost identical, i.e., 688 vs. 692 days.

There is a substantial indication that the subgroups fed diets containing S or HS survived longer than the subgroups fed the other fats, although the differences are not statistically significant at the $P \leq 0.05$ level. The lard-fed rats were the least consistent in their responses to the treatments, the males fed heated lard surviving longer than those fed unheated lard, whereas the females reversed this order. Furthermore, the lard-fed males had the shortest average survival period of all the male

TABLE IV

Incidence of Tumors in Rats Fed Throughout Life Diets Containing Different Fats

Type fat	Number of rats fed[a]	Percent of animals showing tumors: Females, Mammary	Females, Nonmammary, Benign	Females, Nonmammary, Malign.	Males, Nonmammary, Benign	Males, Nonmammary, Malign.
CS	9	44	22	11	33	0
HCS	24	50	4	4	12	8
CO	9	22	0	33	0	22
HCO	24	50	4	13	4	21
L	9	56	11	11	0	11
HL	24	46	0	13	8	13
S	9	45	11	0	0	0
HS	24	46	0	17	4	4
Totals						
Unheated	36	42	11	14	8	8
Heated	96	48	2	12	7	12

[a]For each sex.

subgroups. At least part of the shorter survival of the unheated lard-fed males may be attributed to unexplained early deaths of three of the nine-member life-survivor subgroup. The expected longer survivals of all female subgroups in comparison with the corresponding male subgroups is highly significant ($P \leq 0.01$).

Gross Observations at Autopsy: Organ Weights

The detailed gross observations made at autopsy were distributed rather uniformly among the groups with no unusual observations or conditions attributable to heating of the fats.

The consumption of diets including heated fats did not result in significantly different kidney or adrenal weights when compared with organs from animals fed unheated fats. For those rats routinely killed for tissue samples during the first year of the experiment, the liver-body weight ratios were higher for animals fed heated fat diets than for those fed the corresponding unheated fat diets for all comparisons except that for the S-HS male subgroups. The increases were significant ($P \leq 0.05$) for the male and female subgroups fed the HCS diet, and for the female subgroup fed the HL diet. No significant differences attributable to the heating of the fat components of the diets were found for livers removed from rats found dead or dying, but the liver weights and the liver-body weight ratios of male rats fed the HCS diet were significantly heavier ($P \leq 0.05$) than those for male rats fed diets containing HCO, HL or HS.

Histopathology

Pathological changes were those commonly found in old animals and included respiratory disease, chronic nephritis and periarteritis, the latter occurring mostly in males without relation to diet. In the animals with tumors, the incidence of nephritis was low in those fed hydrogenated vegetable oil (less than 8% compared to 21% on the other diets and present whether fats were heated or unheated).

Table IV gives the incidence of tumors in both sexes and characterizes them as benign or malignant. Of 132 male rats, 23 had tumors, of 132 female rats, 77 were tumor bearers, 61 had mammary tumors and 5 of these had additional tumors located elsewhere.

Mammary tumors were found in 46% of the female rats with a similar incidence among the groups fed different fats. Because of their superficial location they were observed early and were mostly of long duration. The average age at death for mammary tumor bearers varied from 728 days for those fed corn oil to 797 days for those fed hydrogenated vegetable shortening. Tumor weights varied greatly; 15 were under 50 g and 25 were over 250 g. The largest tumor, weighing 835 g, was found in a rat dying at 539 days and had been noted first at 296 days. The body weight after the tumor was removed was 242 g. This was the youngest rat of those dying with mammary tumors. Mammary tumors were often multiple and some were bilateral. The usual picture was that of a benign fibroadenoma. Some showed chronic inflammatory reaction and occasionally abscess formation. Adrenal enlargement was associated with these tumors, of 33 adrenals examined, 24 showed extensive hemorrhage often with cortical lipoidosis. Nonmammary tumors were as frequent in males as in females.

Relatively few tumors developed with diets containing hydrogenated vegetable shortening. Benign tumors predominated in the rats fed cottonseed oil. The highest incidence of malignancy was observed in the animals fed corn oil but too few tumors were involved to establish the significance of these findings. Neoplasms in these rats were mainly of connective tissue origin (sarcomas) rather than of epithelial tissues (carcinomas). Skin tumors, five in male rats and two in females were listed as epitheliomas since they showed excessive growth of cells, formation of keratinized epithelial pearls and local infiltration, but were of low malignancy and probably secondary to chronic ulceration. No distant spread was present except in one 928 day old female with a large tumor between the legs which had some nodules in the mesentery. Subcutaneous tumors were found in nine males and five females. In the male, seven were benign and two sarcomatous. In the female all but one were malignant, of these one was carcinomatous. An adenocarcinoma of the salivary gland might also be added to this group. In none of these were distant metastases found. Abdominal tumors, including those of the gastrointestinal, endocrine and reproductive systems, were found in 10 males and 14 females. In male rats, three were benign and seven malignant, mostly diffuse and involving the gastrointestinal tract; five of these were sarcomatous and two carcinomatous. In female rats there were five benign and nine malignant tumors. The benign tumors included two adenomas of the adrenal gland, two of the pancreas and one uterine fibromyoma. In contrast to males, tumors of the reproductive system were common, six occurring in the uterus and adnexas of which five were malignant. A pancreatic duct carcinoma was the only tumor with distant metastases to the lung. The only primary tumor in the liver was a bile duct carcinoma. Two diffuse lymphosarcomas were found involving most of the abdominal viscera.

Age Incidence of Nonmammary Tumors

The average age at death for males with benign or malignant tumors was over 675 days. Female rats with benign tumors averaged 691 days, those with malignant tumors 546 days. The earlier deaths occurred mostly in those with tumors of the reproductive system.

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Fatty Acids: XXII[1] Partial Synthesis of Racemic Helenynolic Acid From Crepenynic Acid by a Possible Biosynthetic Route and the Discovery of *cis*-9,10-Epoxyoctadec-12-ynoic Acid in *Helichrysum bracteatum* Seed Oil[2]

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ABSTRACT

Racemic helenynolic acid has been prepared from crepenynic acid by epoxidation followed by base-catalyzed rearrangement. This may be the pathway by which helenynolic acid is produced in *Helichrysum bracteatum* seed oil from the crepenynic acid also present. A reinvestigation of the epoxy acids in this oil has shown that *cis*-9,10-epoxyoctadec-12-ynoic acid accompanies the coronaric acid previously identified.

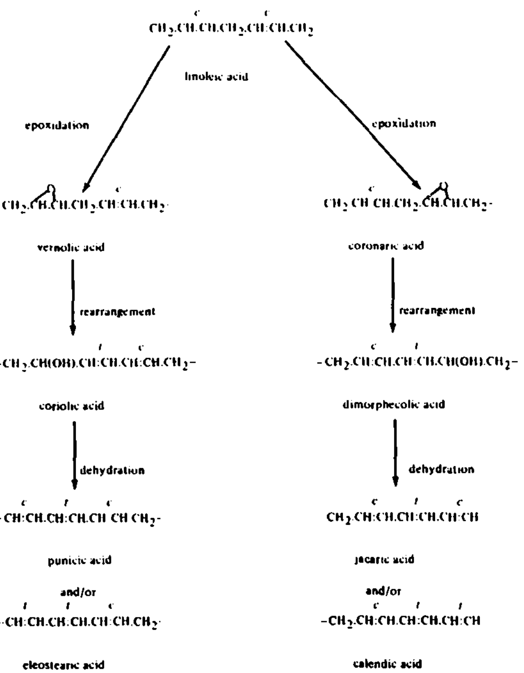
INTRODUCTION

The many long chain acids which have been discovered in recent years include a number of conjugated 9,11,13 and 8,10,12 octadecatrienoates, some hydroxy conjugated octadecadienoates, and several epoxy acids. It has been proposed recently that these are biogenetically related and that the conjugated trienoic acids are produced from the very common linoleic acid via epoxy monoenoic and hydroxy dienoic acids as in Scheme 1 (1,2).

There is no proof of this hypothesis but the smooth conversion of vernolic acid to coriolic acid has been demonstrated in vitro. This change occurs in high yield under mild conditions (0 C, 1 hr) under the influence of lithium diethylamide (2). Attention has been drawn to the correlation of absolute configuration which should exist between these natural acids and it has been suggested that other stereoisomers await discovery (2).

Helichrysum bracteatum seed oil is known to contain several unusual acids including (in addition to palmitic, stearic, oleic and linoleic) coronaric acid, hydroxy dienoic acids (including possibly α-dimorphecolic), crepenynic acid, and helenynolic acid, this last being a new hydroxy enynoic acid (3-5). (Following the designation of natural eleostearic acid ($9c11t13t$) as the α isomer and the all *trans* acid as the β-isomer, we use (14,15) the symbols α and β with coriolic and dimorphecolic acids to indicate the *cis,trans* and *trans,trans* isomers respectively). If α-dimorphecolic acid arises from linoleic acid via coronaric acid then it seems likely that helenynolic acid might be formed from crepenynic acid via an undiscovered epoxy acetylenic acid.

We have now shown that crepenynic acid readily furnishes racemic helenynolic acid by epoxidation and rearrangement and, encouraged by this, we have re-examined the epoxy acids of *Helichrysum bracteatum* seed oil and shown them to contain the hitherto unknown *cis*-9,10-epoxyoctadec-12-ynoic acid.



SCHEME 1

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EXPERIMENTAL PROCEDURES

All solvents were dried and distilled before use. Petroleum refers to the fraction of bp 40-60 C.

TLC was carried out with thin layers of silica (0.3 mm for analytical purposes and 1.0 mm for preparative purposes). When required, silver nitrate (15%) was incorporated into the silica layers. The developing solvent was ether mixed with benzene or petroleum and these mixtures are indicated by symbols such as PE30 indicating a 70:30 mixture of petroleum and ether.

A Pye 104 was used for GLC. It was fitted with a column (5 ft x 1/4 in.) packed with Gas Chrom Z (70-80 mesh) coated with diethylene glycol succinate (DEGS 20%) or Apiezon L (ApL 5%) and normally operated at 190 C or 210 C, respectively. In the following account symbols such as DEGS, 20.0 indicate a carbon number (equivalent chain length) of 20.0 on a DEGS column.

Infrared spectra were run on Perkin Elmer spectrophotometers (137,237, or 261) using thin films in sodium chloride discs or 1% solutions (carbon disulphide) in 1 mm path-length cells. Ultraviolet spectra were recorded in methanol solution with a Unicam SP/800 spectrophotometer. A Perkin Elmer R10 spectrometer (60 Mc/sec) was used to record NMR spectra on 15% solutions in carbon tetrachloride.

Methylation was carried out with boron trifluoride-methanol (6) and von Rudloff oxidation as recommended by Tulloch and Craig (7).

Conversion of Methyl Crepenynate to Racemic Methyl Helenynolate

Afzelia cuanzensis seed oil (2.36 g) was refluxed for 15 min with methanolic sodium methoxide (25 ml, 0.1 N) and the methyl esters (2.28 g) recovered by petroleum extraction of the acidified reaction mixture. The esters contained about 40% of methyl crepenynate (DEGS, 21.6) and this was isolated by preparative silver ion TLC (PE25). GLC showed it to be pure, apart from methyl linoleate (3%). Methyl crepenynate (285 mg, 1 mmole) reacted overnight with an ethereal solution of monoperphthalic acid (5 ml, 2.2 mmole) and the monoepoxide (202 mg; DEGS, 26.0) was readily separated from unreacted crepenynate (24 mg) and diepoxide (10 mg) by preparative TLC (PE30).

This epoxy ester (240 mg) was also prepared (more conveniently) directly from *Afzelia* esters (1.0 g) by reaction with a 10-fold excess of monoperphthalic acid. Preparative TLC (PE30) gave a mixture of epoxystearate and epoxyoctadecynoate which was then separated by preparative silver ion TLC.

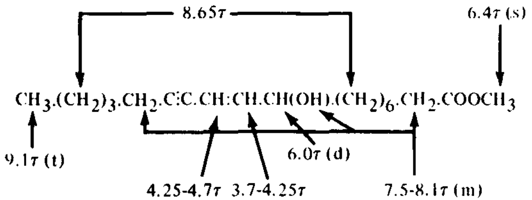
In a nitrogen atmosphere, diethylamine (0.4 ml) in anhydrous ether (10 ml) was slowly added, dropwise, to an ice cold solution of phenyl-lithium (8) (5 ml, 4 mmole) in anhydrous ether (10 ml). After 10 min, epoxy ester (250 mg) in anhydrous ether (10 ml) was added, and after stirring for 1 hr at 0 C, the reaction product (270 mg) was recovered by ether extraction. This was separated by preparative TLC (BE25) into four main fractions: epoxy ester (20 mg, 9%), hydroxy ester (120 mg, 53%) and (presumably) epoxy amide (28 mg, 12%) and hydroxy amide (60 mg, 26%).

Proof of Structure

The major product of the rearrangement reaction was shown to be methyl 9-hydroxy-octadec-*trans*-10-en-12-ynoate on the following evidence:

(a) Compared with methyl 9-hydroxy-octadeca-*trans*-10, *cis*-12-dienoate the rearrangement product had a slightly lower R_f value on silica (PE40) and a slightly higher value on silica impregnated with silver nitrate (BE25). After conversion to its trimethysilyl ether with hexamethyldisilazane and trimethylchlorosilane it gave a single peak on GLC (DEGS, 23.4).

(b) The ester had strong absorption bands at 3595, 1730 and 950 cm^{-1} in its IR spectrum. Its UV spectrum showed an absorption peak at 228 mμ ($E^{1\%}_{1cm}$ 600) and an inflexion at 238 mμ ($E^{1\%}_{1cm}$ 510). The NMR spectrum is summarized:



(c) The rearrangement product (20 mg), hydrogenated in methanol solution (5 ml) with palladium-charcoal (10%, 20 mg), gave a mixture of methyl hydroxy- and oxostearates (DEGS, 25.9 and 24.9 respectively). Dissolved in acetic acid (2 ml), this mixture (15 mg) was stirred at room temperature for 2 hr with a solution of chromium trioxide (120 mg) in acetic acid (2 ml). Thereafter the mixture was diluted with water (25 ml), treated with sulfur dioxide to destroy excess of oxidant, and extracted with petroleum (2 x 10 ml). After esterification, GLC showed the presence of methyl octanedioate and nonanedioate.

(d) von Rudloff oxidation gave hexanoic and nonanedioic acids.

(e) The hydroxy ester (43 mg), dissolved in ether (3 ml), was added to a suspension of lithium aluminium hydride (200 mg) in anhydrous ether (2 ml), and the mixture refluxed for 2 hr. The recovered product (37 mg) was purified by preparative TLC (PE60) and the major component (33 mg) was shown to be an allene by its IR absorption at 1950 cm^{-1}.

(f) Refluxed with methanolic hydrogen chloride (7 ml, 0.1 N) and isolated by preparative TLC (PE30), the hydroxy ester (24 mg) furnished an ether (IR absorption at 1080 and 1100 cm^{-1}) as major product (18 mg). GLC (DEGS, 25.2) indicated that some minor components were also present.

(g) The hydroxy ester (40 mg) was stirred with potassium azodicarboxylate (1.2 g) in anhydrous methanol (3 ml) and a mixture of methanol-acetic acid-water (1:1:1) was slowly added, dropwise, until the yellow color disappeared. The product (34 mg) was divided into three fractions by preparative silver ion TLC (BE15): H1; 17 mg; 63%; DEGS, (TMSi derivative) 23.4; IR absorption at 3595 and 950 cm^{-1} unreacted hydroxy enyne: H2; 3 mg; 11%; DEGS, (TMSi derivative) 20.8; IR absorption at 3595, 980 and 945 cm^{-1}; hydroxy *cis,trans* diene: H3; 7 mg; 26%; DEGS, (TMSi derivative) 21.6; IR absorption at 3595 cm^{-1}, hydroxy yne. Von Rudloff oxidation of H1 and of H2 gave hexanoic and nonanedioic acids; oxidation of H3 gave hexanoic acid and (presumably) the lactone of 4-hydroxydodecanedioic acid (IR absorption at 1770 and 1730 cm^{-1}; DEGS, 29.0; ApL, 17.2). Oxidation of methyl 9-hydroxyoctadec-12-enoate gave products with the same chromatographic and spectroscopic properties.

Isolation of an Acetylenic Epoxy Acid From Helichrysum bracteatum Seed Oil

Helichrysum bracteatum seeds (9.8 g) extracted with petroleum gave a yellow oil (2.02 g) which was shaken at room temperature overnight with anhydrous methanolic sodium methoxide (25 ml, 0.1%). The methyl esters (1.67 g) were recovered and separated into four fractions by preparative TLC (PE30): A, 1.01 g, 68%; B, 0.21 g, 14%; C, 0.19 g, 13%; D, 0.08 g, 5%. Fraction B, with the same R_f value as methyl 12,13-epoxyoleate showed only three peaks in its GLC: X; 6%; DEGS, 24.0: Y; 69%; DEGS, 24.6: Z; 25%; DEGS, 26.0. Attempts to separate these by silver ion TLC were unsuccessful. Fraction B (200 mg) was therefore treated overnight at room temperature with excess of monoperphthalic acid in ether (5 ml, 2.2 mole). The product was separated by preparative TLC (PE30) into monoepoxide (56 mg) and diepoxide (121 mg). The monoepoxide contained only two components [DEGS, 24.0 (20%) and 26.0 (80%)] and these were separated by preparative silver ion chromatography (PE30) into an upper (11 mg) and lower (42 mg) band.

Proof of Structure

Compound Z was shown to be identical with the methyl *cis*-9,10-epoxyoctadec-12-ynoate previously obtained by epoxidation of methyl crepenynate.

(a) The natural and synthetic esters were identical in their behavior on TLC (PE30), GLC (DEGS, 26.0; ApL, 19.1) and in their infrared and NMR spectra (no signal for olefinic proton, broad multiplet centered on 7.27τ for epoxy ring protons).

(b) The natural ester (20 mg) was subjected to acetolysis (9) by reaction first with acetic acid (2 ml) and then with aqueous methanolic (1:4) sodium hydroxide (5 ml, 8%). After recovery (18 mg), part of the product (10 mg) was hydrogenated with palladium-charcoal. Von Rudloff oxidation furnished hexanoic and nonanedioic acids from the nonhydrogenated ester and nonanoic and nonanedioic acids from the reduced product.

The acetylenic dihydroxy ester, the saturated dihydroxy ester, and methyl *threo*-9,10-dihydroxystearate showed identical behavior on a TLC plate impregnated with boric acid (5%, PE50) (10).

(c) Base-catalyzed isomerization (lithium diethylamide) of the natural ester (17 mg) gave a product (27 mg) from which hydroxy ester (7 mg) was isolated by preparative TLC (PE45). This had an ultraviolet spectrum (λ max 228 mμ, $E^{1\%}_{1cm}$ 500; 238 mμ $E^{1\%}_{1cm}$ 430) and an IR spectrum (absorption at 3595 and 950 cm^{-1}) identical with those obtained from the product of a similar reaction on the synthetic epoxy ester.

Component X was shown to be methyl *cis*-9,10-epoxystearate. After acetolysis and hydrolysis it gave methyl dihydroxystearate which was oxidized to nonanoic and nonanedioic acids and which behaved in the same way as authentic methyl *threo*-9,10-dihydroxystearate on thin layer plates treated with boric acid.

Examination of Dimorphotheca pluvialis ringens and D. aurantiaca Seed Oils

Seeds were extracted with petroleum and the oils converted to methyl esters by overnight reaction with cold dilute sodium methoxide. Epoxy esters were then isolated by TLC (PE30)

and examined further by GLC, silver ion TLC, and IR spectroscopy.

The esters from *D. pluvialis ringens* seed oil gave an epoxide fraction of only 1%. This was epoxystearate (10%) and epoxyoctadecenoate (90%) but the latter showed no significant absorption in the 900-1000 cm^{-1} region of its IR spectrum.

D. aurantiaca seed oils also gave about 1% of epoxy esters comprising saturated (15%) and unsaturated (85%) components. The latter showed no evidence of *trans* saturation in its IR spectrum.

DISCUSSION

We have already shown (2) that suitably constructed epoxides (1) rearrange, under the influence of lithium diethylamide, to the *trans* enol (2). Under the conditions examined this occurs only when the CH_2 group α to the epoxide is activated by some adjacent group X. Reaction proceeds smoothly when X is a double bond and not at all when X is a

$$-\overset{O}{CH.CH}.CH_2.X \xrightarrow{LiNEt_2} -CH(OH).\overset{t}{CH{=}CH}.X$$

(1) (2)

saturated polymethylene chain. It was of interest to know whether this reaction would occur when X is a triple bond since this would make possible a partial synthesis of racemic helenynolic acid from crepenynic acid and provide (perhaps) a chemical analogy for a possible biosynthetic pathway.

$$\underset{(13)}{-C{\equiv}C}.CH_2.\overset{c}{CH{=}\underset{(9)}{CH}}- \longrightarrow C{\equiv}C.CH_2.\overset{O}{CH.CH} \longrightarrow C{\equiv}C.\overset{t}{CH{=}CH}.CH(OH)$$

Crepenynic acid   Helenynolic acid

Using *Afzelia cuanzensis* (11) as a source of crepenynic acid the desired epoxy acid was prepared by epoxidation with monoperphthalic acid. Rearrangement occurred at 0 C in 1 hr and a hydroxy ester (53%) was isolated by TLC. As with the olefinic compounds already examined the yield of ester is reduced by a competitive reaction leading to hydroxy *N,N*-diethylamide (20%). The IR and UV spectra of the hydroxy ester showed the presence of a conjugated *trans*-enyne system and a hydroxyl group. The NMR spectrum was the same as that reported for natural helenynolic ester (4). The hydroxyl group was attached to C_9 since oxidation of the perhydro hydroxy ester gave octanedioic and nonanedioic esters. Since von Rudloff oxidation gave hexanoic and nonanedioic acids the hydroxy enyne system must lie between C_9 and C_{13}. The synthetic ester was reduced by lithium aluminium hydride to an allene and etherified, rather than dehydrated, with methanolic hydrogen chloride as described by Powell et al. (4) for the natural acid. Unsaturation was shown to be 10-en-12-yne by partial reduction with di-imide (generated from azo dicarboxylic acid) which furnished, among other products, a hydroxy ynoic acid oxidized to hexanoic acid and (probably) the lactone of 4-hydroxy-dodecanedioic acid. An authentic sample of methyl 9-hydroxyoctadec-12-enoic acid gave the same oxidation products.

If helenynolic acid is produced naturally from crepenynic acid by an enzyme-catalyzed rearrangement of the epoxide it seemed possible that the epoxide of crepenynic acid might occur in *Helichrysum bracteatum* seed oil. The epoxy esters in this oil were readily isolated by TLC, and GLC showed the presence of three components which, from their carbon numbers, could be saturated, olefinic, and acetylenic epoxy esters. Attempts to separate these by silver ion chromatography were not successful but this difficulty was overcome by treating the natural epoxy esters with excess of monoperphthalic acid. The olefinic epoxide was converted to a diepoxide but the saturated and acetylenic epoxides were unchanged. After separation from the diepoxide the two monoepoxides could be separated by silver ion TLC.

The epoxyoctadecenoate (about 10% of total esters) was not examined further and is presumably the methyl coronarate already reported. The saturated epoxide (1%) was shown to be methyl *cis*-9,10-epoxystearate and the acetylenic epoxide (3%) was identified as methyl *cis*-9,10-epoxyoctadec-12-ynoate and was thus identical with epoxidized crepenynic acid.

The natural and synthetic acids were identical in their chromatographic (TLC and GLC) and spectrosopic (IR, NMR) behavior. The natural epoxy ester, by acetolysis, hydrolysis and re-esterification, gave an acetylenic dihydroxy ester, some of which was hydrogenated to a saturated dihydroxy ester. Oxidation of the unsaturated and saturated dihydroxy esters gave hexanoic and nonanedioic acids and nonanoic and nonanedioic acids respectively showing the position of the epoxide [C_9-C_{10}] and of the unsaturated center (Δ^{12}). Since the saturated and unsaturated dihydroxy esters run with methyl *threo*-9,10-dihydroxystearate on boric acid impregnated TLC plates (10) all three must be *threo* diols and the epoxide from which two of them were derived must be *cis*. Finally, base-

catalyzed rearrangement gives a product identical with that previously obtained from synthetic epoxidized crepenynic ester. This product is presumably the same as natural helenynolic ester.

Since methyl helenynolate has the 9D configuration we expect the new acetylenic epoxy acid to have the 9D, 10D configuration and, by analogy, we predict that coronaric acid and the hydroxy dienoic acid also present in this seed oil may be 9D, 10D-epoxyoctadec-*cis*-12-enoic acid and 9D-hydroxyoctadeca-*trans*-10, *cis*-12-dienoic acid (α-dimorphecolic). This coronaric acid would then be the stereoisomer of the 9L, 10L isomer recognized in *Xeranthemum annuum* seed oil (12).

β-Dimorphecolic acid (9-hydroxyoctadeca-*trans*-10, *trans*-12-dienoic acid) can be fitted into our biogenetic proposals (1,2) either by postulating a *cis,trans* stereomutation at some stage or by starting with the 9*c*,12*t* or 9*t*,12*t* stereoisomer of linoleic acid. Morris and Marshall (13) have recently shown that *Dimorphotheca aurantiaca* seed oil contains both β-dimorphecolic acid and the 9*c*,12*t* isomer of linoleic acid. Encouraged by our success with *Helichrysum bracteatum* seed oil we examined the seed oils of *D. aurantiaca* and *D. pluvialis ringens* to see if these contained any epoxy *trans* monoenoic acids. Both contained about 1% of epoxy esters which were mainly olefinic but we could find no infrared evidence of *trans* unsaturation.

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Cholesterol Absorption With Different Fats Following Thoracic Duct Cannulation of the Rat[1]

ABSTRACT

Radioactive cholesterol in three unsaturated fats was absorbed into the lymph to the same extent as cholesterol in three saturated fats or in fats which had been stripped of sterols. Absorption on the first day following cannulation was less than half that on subsequent days.

The degree to which exogenous cholesterol may participate in accumulation of excessive cholesterol in mammalian tissues is related to the amount of fat which accompanies the cholesterol in the diet and also may be related to the source and degree of unsaturation of the dietary fat. In this paper, we have observed by direct analysis of the thoracic duct lymph the relative amounts of labeled cholesterol recovered when solutions of the cholesterol in different fats are placed in the stomach of the rat.

The experimental conditions were similar to those which Sylven and Borgstrom (1,2) employed in their examination of cholesterol absorption from triolein and from triglycerides of different chain length. The labeled cholesterol was either ^{3}H-cholesterol (Amersham-Searle) or 4-^{14}C-cholesterol (New England Nuclear Corp.) of radiochemical purity 96+%. The following test fats were contributed by suppliers: safflower oil (Pacific Vegetable Corp., Richmond, Calif.); beef tallow (Swift & Co., Chicago); hydrogenated coconut oil (Procter & Gamble, Cincinnati); lard and corn oil which has been stripped of sterols by molecular distillation (Distillation Products Co., Rochester, N.Y.). The following were purchased commercially: triolein (Matheson, Coleman and Bell); cottonseed oil (Wesson Oil); corn oil (Mazola); lard (Stark & Wetzel). Rats of the Wistar strain (150-250 g) were injected with 2.5 mg/100 g body weight of sodium pentobarbital (Nembutal) and the thoracic duct

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TABLE I

Changes in Ability of the Rat to Absorb Cholesterol Following Cannulation of Thoracic Duct[a]

Variables					Total radioactive cholesterol (μmoles) in lymph during 0 to 8 hr		
Sex	Preoperative treatment	Anesthetic	Test dosage	Heparin	Day 1	Day 2	Day 3
M	Not fasted	Pentobarbital	Oral	Once	4.3 (13)	10.4 (11)	10.7 (8)
F	Not fasted	Pentobarbital	Oral	Once	3.2 (14)	5.7 (8)	5.9 (2)
F	Not fasted	Pentobarbital	Oral	Twice	3.6 (5)	7.1 (5)	9.7 (4)
M	Fasted	Pentobarbital	Oral	Once	4.9 (4)	11.0 (3)	---
M	Fasted	Ether	Stom. cann.	Twice	2.8 (7)	8.4 (6)	---
M	Fasted	Pentobarbital	Stom. cann.	Twice	3.1 (12)	11.2 (10)	---
F	Fasted	Pentobarbital	Stom. cann.	Twice	2.6 (10)	7.2 (4)	---
M	Fasted	Pentobarbital	Stom. cann.	Twice	None[b]	11.5 (3)	10.9 (3)

[a]After surgery to secure cannulae in the thoracic duct (and in the stomachs of some animals), rats were held in restraining cages overnight with free access to water containing 0.64% NaCl and 0.04% KCl. On the subsequent three days all except the last group in the Table received, in 0.8 ml of safflower via stomach intubation or cannula, 2.5 μC of ^{3}H-cholesterol (Day 1), 0.5 μC of 4-^{14}C-cholesterol (Day 2) and 2.5 μC of ^{3}H-cholesterol (Day 3). Cholesterol was extracted from the collected lymph with acetone-ethanol (1:1), then radioactivity was determined by scintillation counting. Heparin, 125 USP units, was injected intraperitoneally during surgery and, when given twice, 30 hr later. All numbers are mean values of the number of rats shown in parentheses.

[b]No cholesterol was added to the safflower oil on Day 1; ^{3}H-cholesterol was added on Day 2 and 4-^{14}C-cholesterol on Day 3.

TABLE II

Recovery From Lymph of Radioactive Cholesterol Given in Different Fats to Male Rats[a]

Test fat	No. of experiments		Total radioactive cholesterol (μmoles) in lymph during	
	Day 2 rats	Day 3 rats	0-8 hr	0-24 hr
Triolein	6	6	10.1 ± 1.5	19.6 ± 2.5
Safflower oil	7	3	10.8 ± 1.4	16.6 ± 1.6
Tallow	5	6	9.8 ± 1.5	15.7 ± 1.6
Cottonseed oil	13	10	8.7 ± 0.9	12.9 ± 1.0
H. coconut oil	11	13	6.0 ± 0.7	9.6 ± 0.9
Lard	---	9	9.8 ± 0.9	14.3 ± 1.1
Stripped lard	9	---	9.3 ± 1.5	14.4 ± 1.8
Corn oil	---	10	10.7 ± 0.8	14.7 ± 0.8
Stripped corn oil	10	---	9.9 ± 1.1	15.0 ± 1.2

[a]Rats were fasted overnight, their thoracic ducts and stomachs were cannulated under pentobarbital anesthetic, then each was given an injection of heparin. The following day (Day 1) each rat was given 0.8 ml of the test fat without added cholesterol and a second injection of heparin. The next day (Day 2) each rat was given by stomach cannula 50 μmoles of cholesterol, containing 2.5 μC of ^{3}H-cholesterol, in 0.8 ml of the test fat. The following day (Day 3) 50 μmoles of cholesterol, containing 0.5 μC of 4-^{14}C-cholesterol, was given in a different test fat except in the case of triolein. Tests with the stripped fat preceded those with the comparable intact fat in each case. Values are expressed as mean and standard error for the total number of Day 2 and Day 3 rats.

was cannulated (3) with polyethylene tubing, (3002 Irvington size 24, black). Stomachs of fasted rats also were cannulated (4) with polyethylene tubing PE-60. To inhibit clotting of lymph, 125 USP units of heparin was injected intraperitoneally. Rats which produced 30 ml or more of lymph overnight were used for the experiments. A test dose of labeled cholesterol in 0.8 ml of fat at 40 C was administered by gastric intubation or via the stomach cannula. The cannula was rinsed with 2.0 ml of 0.9% NaCl solution. After administering a test dose, lymph was collected over periods of 0-8 hr and 8-24 hr in flasks containing heparin, 200 USP units per flask. Cholesterol was extracted from a portion of the collected lymph by the method of Sperry and Webb (5). A portion of the extract was then placed in a scintillation vial, the solvent evaporated and 15 ml of toluene scintillation fluid was added. Scintillation counting efficiencies were determined by the channels ratio method and absorbed exogenous cholesterol was calculated from radioactivity data.

Absorption of labeled cholesterol, as measured in the lymph, was usually less than half as much on the first day following surgery as on the second and third days (Table I). This slow increase of ability to absorb cholesterol following surgery has not been reported previously. It appears not to be a function of sex, fasting, type of anesthetic, heparin injection or mode of administration of the test dosage. It may reflect a lag in recovery of normal physiological processes following surgery. Absorption during the second and third days was essentially constant. Therefore, for tests of the influence of different fats upon absorption in subsequent work all animals were given an additional day for recovery before test dosages were administered.

The degree of unsaturation appears not to be a factor in either the rate or extent of absorption of cholesterol, as indicated by its appearance in thoracic duct lymph. Thus, safflower oil, corn oil and cottonseed oil did not produce results measurably different from those with triolein, beef tallow or lard (Table II). Our observations with hydrogenated coconut oil confirm recent findings (2) that cholesterol absorption from a fat is directly related to the chain length of the fatty acids in the triglyceride of the fat. Our results for cholesterol absorption during a 24 hr period are slightly lower than those of Sylven and Borgstrom (1,2) but the relative total amounts of cholesterol absorbed from coconut oil and triolein are about the same as theirs. Stripping of sterols and other relatively volatile material from the triglycerides of either lard or corn oil also produced no measurable effects upon cholesterol absorption. Earlier evidence (6,7) that plant sterols may specifically inhibit cholesterol absorption from vegetable oils has not been supported by recent work (8) nor by the present studies. It is possible that sitosterol and other plant sterols are less effective inhibitors of cholesterol absorption than was formerly believed.

From the data presented in this paper it appears that any hypocholesterolemic effect of highly unsaturated fats is not due to suppressed absorption of cholesterol.

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Frieda Castor performed the cannulations.

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Tropaeolum speciosum Seed Fat: A Rich Source of *cis*-15-Tetracosenoic and *cis*-17-Hexacosenoic Acids

ABSTRACT

Tropaeolum speciosum seed fat contains 41.6 wt % *cis*-15-tetracosenoic acid and 7.6 wt % *cis*-17-hexacosenoic acid. Unlike nasturtium seed fat (*Tropaeolum majus*), the 22:1, 24:1 and 26:1 in *T. speciosum* triglycerides are almost entirely esterified at the 1,3 positions.

The common garden nasturtium (*Tropaeolum majus*) contains the highest level of erucic acid (~79 mole %) found in any seed fat (1) and produces unique triglycerides having substantial amounts of 22:1 esterified at the 2 position (2). In view of these unusual characteristics, it was of interest to study the fatty acid compositions of other species from the small Tropaeolaceae family. Examination of *Tropaeolum speciosum* seed fat has revealed that it is a rich new source of *cis*-15-tetracosenoic and *cis*-17-hexacosenoic acids. A detailed characterization of *T. speciosum* fatty acids and triglycerides is presented here.

Tropaeolum speciosum (perennial flame flower) seeds were purchased from Harry E. Saier, Dimondale, Mich., and from the Geo. W. Park Seed Co., Greenwood, S.C. After damaged seeds and foreign material had been removed, the seeds were ground in a Waring blender, placed in a paper thimble, and extracted with petroleum ether for 4 hr on a Soxhlet extraction apparatus. The seeds contained 26.0% fat, wet basis. A portion of the total lipid was converted into the corresponding methyl esters by KOH-catalyzed methanolysis (3). The fatty acid composition was determined by gas chromatography at 185 C on 1.82 m x 2.4 mm i.d. columns packed with 10% EGSSX or 10% EGSSY on 100/120 mesh Gas Chrom Q (Applied Science Laboratories). Fifteen peaks were observed in the resultant chromatogram. These were identified (Table I) by co-chromatography with pure standards, by graphing the logarithm of the retention times vs. carbon number, and by sample hydrogenation.

The identity of *cis*-15-tetracosenoic (nervonic) acid was verified by several methods. Infrared spectroscopy of the methyl esters prepared from *T. speciosum* seed fat showed no absorption band in the 10.0-10.5 μ region; thus all unsaturation was presumed to have a *cis*-configuration. A fraction containing $>95\%$ 24:1 was isolated from the total methyl esters by preparative GLC on a 1.82 m x 12 mm i.d., 15% SE-30 column operated at 300 C. After purification by preparative thin layer chromatography (petroleum ether-diethyl ether-acetic acid, 96:3:1), the 24:1 was subjected to reductive ozonolysis by the procedure of Stein and Nicolaides (4). GLC of the products on an EGSSX column at 85 C and 185 C identified nonanal and a C_{15} aldehyde-ester as the sole products. Thus *T. speciosum* seed fat contains 41.6 wt % *cis*-15-tetracosenoic acid.

The identity of *cis*-17-hexacosenoic acid was established in a similar manner. After preparative GLC and TLC to isolate the 26:1, reductive ozonolysis produced only nonanal and a C_{17} aldehyde-ester. Therefore *T. speciosum* seed fat

From the data presented in this paper it appears that any hypocholesterolemic effect of highly unsaturated fats is not due to suppressed absorption of cholesterol.

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Tropaeolum speciosum Seed Fat: A Rich Source of *cis*-15-Tetracosenoic and *cis*-17-Hexacosenoic Acids

ABSTRACT

Tropaeolum speciosum seed fat contains 41.6 wt % *cis*-15-tetracosenoic acid and 7.6 wt % *cis*-17-hexacosenoic acid. Unlike nasturtium seed fat (*Tropaeolum majus*), the 22:1, 24:1 and 26:1 in *T. speciosum* triglycerides are almost entirely esterified at the 1,3 positions.

The common garden nasturtium (*Tropaeolum majus*) contains the highest level of erucic acid (~79 mole %) found in any seed fat (1) and produces unique triglycerides having substantial amounts of 22:1 esterified at the 2 position (2). In view of these unusual characteristics, it was of interest to study the fatty acid compositions of other species from the small Tropaeolaceae family. Examination of *Tropaeolum speciosum* seed fat has revealed that it is a rich new source of *cis*-15-tetracosenoic and *cis*-17-hexacosenoic acids. A detailed characterization of *T. speciosum* fatty acids and triglycerides is presented here.

Tropaeolum speciosum (perennial flame flower) seeds were purchased from Harry E. Saier, Dimondale, Mich., and from the Geo. W. Park Seed Co., Greenwood, S.C. After damaged seeds and foreign material had been removed, the seeds were ground in a Waring blender, placed in a paper thimble, and extracted with petroleum ether for 4 hr on a Soxhlet extraction apparatus. The seeds contained 26.0% fat, wet basis. A portion of the total lipid was converted into the corresponding methyl esters by KOH-catalyzed methanolysis (3). The fatty acid composition was determined by gas chromatography at 185 C on 1.82 m x 2.4 mm i.d. columns packed with 10% EGSSX or 10% EGSSY on 100/120 mesh Gas Chrom Q (Applied Science Laboratories). Fifteen peaks were observed in the resultant chromatogram. These were identified (Table I) by co-chromatography with pure standards, by graphing the logarithm of the retention times vs. carbon number, and by sample hydrogenation.

The identity of *cis*-15-tetracosenoic (nervonic) acid was verified by several methods. Infrared spectroscopy of the methyl esters prepared from *T. speciosum* seed fat showed no absorption band in the 10.0-10.5 μ region; thus all unsaturation was presumed to have a *cis*-configuration. A fraction containing $>95\%$ 24:1 was isolated from the total methyl esters by preparative GLC on a 1.82 m x 12 mm i.d., 15% SE-30 column operated at 300 C. After purification by preparative thin layer chromatography (petroleum ether-diethyl ether-acetic acid, 96:3:1), the 24:1 was subjected to reductive ozonolysis by the procedure of Stein and Nicolaides (4). GLC of the products on an EGSSX column at 85 C and 185 C identified nonanal and a C_{15} aldehyde-ester as the sole products. Thus *T. speciosum* seed fat contains 41.6 wt % *cis*-15-tetracosenoic acid.

The identity of *cis*-17-hexacosenoic acid was established in a similar manner. After preparative GLC and TLC to isolate the 26:1, reductive ozonolysis produced only nonanal and a C_{17} aldehyde-ester. Therefore *T. speciosum* seed fat

TABLE I

Fatty Acid Composition of *Tropaeolum speciosum* Seed Fat

Fatty acid	Total lipids wt %	Triglycerides mole %	2 Position of triglycerides mole %
14:0	Trace	Trace	---
16:0	0.7	1.2	0.6
16:1	0.1	0.2	0.6
17:1	0.1	0.1	0.2
18:0	Trace	Trace	---
18:1ω9	29.7	33.5	89.1
18:2ω6	1.4	2.7	6.3
18:3	0.5	0.9	2.3
20:1	0.4	0.6	0.3
22:0	0.2	0.3	---
22:1ω9	16.8	15.4	0.3
24:0	0.2	0.2	---
24:1ω9	41.6	36.3	0.3
26:1ω9	7.6	7.6	---
28:1	0.7	1.0	---

contains 7.6 wt % *cis*-17-hexacosenoic acid.

The 22:1 was also isolated by preparative GLC and TLC. Ozonolysis gave only nonanal and a C_{13} aldehyde-ester, identifying erucic acid. A C_{18} fraction was similarly purified and subjected to ozonolysis. The products were almost entirely nonanal and a C_9 aldehyde-ester, identifying oleic acid. A small amount of hexanal was also observed, no doubt originating from the linoleic acid present. The position of the double bond in the minor monoene acids was not established, but it seems probable that the 20:1 and 28:1 are also of the ω9 series of acids.

Since *T. majus* is unique among seed fats in having large amounts of 22:1 esterified at the 2 position of the triglycerides, it was also of interest to examine *T. speciosum* triglycerides by lipase hydrolysis. The triglycerides were isolated from the total lipids by column chromatography on activated Florisil (5). A portion of the triglycerides was converted to methyl esters and analyzed for its fatty acid composition, and another portion was subjected to lipolysis by pancreatic lipase using the procedure of Luddy et al. (6). The resultant 2-monoglycerides were isolated by preparative TLC, converted to methyl esters, and their fatty acid composition was determined by GLC. The compositions found are reported in mole per cent in Table I. It is clear that 22:1, 24:1 and 26:1 are almost entirely esterified at the 1,3 positions of *T. speciosum* triglycerides. This same type of positioning occurs in *T. peregrinum* seed triglycerides which contain 9.6 mole % 20:1 and 31.9 mole % 22:1 (Litchfield, unpublished data). On the basis of these results, *T. majus* remains unique in its positioning of substantial 22:1 at the 2 position of its seed triglycerides.

Although *cis*-15-tetracosenoic acid is commonly found in animal sphingolipids, it is an unusual component in seed fats. Three *Ximenia* species contain 3-7% nervonic acid (7). Some 24:1 is found in Cruciferae seed fats, but it rarely amounts to more than 1-2% of the total fatty acids (8). An exception is *Lunaria annua (=L. biennis)* in which Wilson et al. (9) found 21 wt % 24:1ω9. *T. speciosum* contains 41.6 wt % nervonic acid, the highest level yet reported in any seed fat. The longer chain monoenes in *T. speciosum* seed fat (7.6 wt % 26:1ω9 and 0.7 wt % 28:1) are rarely found in seeds, although 3-9 wt % 26:1ω9 and 5-12 wt % 28:1ω9 are known to occur in various *Ximenia* seed fats (7). The commercial availability of *T. speciosum* seeds and the high levels of 24:1ω9 and 26:1ω9 in this seed fat make it a very useful starting material for the laboratory preparation of pure *cis*-15-tetracosenoic and *cis*-17-hexacosenoic acids.

The fatty acid composition of *T. speciosum* seed fat suggests that the enzyme systems for synthesizing oleic acid and for elongating it into C_{20}-C_{28} ω9 monoenes are extremely active in this seed (total ω9 monoene = 94.3 mole %). Apparently this is a typical characteristic of Tropaeolaceae seed fats (*T. majus* contains 98.7 mole % monoenes, *T. peregrinum* 96.5 mole %) and distinguishes them from seed fats of other plant families.

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Cycloheximide and Chloramphenicol: Effect on Rat Liver Acetate Metabolism[1]

ABSTRACT

Conversion of isotopic acetate to cholesterol, to fatty acids and to CO_2 by rat liver homogenates is dramatically curtailed within 3 hr following injection of chloramphenicol or cycloheximide. Cholesterol synthesis from mevalonate remains unaltered, indicating that this does not imply generalized depression of all metabolic processes. This apparent shutdown of acetate metabolism suggests that caution be exercised when these drugs are employed as specific inhibitors of protein synthesis.

Cycloheximide and chloramphenicol, widely used as specific, reversible inhibitors of protein synthesis, block transfer of amino acid residues from aminoacyl-t-RNA into polyribosome-bound polypeptide (1,2). Chloramphenicol, a potent inhibitor for microorganisms, is relatively ineffective in mammalian systems other than mitochondria (3). Cycloheximide readily blocks synthesis in yeast or mammalian systems (2) but is a relatively ineffective inhibitor of bacterial or mammalian mitochondrial protein synthesis. It is perhaps less widely known that these drugs affect processes other than protein synthesis. Chloramphenicol inhibits ion accumulation by maize mitochondria (4) and NADH oxidation by beef heart mitochondria at a step prior to cytochrome b (5), while cycloheximide blocks ACTH-stimulated conversion of cholesterol to pregnenelone in rat adrenal gland (6). While attempting to use these drugs as protein synthesis inhibitors, we noted that both drugs rapidly and profoundly depress three major routes of acetate metabolism in rat liver. Our results suggest that caution should be exercised in interpreting effects of these drugs solely in terms of inhibition of protein synthesis. This is particularly true for metabolic pathways or reactions where acetate serves as an amphibolic intermediate.

Female Wistar rats (100 g) injected intraperitoneally with 0.9% saline, chloramphenicol succinate (750 mg/kg) in saline, or cycloheximide (2 mg/kg) in saline were killed 0-6 hr after injection. Liver homogenates prepared in buffer (The buffer was: 50 mM potassium phosphate (pH 7.0), 250 mM sucrose, 7.5 mM $MgCl_2$, 1.5 mM glutathione, 30 mM nicotinamide, 150 mM KCl and 1 mM EDTA; 2.5 ml of buffer were used per gram wet tissue.) were centrifuged 10 min at 700 X *g*, and the supernatant liquid centrifuged 15 min at 10,000 X *g*. Cholesterol synthesis, fatty acid synthesis and CO_2 production were then studied (7) using the 10,000 X *g* supernatant liquid as enzyme.

Cholesterol synthesis from acetate by liver homogenates was depressed within 2 hr fol-

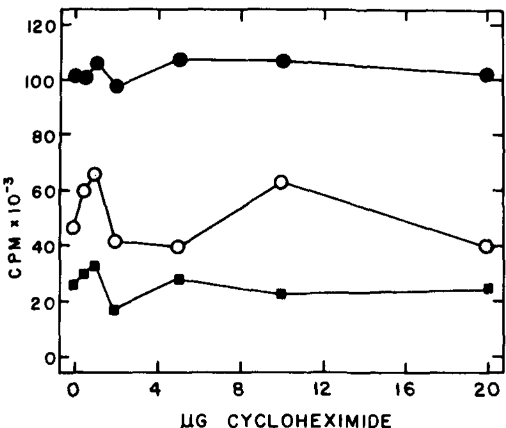

FIG. 1. Effect of addition of varying concentrations of cycloheximide in liver homogenates prepared from untreated animals. The assay and analysis conditions were those of Table I. Incorporation of isotope from 1-^{14}C-acetate into cholesterol (o), fatty acids (■), and CO_2 (●) was measured.

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Cholesterol synthesis from acetate by liver homogenates was depressed within 2 hr fol-

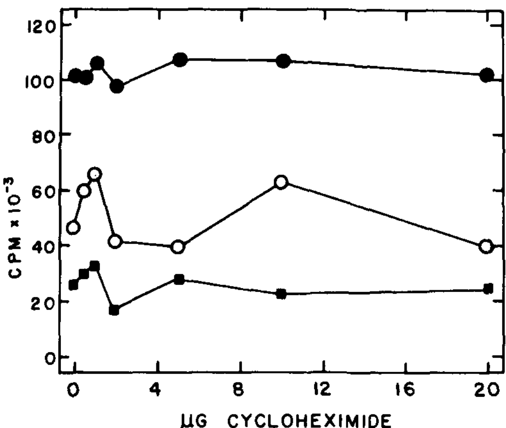

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TABLE I

Cholesterol, Fatty Acid and CO_2 Formation From 1-^{14}C-Acetate and From 2-^{14}C-Mevalonate in Liver Homogenates From Cycloheximide-Treated Rats[a]

Elapsed time after injection, hr	Radioactivity (cpm x 10^{-3}) incorporated into				
	Cholesterol from		Fatty acids from acetate	CO_2 from	
	Acetate	Mevalonate		Acetate	Mevalonate
0	32.5 ± 4.9	21.9 ± 0.5	169 ± 29	300 ± 6	13 ± 2
2	9.0 ± 2.2	24.3 ± 1.3	43 ± 5	216 ± 2	13 ± 1
4	7.9 ± 1.1		15 ± 8	182 ± 4	17 ± 0.1
6	0.4 ± 0.03	27.3 ± 5.9	4.3 ± 0.3	68 ± 4	13 ± 0.3

[a]Incubation flasks (25 ml) contained 2.5 ml 10,000 X *g* supernatant liquid, 4 μmoles ATP, 1.0 μmole CoA, 9 μmoles glucose-1-phosphate, 2 μmoles NADP, 2 μmoles glutathione and either 3 μC (4.7 μg) of 1-^{14}C-acetate or 0.1 μC (4.4 μg) of 2-^{14}C-mevalonate. A removable center well contained 0.5 ml of Hyamine 10X (Packard) to trap CO_2. Incubation was for 2 hr at 37 C. Fatty acids, cholesterol and CO_2 were then isolated and counted as described by Siperstein and Guest (7). Data are mean values, and standard deviation for triplicate determinations.

lowing injection of cycloheximide (Table I). By contrast, cholesterol synthesis from mevalonate was unaffected. Comparable results were obtained using livers from chloramphenicol-treated rats (Table II). We next inquired whether other metabolic pathways originating with acetate were similarly affected. We found (Table I) that biosynthesis not only of cholesterol but of fatty acids was depressed within 2 hr by cycloheximide pretreatment. CO_2 production from acetate also was depressed in the same time interval. These effects were not observed when cycloheximide was added to homogenates of liver from untreated rats (Fig. 1), nor were the effects of injection of cycloheximide reversed by addition to incubation mixtures of from 1 to 80 μmoles of glutathione.

These data demonstrate prompt depressant effects of injected cycloheximide or chloramphenicol on three sequences of metabolic reactions, measured in vitro, originating with acetate: the biosynthetic pathways leading to cholesterol and to fatty acids, and the citric acid cycle. Although catabolism (CO_2 production) also is affected within 3 hr, the biosynthetic pathways are more drastically curtailed. The results cannot be attributed to a general toxic effect of the admittedly large doses of antibiotics administered, for neither cholesterol synthesis nor CO_2 production from mevalonate was significantly affected. Direct enzyme inhibition also can be ruled out by our failure to demonstrate any effect of cycloheximide added in vitro on the above processes. Addition of glutathione was tested because Munro et al. (8) have reported reversal by glutathione of the purely inhibitory effects of cycloheximide on in vitro protein synthesis by rat liver. We noted no such reversal.

We suggest two explanations. The in vivo acetate pool size may be vastly increased by these drugs, diluting out added ^{14}C-acetate. Alternatively, a participating enzyme, possibly acetate thiokinase, is rapidly degraded in vivo. Inhibition by drugs of synthesis of new

TABLE II

Cholesterol Synthesis From Acetate and From Mevalonate in Liver Homogenates From Chloramphenicol-Treated Rats[a]

Elapsed time after injection, hr	Radioactivity (cpm x 10^{-3}) in isolated cholesterol after incubation with	
	1-^{14}C-acetate	2-^{14}C-mevalonate
Saline, 0	25.8 ± 10.1	29.3 ± 3.2
Chloramphenicol, 0	26.3 ± 7.7	42.1 ± 14.4
Chloramphenicol, 3	0.3 ± 0.06	27.2 ± 4.9
Chloramphenicol, 6	2.6 ± 0.2	27.9 ± 4.0
Chloramphenicol, 9	1.2 ± 0.5	25.1 ± 1.4
Chloramphenicol, 12	8.5 ± 1.3	37.6 ± 5.2
Chloramphenicol, 15	0.5 ± 0.1	36.6 ± 3.7

[a]Incubation and assay conditions are those of Table I. Data again represent mean value, and standard deviation for triplicate incubations.

enzyme would then cause decreased acetate utilization.

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Monounsaturated Fatty Acids of Mouse Skin Surface Lipids

ABSTRACT

The occurrence of the various positional isomers of monounsaturated fatty acids of mouse skin surface lipids was studied by conventional oxidative cleavage-gas liquid chromatography techniques. The pattern observed could be accommodated by the biosynthetic scheme proposed elsewhere for rat skin fatty acids, except that 18:Δ9 replaced 16:Δ9 as the main precursor.

Observations by Nikkari and Haahti that rat skin surface lipids possessed fatty acids of unusually great chain length (1) were extended to mouse skin lipids (surface and epidermal) in an earlier communication (2). In the mouse, the monounsaturated nature of these chains, and their existence in the sterol ester fraction, were demonstrated. More recently, Nicolaides and Ansari have investigated the relative amounts of the various double bond positional isomers in the monoene fatty acids of rat surface lipids (3). They concluded that even-carbon chains were biosynthesized by desaturation of saturated C_{14} through C_{20} chains at the ninth carbon, followed by chain elongation or shortening by C_2 units, the former being strongly preferred. Their results suggested that elongation of 16:Δ9 was the major pathway:
16:Δ9 → 18:Δ11 → 20:Δ13 → etc. because the ω7 series of isomers formed by this procedure generally accounted for 80-90% of the isomers at each chain length.

The present report describes results for mouse surface lipids. A large pooled sample (700 mg) was collected from 20 albino mice, strain BAL/Bc, with acetone over a period of four weeks, and the fatty acids were obtained as methyl esters by techniques already described (2). The monoene ester fraction was isolated by the method of Anderson and Hollenbach (4). Individual esters were obtained by preparative GLC using a column (180 X 0.6 cm) packed with 4% OV-1 phase on Chromosorb G (70-80 mesh) which was temperature-programmed from 130 C to 300 C at 4° per minute. Samples of 2 mg were injected and emerging esters condensed in glass tubes. The purity of each ester was checked on an analytical GLC column (300 X 0.2 cm) packed with 3% OV-1 on Chromosorb W (100-120 mesh) and programmed 130-300 C, before oxidative degradation by the method of Chang and Sweeley (5). Fission fragments from esters with 14 through 22 carbons were analyzed on a GLC column (300 X 0.2 cm) packed with 7% F-60/1% EGSP-Z on Gas Chrom P (80-100 mesh) which was programmed 55-210 C at 4° per minute. Fragments from esters C_{24} through C_{34} were analyzed on the 3% OV-1 column described above, and programmed 55-270 C. In both cases, relative amounts of positional iso-

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TABLE I

Occurrence of Double Bond Isomers in Monoene Fatty Acids of Mouse Surface Lipids

Percentage of monoenes	Fatty acid	Isomer	Percentage of isomers
0.3	C_{14}	5	51
		7	26
		9	21
9.8	C_{16}	7	86
		9	14
13.2	C_{18}	9	90
		11	10
21.5	C_{20}	9	5
		11	76
		13	19
15.5	C_{22}	9	4
		11	4
		13	74
		15	15
4.2	C_{24}	13	3
		15	82
		17	14
2.5	C_{26}	15	3
		17	85
		19	11
3.1	C_{28}	17	2
		19	86
		21	10
5.6	C_{30}	19	3
		21	83
		23	14
10.8	C_{32}	21	3
		23	85
		25	11
8.5	C_{34}	23	3
		25	85
		27	11

mers were calculated from peak areas as before (6).

The composition of the monoene fatty acids of mouse surface lipids was found to resemble that of the rat, when each chain length was expressed as a percentage of the whole. Results for even-carbon acids are given in Table I. Odd-carbon and branched acids formed 5.1% of the total and were not included in this study. The only notable difference between the pattern for the mouse (Table I) and the rat (3) lies in the fact that 20:1 is most abundant in the mouse and 18:1 in the rat.

Examination of the relative amounts of positional isomers, however, suggests that although the same chain elongation processes are obviously operating in mouse as well as rat skin, the main precursor is 18:Δ9 instead of 16:Δ9. The principal pathway is thus: 16:Δ7 ← 18:Δ9 → 20:Δ11 → 22:Δ13 →, etc., and isomers of the ω9 series predominate, while the ω7 series is of secondary importance. This is the exact reversal of the pattern for the rat. The general result is that, for any given chain length, the principal isomer in the case of the mouse has its double bond located nearer to the carboxyl by two carbons than the corresponding isomer in the rat. Elongation of 14:Δ9, giving rise to a ω5 series, was not detected.

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[Received September 15, 1969]

The Reduction of Alkyl Sulfate to Alkane With Lithium Aluminum Hydride

ABSTRACT

A general reaction is described for the reduction of alkyl sulfate esters to alkanes by use of lithium aluminum hydride. The method has been applied to sodium dodecyl sulfate and sodium hexadecyl sulfate.

Shortly after the observation of Schmid and Karrer (1) that aliphatic toluene sulfonic esters, like alkyl halides, are reduced to the corresponding alkanes by lithium aluminum hydride, Karrer and Jucker (2) attempted the same reaction on phosphate esters. Unlike the tosylates the hydride attacked the phosphorous

TABLE I

Occurrence of Double Bond Isomers in Monoene Fatty Acids of Mouse Surface Lipids

Percentage of monoenes	Fatty acid	Isomer	Percentage of isomers
0.3	C_{14}	5	51
		7	26
		9	21
9.8	C_{16}	7	86
		9	14
13.2	C_{18}	9	90
		11	10
21.5	C_{20}	9	5
		11	76
		13	19
15.5	C_{22}	9	4
		11	4
		13	74
		15	15
4.2	C_{24}	13	3
		15	82
		17	14
2.5	C_{26}	15	3
		17	85
		19	11
3.1	C_{28}	17	2
		19	86
		21	10
5.6	C_{30}	19	3
		21	83
		23	14
10.8	C_{32}	21	3
		23	85
		25	11
8.5	C_{34}	23	3
		25	85
		27	11

mers were calculated from peak areas as before (6).

The composition of the monoene fatty acids of mouse surface lipids was found to resemble that of the rat, when each chain length was expressed as a percentage of the whole. Results for even-carbon acids are given in Table I. Odd-carbon and branched acids formed 5.1% of the total and were not included in this study. The only notable difference between the pattern for the mouse (Table I) and the rat (3) lies in the fact that 20:1 is most abundant in the mouse and 18:1 in the rat.

Examination of the relative amounts of positional isomers, however, suggests that although the same chain elongation processes are obviously operating in mouse as well as rat skin, the main precursor is 18:Δ9 instead of 16:Δ9. The principal pathway is thus: 16:Δ7 ← 18:Δ9 → 20:Δ11 → 22:Δ13 →, etc., and isomers of the ω9 series predominate, while the ω7 series is of secondary importance. This is the exact reversal of the pattern for the rat. The general result is that, for any given chain length, the principal isomer in the case of the mouse has its double bond located nearer to the carboxyl by two carbons than the corresponding isomer in the rat. Elongation of 14:Δ9, giving rise to a ω5 series, was not detected.

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A general reaction is described for the reduction of alkyl sulfate esters to alkanes by use of lithium aluminum hydride. The method has been applied to sodium dodecyl sulfate and sodium hexadecyl sulfate.

Shortly after the observation of Schmid and Karrer (1) that aliphatic toluene sulfonic esters, like alkyl halides, are reduced to the corresponding alkanes by lithium aluminum hydride, Karrer and Jucker (2) attempted the same reaction on phosphate esters. Unlike the tosylates the hydride attacked the phosphorous

atom producing phosphine, phosphine oxides and the corresponding aliphatic alcohol. This applied to a monoalkyl phosphate (cetyl phosphate) and a dialkyl phosphate (lecithin). Several attempts have been made to cleave sulfate groups from sugars with lithium aluminum hydride (3-6). In one case (5,6) the lithium aluminum hydride appeared to have no effect on methyl β-D-galactopyranoside 6-(ammonium sulfate) after refluxing in tetrahydrofuran for 70 hr. Apparently the original sugar sulfate was isolated from the reaction mixture. In Grant and Holt's study (3,4), however, the sulfate group was removed from a variety of methyl glycoside sulfates, including di-*O*-isopropylidene-α-D-galactopyranose 6-sulfate. In this case the product was not the deoxysugar, which results from tosylate cleavage (7), but the original sugar. This data suggests that the sulfur atom is attacked as is the case with phosphate esters (2).

In connection with structural studies on natural alkyl sulfates in the phytoflagellate *Ochromonas danica* (8-10) the author investigated the reaction of lithium aluminum hydride on typical alkyl sulfates as a means of cleaving the sulfate group. The first laboratory attempts of this reaction produced largely dodecanol and small amounts of dodecane. The absence of reduced sulfur, however, made it apparent that hydrolysis had occurred rather than reduction of sulfate. Rigorous drying conditions were employed which permitted a high yield of alkane, a yield comparable to lithium aluminum hydride reduction of tosyl esters.

Sodium dodecyl sulfate (SDS) was obtained from Mann Research Laboratories. Sodium hexadecyl sulfate was synthesized from sulfotrioxide pyridine in the usual manner (11). The alkyl sulfates were dried at 80 C at 3 mm overnight and stored for two weeks at 60 C in a vacuum disiccator over concentrated sulfuric acid. Dodecane was obtained from K and K Laboratories. Thin layer chromatography (TLC) was conducted in Supelcosil 12B (Supelco Laboratories, Belafonte, Pa.) using hexane as the solvent. Gas chromatographs were obtained on a column of 3% JXR on Gas Chrom Q (Supelco) at 100 C by procedures published elsewhere (12).

SDS (144 mg, 0.5 mmole) was added to a suspension of lithium aluminum hydride (34 mg, 1.0 mmole an eightfold excess) in tetrahydrofuran (3.0 ml, previously distilled from lithium aluminum hydride). The mixture was refluxed for 24 hr after which 5 ml of water was cautiously added with cooling followed by clarification of the suspension by the dropwise addition of 6N hydrochloric acid. The product was extracted with three successive extractions of 10 ml portions of hexane. The combined hexane was back washed with water, dried over sodium sulfate and evaporated under reduced pressure. The product (81 mg) was examined with TLC and GLC. The latter procedure showed 98% dodecane and traces of decane and tetradecane judging from the relative retention data and peak size. A sample of the sodium dodecyl sulfate was solvolysed in moist dioxane (10). The dodecanol was examined by gas chromatography and found to contain the same contaminants in the same proportions. The infrared spectrum was taken in carbon tetrachloride with a Perkin Elmer Infracord Model 137 infrared spectrophotometer.

An identical procedure was applied to sodium hexadecyl sulfate. In this case the yield was 98.7% hexadecane.

The infrared spectrum of the product of lithium aluminum hydride reduction of SDS showed absorption bands typical of alkanes (2950, 2925, 2875, 1470, 1380, 723 cm^{-1}) and was virtually identical to that of authentic dodecane. Likewise thin layer and gas chromatographic behavior of the two substances confirmed the assignment. These results show that sulfate esters are cleaved by hydride attack with lithium aluminum hydride. The mechanism is presumably that of a Walden inversion as in all other hydride reductions on tetrahedral carbon atoms (13) including the tosylates. This reaction opens the possibility of using the sulfate as an intermediate in the reduction of hydroxyl groups and in stereospecific labeling of hydrogen atoms with deuterium or tritium (14).

The results of repeated runs with alkyl sulfate prepared under a variety of drying conditions suggested that the amount of alcohol obtained was directly proportional to the amount of water present in the alkyl sulfate preparation. This technique could therefore provide a convenient method for assaying the water in an aliphatic sulfate preparation.

This report would appear to contradict previous reports on the reductive cleavage of sulfate esters with lithium aluminum hydride in that these reports did not find the reduced carbon, but an alcohol (3-6). It is clear, however, from the above that the sulfate ester preparations previously used contained small amounts of water, probably a mole of crystallization. It would appear that a vigorously dried sugar sulfate preparation could be reduced to the deoxy sugar and the position of the sulfate thereby identified.

Reduction of natural sulfatides and steroid sulfates with lithium aluminum hydride would

yield more volatile products than hydrolysis or solvolysis thereby facilitating the use of gas chromatography in the analysis of these substances. Such an attempt was made on the 1,14-docosanediol-1,14-disulfate of *Ochromonas danica* (10). Unfortunately the presence of chlorohydrin sulfates (15) in this sulfatide mixture, especially *threo*-(R)-13-chloro-1-(R)-14-docosanediol 1,14-disulfate, produced a complex and uninterpretable product.

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[Received May 24, 1969]

The Densities of Three Classes of Marine Lipids in Relation to Their Possible Role as Hydrostatic Agents

ABSTRACT

Wax esters, diacyl glycerol ethers and triglycerides were isolated from marine oils by preparative thin layer chromatography. Their densities indicated that wax esters would nearly equal squalene in effectiveness as a buoyant agent and that diacyl glycerol ethers would surpass triglycerides. Their compositions were listed to permit comparison with lipids from other sources.

Lipids have long been thought to serve as hydrostatic agents by which some fatty marine animals attain neutral buoyancy (1). However, an evaluation of the relative effectiveness of the several lipid classes which might contribute buoyancy has been hampered by a lack of data on their densities. Of the more common lipids, only the densities of squalene, two wax esters and five triglycerides are reported (2,3). No data are given for diacyl glycerol ethers. In contrast, there is considerable information on the densities of marine oils (4) but it is of little value in this regard because it generally refers to oils consisting of several lipid classes. This communication reports the densities of wax esters, diacyl glycerol ethers and triglycerides isolated from oils of marine origin. Their composition is included to allow estimates to be made of the densities of similar classes of lipids from other sources.

Individual classes of lipids were isolated by preparative TLC from the following oils: wax esters from the stomach oil of the wandering albatross, *Diomedea exultans* (5); diacyl

TABLE I

Densities of Classes of Lipids Isolated by Preparative TLC

Lipid	Source	Density 21/4
Wax esters (2)	Albatross stomach oil	0.8578 (average)
Diacyl glycerol ethers (2)	Shark liver oil	0.8907 (average)
Triglycerides-1	Antarctic krill	0.9168
Triglycerides-2	Fish body oil	0.9154

yield more volatile products than hydrolysis or solvolysis thereby facilitating the use of gas chromatography in the analysis of these substances. Such an attempt was made on the 1,14-docosanediol-1,14-disulfate of *Ochromonas danica* (10). Unfortunately the presence of chlorohydrin sulfates (15) in this sulfatide mixture, especially *threo*-(R)-13-chloro-1-(R)-14-docosanediol 1,14-disulfate, produced a complex and uninterpretable product.

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TABLE II

Composition in Weight Per Cent of Saponified Lipids as Determined by GLC[a]

Component	Fatty acid methyl esters				α-Glycerol ethers[b]	Alcohols[c]
	W.E.	G.E.	Trig-1	Trig-2		
14:0			5.4	4.4	2.0	4.0
15:0						2.1
16:0		8.9	28.0	16.9	24.6	43.5
16:1		2.6	7.3	7.4	8.9	4.3
17:0						2.4
17:1					6.3	
18:0			2.9	6.3	4.2	5.4
18:1	90.7	57.2	29.0	28.7	52.7	29.6
18:2			2.9			
20:1	5.1	18.1	5.4	5.6		8.8
20:5			12.5	7.0		
22:1		11.5				
22:5				2.6		
22:6			6.1	8.1		

[a]Components $<2\%$ are omitted.
[b]As isopropylidene derivatives.
[c]As trifluoroactetates.

glycerol ethers from the liver oil of the black shark, *Dalatias licha* (5); triglycerides-1 from the lipids of antarctic krill, *Euphausia superba*; triglycerides-2 from fish body oil of tarakihi, *Cheilodactylus macropterus*. After elution, the lipids were freed of solvent by dry nitrogen over a warm water bath until they reached constant weight. The densities, relative to water at 4 C, were determined at room temperature (ca. 21 C) in a 0.1 ml pipette which had been standardized with distilled water. The densities listed (Table I) are the averages of six weighings. Duplicate determinations of the densities of two lots of wax esters and diacyl glycerol ethers agreed within $\pm$ 2 X 10^{-4}. The measured densities of TLC-purified squalene (Eastman Kodak 90%) and ethylene glycol (May & Baker 95%) were within $\pm$ 3 X 10^{-4} of the values listed (3).

The lipids were saponified and the fatty acids methylated by BF_3-methanol (6). The fatty alcohols were converted to trifluoroacetates (7) and the α-glycerol ethers to isopropylidene derivatives (8). The lipids were analyzed by GLC using a Pye Model 104 instrument equipped with flame ionization detectors and 180 X 0.6 cm stainless steel columns filled with acid washed, silanized, 80-100 mesh Celite containing 5% Apiezon L or 10% diethylene glycol succinate. The derivatives were identified by comparison of the semilog plots of their relative retention values with those of reference compounds. The percentage compositions were calculated from peak areas obtained with a Disc Integrator.

Some of the bathypelagic fishes and crustaceans contain large amounts of wax esters (9) which have been regarded as possible buoyant agents (10). Oleyl oleate and cetyl palmitate have densities of 0.8600 30/4 and 0.8324 50/4 respectively (2,3), but under physiological conditions the amount of saturated wax esters would be limited by their high melting points. The wax esters of this study had an average density of 0.8578. This value is close to that of oleyl oleate and reflects the characteristic predominance of monoenes in naturally occurring wax esters. It is also similar to that of squalene, 0.8562 20/4 (3), and illustrates the hydrocarbon character of wax esters which has been discussed by Mead et al. (11).

Diacyl glycerol ethers occur in large amounts in the livers of many elasmobranch fishes (12) where they undoubtedly contribute buoyancy, though they may also serve as metabolic reserves. The fractions isolated in this study had an average density of 0.8907. Their composition (Table II) was similar to that of the wax esters in having unsaturation limited to monoenes which comprised 89.4% of the fatty acids and 69% of the α-glycerol ethers.

The two triglyceride fractions had densities which were very similar to that of triolein, 0.915 20/4 (3). The triglyceride iodine values of 140 (calculated) and 150 (Wijs) are in the middle of the range found in marine oils (ca. 100-200), suggesting that their densities are nearly average. Bailey (4) gives a range of densities for fish body oils which extends somewhat higher, 0.913 to 0.937 (temperature

15.56 C). Although the density of a more highly unsaturated oil (I.V. 190, non-sap 1.4%) can reach 0.927 (4) the upper limit of this range probably refers to oils containing relatively large amounts of cholesterol (D 20/4 = 1.067) or short chain acids such as isovaleric (D 15/4 = 0.9373).

These results reveal that squalene and wax esters would be nearly equal in effectiveness as hydrostatic agents and greatly surpass diacyl glycerol ethers and triglycerides. Pristane, having a density of 0.78 (temperature not specified) (13), is probably the most effective of the naturally occurring buoyant agents but it rarely occurs in significant amounts.

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Plasma Cholesterol Turnover and Esterification in the Pigeon

ABSTRACT

The plasma cholesterol turnover and serum cholesterol esterifying system was studied in White Carneau pigeons. Eight pigeons received a single injection of 1,2-^{3}H-cholesterol intravenously and the decline in plasma cholesterol specific activity was measured at intervals from 1-64 days. Kinetic analysis of the plasma cholesterol die-away curves indicates that plasma cholesterol turnover in the pigeon conforms to a 2 pool model. The mass of pool A (cholesterol in blood and those tissues which are rapidly equilibrated with blood) in pigeons maintaining consistently high serum cholesterol levels (≈900 mg/100 ml) was 988 mg and the daily fractional turnover of pool A was 19.7% compared with 676 mg and 12.2% found in pigeons maintaining consistently low (≈400 mg/100 ml) serum cholesterol levels. Serum cholesterol esterifying activity, in vitro, showed a positive correlation (rxy=0.806) with serum cholesterol concentration in the pigeon. The pigeon differs, in this regard, from the chicken, rat and rabbit in which a negative correlation has been reported.

Individual differences in the control of serum cholesterol levels of monkeys fed an atherogenic diet have been investigated in this laboratory (1,2). The findings indicated that, in general, animals maintaining the highest serum cholesterol levels have lower fractional turnover rates of cholesterol in the more rapidly miscible pool. In work with the pigeon, it was obvious that individual birds also vary greatly in their responses to a cholesterol-containing diet. In an attempt to study this phenomenon more closely we have examined the plasma cholesterol turnover in two groups of pigeons especially selected for their ability to maintain either consistently high (hyperresponders) or consistently low (hyporesponders) serum cholesterol levels when maintained on an atherogenic diet (pigeon pellets coated with a mixture of lard and cholesterol, to give a final concen-

15.56 C). Although the density of a more highly unsaturated oil (I.V. 190, non-sap 1.4%) can reach 0.927 (4) the upper limit of this range probably refers to oils containing relatively large amounts of cholesterol (D 20/4 = 1.067) or short chain acids such as isovaleric (D 15/4 = 0.9373).

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Plasma Cholesterol Turnover and Esterification in the Pigeon

ABSTRACT

The plasma cholesterol turnover and serum cholesterol esterifying system was studied in White Carneau pigeons. Eight pigeons received a single injection of 1,2-^{3}H-cholesterol intravenously and the decline in plasma cholesterol specific activity was measured at intervals from 1-64 days. Kinetic analysis of the plasma cholesterol die-away curves indicates that plasma cholesterol turnover in the pigeon conforms to a 2 pool model. The mass of pool A (cholesterol in blood and those tissues which are rapidly equilibrated with blood) in pigeons maintaining consistently high serum cholesterol levels (≈900 mg/100 ml) was 988 mg and the daily fractional turnover of pool A was 19.7% compared with 676 mg and 12.2% found in pigeons maintaining consistently low (≈400 mg/100 ml) serum cholesterol levels. Serum cholesterol esterifying activity, in vitro, showed a positive correlation (r_{xy}=0.806) with serum cholesterol concentration in the pigeon. The pigeon differs, in this regard, from the chicken, rat and rabbit in which a negative correlation has been reported.

Individual differences in the control of serum cholesterol levels of monkeys fed an atherogenic diet have been investigated in this laboratory (1,2). The findings indicated that, in general, animals maintaining the highest serum cholesterol levels have lower fractional turnover rates of cholesterol in the more rapidly miscible pool. In work with the pigeon, it was obvious that individual birds also vary greatly in their responses to a cholesterol-containing diet. In an attempt to study this phenomenon more closely we have examined the plasma cholesterol turnover in two groups of pigeons especially selected for their ability to maintain either consistently high (hyperresponders) or consistently low (hyporesponders) serum cholesterol levels when maintained on an atherogenic diet (pigeon pellets coated with a mixture of lard and cholesterol, to give a final concen-

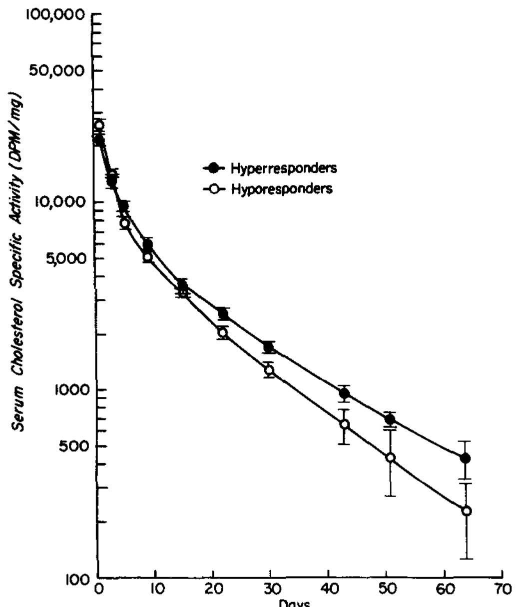

FIG. 1. Serum 1,2-^{3}H-cholesterol die-away curves for White Carneau pigeons. Each value represents the mean and standard error of the mean from four pigeons.

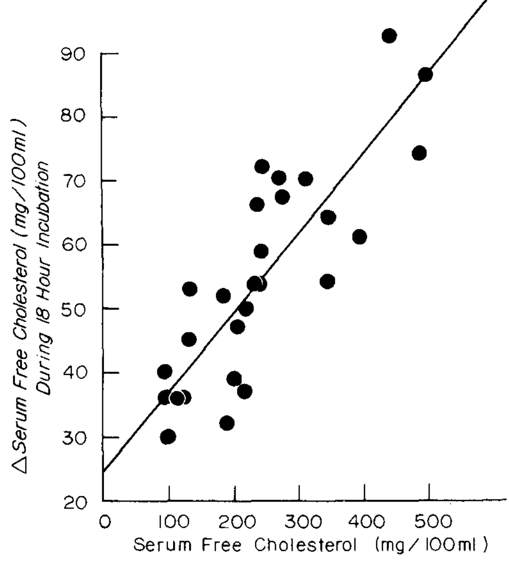

FIG. 2. Relationship between serum cholesterol concentration and cholesterol esterifying activity of serum. Line shown is the linear regression line, rxy=0.806.

tration of 0.25% cholesterol and 5% lard; Purina Pigeon Pellets from Ralston-Purina Co., St. Louis, Mo.). The results are presented in this report. In addition, data are presented which suggest that the serum cholesterol esterification system in the pigeon differs from that of some other animals (3) in that the activity appears to increase rather than decrease, with hypercholesteremia.

A group of 2-month old White Carneau (WC) pigeons was fed the atherogenic diet for 11 weeks; serum cholesterol levels were determined weekly (4). At the end of 11 weeks, it was found that four of the pigeons (two males and two females) were hyporesponders and had maintained essentially the same serum cholesterol levels they had prior to cholesterol feeding (299±11 vs. 355±60 mg/100 ml) while four more were hyperresponders (two males, two females) whose serum cholesterol levels had increased significantly ($P < 0.001$) above the values prior to cholesterol feeding (305±9 vs. 801±82 mg/100 ml). In order to study cholesterol turnover in these pigeons each was injected intravenously with 0.5 ml of a Tween-20-ethanol-saline (0.5:1.5:3.0 v/v/v) suspension containing 0.1 mg 1,2-^{3}H-cholesterol (sp. act. 117 μc/mg). Blood samples were obtained from the alar vein at intervals from 1-64 days after tracer administration and the decline in serum cholesterol specific activity was measured. Radioactivity of serum cholesterol samples was assayed by counting in a Beckman DPM-100 (Beckman Instruments, Inc., Fullerton, Calif.), a portion of the isopropanol extract of serum which was used also for the total serum cholesterol determination (4). Serum cholesterol levels of hypo- and hyperresponders remained essentially stable during the 64 days of the turnover study (the means and standard errors of 10 determinations made during this period were 442±26 and 936±42 mg/100 ml, respectively).

The serum isotope die-away curves for the two groups of pigeons are shown in Figure 1. The double exponential curves derived by analysis of the data with an IBM 1620 computer (International Business Machines Corp., White Plains, N.Y.) indicate that turnover of serum cholesterol in the pigeon appears to conform to a 2 pool model (5,6). For this reason, the kinetic analysis for 2 pool systems (5,6) was applied to the data; the results are presented in Table I. The terms used here have been defined previously (1,5,6).

The slopes of the linear portions (day 22-64) of the two die-away curves were significantly different ($P < 0.02$, t-test).

The mass of pool A (M_A), cholesterol in blood and those tissues which are rapidly equilibrated with blood, was smaller in the hyporesponders than in hyperresponders (676 vs. 988 mg) as were the half-lives of the first and

TABLE I

Kinetic Analysis of Serum Die-away Curves in White Carneau Pigeons Following Injection of 1,2-^{3}H-Cholesterol

Group	C_A[a]	C_B[b]	t-1/2, days First exponential	t-1/2, days Second exponential	k_{AA}[c]	M_A[d] (mg)	PR_A[e] (mg/day)	$F.T.O_A$[f] (%)
	(dpm/mg)	(dpm/mg)						
Hypo-responders	31000	6943	1.5	12.7	-.3747	676	133	19.7
Hyper-responders	19679	6278	2.4	16.1	-.2098	988	120	12.2

[a,b]y-Intercepts of first and second exponentials composing the serum cholesterol die-away curve.

[c,d,e]See text.

[f]$F.T.O_A$, % = fractional turnover of pool A expressed as a percentage of the size (mg) of pool A.

second exponentials (1.5 and 12.5 vs. 2.4 and 16.1 days). In addition the rate constant for removal of cholesterol from pool A (k_{AA}), production rate (PR_A, represents the rate of entry of cholesterol into pool A, excluding recycled material originating in pool A) and daily fractional turnover of pool A ($F.T.O._A$) were all greater in the hyporesponders and are compatible with the smaller size of pool A and the lower serum cholesterol levels found in this group.

The mechanism by which hyporesponders maintain low levels of serum cholesterol cannot be determined from these data since whole body cholesterol turnover cannot be obtained from the 2 pool model (6). However, it is clear that individual response to cholesterol-containing diets is quite variable. Since this phenomenon has been observed in pigeons and in nonhuman primates, it may also be a factor involved in hypercholesteremia in the human being.

Individual serum samples (from noncholesterol-fed or cholesterol-fed WC) containing penicillin and streptomycin (446 IU and 0.2 mg/ml respectively) were incubated in stoppered 25 ml Erlenmeyer flasks for 18 hr at 37 C in a metabolic shaker. Free cholesterol concentrations were measured (7) immediately prior to incubation (0 time) and after 18 hr incubation. The decrease in serum free cholesterol concentration between 0-18 hr incubation was taken as a measure of the cholesterol esterifying activity of the serum (3,8). The results are shown in Figure 2. The increase in serum cholesterol esterifying activity correlated highly (rxy=0.806) with serum cholesterol concentration. By contrast, hypercholesteremia decreased the esterification rate of serum cholesterol in the rabbit, rat and chicken in vitro (3). These results do not appear to be a function of age or sex since the esterifying activity of the serum from 8-month-old male and female hyporesponders was similar to that found in the 2-month-old noncholesterol-fed male and female pigeons with similar serum cholesterol levels.

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Incorporation of U-^{14}C-Leucine Into the Serum Lipoproteins and Proteins of Partially Hepatectomized Rats

ABSTRACT

The incorporation of U-^{14}C-leucine into five classes of serum lipoproteins and into lipoprotein-free serum proteins was investigated in partially hepatectomized, sham operated and normal rats. The serum concentration of very low density lipoprotein and the predominant subfraction of high density lipoprotein (HDL_2) was considerably reduced in partially hepatectomized rats as compared with both normal and sham operated rats. The incorporation of U-^{14}C-leucine into HDL_2 of partially hepatectomized rats was only one third and one fourth of that observed with normal and sham operated rats, respectively. On the other hand, the incorporation of the label into low density lipoproteins and into serum proteins was slightly greater in partially hepatectomized rats as compared with normal rats. In sham operated animals, the incorporation into most fractions was considerably greater than those observed with lobectomized and normal rats.

There have been no investigations on the synthesis of the subclasses of serum high density lipoproteins (HDL) in normal rats or on the synthesis of serum lipoproteins in partially hepatectomized rats (1). In this communication data is presented on the incorporation of U-^{14}C-leucine into five classes of serum lipoproteins and into lipoprotein-free serum proteins in partially hepatectomized, sham operated and normal rats.

Partial hepatectomy was performed on six male rats (av. wt. 298 g, Holtzman strain) as described earlier (1). Sham operation was conducted on six male rats (av. wt. 299 g). Six other nonoperated male rats (av. wt. 313 g) were used as external controls. The animals were fasted 24 hr prior to the operation and were not fed postoperatively. Twenty hours after the operation, 5 μc of U-^{14}C-leucine (24 mc/mmole, Volk Radiochemical Co., Calif.) was injected intraperitoneally into each animal and the blood obtained from the abdominal aorta 2.5 hr after injection.

For ultracentrifugal isolation of the serum lipoproteins, 2 ml of serum from every rat in each group was used to fill two pollyallomer tubes of 6.0 ml capacity. The fractions isolated at 10 C using a type 40.3 rotor were: very low density lipoprotein (VLDL) $d < 1.006$; low density lipoprotein (LDL), $1.006 < d < 1.050$; high density lipoprotein (HDL_1), $1.050 < d < 1.063$ (2); high density lipoprotein (HDL_2), $1.063 < d < 1.125$; high density lipoprotein (HDL_3), $1.125 < d < 1.21$; and bottom fraction proteins (BFP), $d > 1.21$. The time of centrifugation at 114,480 x g was 19 hr for VLDL, LDL and HDL_1 and 23 hr for HDL_2 and HDL_3. The ultracentrifugal fractions were dialysed exhaustively against physiological saline containing 0.005% versene and 0.05% unlabeled leucine. An aliquot of the dialysed samples were solubilized in NCS reagent (Amersham/Searle, Ill.) and counted at approximately 75% efficiency in a Tricarb Scintillation spectrometer (3,4). By counting the lipid extracts of the various fractions it was established that U-^{14}C-leucine was almost entirely incorporated (between 90% to 95%) into the protein moieties of the lipoproteins. Protein and lipid determinations were made as described earlier (1).

As observed earlier, the level of LDL was increased as a result of either partial hepatectomy or sham operation (Table I). The serum concentration of HDL_1 and HDL_3 were approximately the same in the three groups while that of VLDL was less in lobectomized rats. The concentrations of BFP were somewhat less in the operated rats. The present results have demonstrated that the striking decrease in total HDL observed earlier (1) in partially hepatectomized rats was almost entirely due to the predominant subfraction of high density lipoproteins (HDL_2).

The highest specific activity (dpm/mg protein) in normal rats was in the LDL fraction and was very close to the specific activity of the HDL_2 fraction. The specific activity of HDL_1 was less than that of LDL or HDL_2 but was considerably greater than that of the HDL_3 fraction. The specific activities of LDL and HDL_2 were between sevenfold to ninefold of that observed for BFP.

In view of the changes in serum lipoprotein concentrations in operated animals as compared with normal animals, it is perhaps more useful to compare the data on total incorporation (dpm/ml fraction) rather than the data on specific activities (dpm/mg protein). In sham operated rats, the total incorporation into most fractions was greatly increased as compared with both lobectomized and normal rats. It is possible that certain serum lipoproteins and

TABLE I

Incorporation of U-^{14}C-Leucine *Into* Serum Lipoproteins and Proteins in Sham Operated, Partially Hepatectomized and Normal Rats

Treatment	Total no. of rats	Lipoprotein fraction	TL[a] (mg/100 ml serum)	Protein (mg/100 ml serum)	LP[b] (mg/100 ml serum)	T[c]	SA[d]
Sham Operation	6	VLDL	43	9[e]	52	1083	2222
Partial Hepatectomy	6	VLDL	23	6[e]	29	533	1588
Nonoperated Control	6	VLDL	57	7[e]	64	691	1721
Sham Operation	6	LDL	42	12	54	2133	3814
Partial Hepatectomy	6	LDL	47	12	59	1564	2898
Nonoperated Control	6	LDL	29	7	36	1295	3870
Sham Operation	6	HDL_1	23	8	31	833	2535
Partial Hepatectomy	6	HDL_1	19	9	28	676	2029
Nonoperated Control	6	HDL_1	20	8	28	864	2818
Sham Operation	6	HDL_2	81	47	128	6050	3741
Partial Hepatectomy	6	HDL_2	33	17	50	1559	2623
Nonoperated Control	6	HDL_2	65	40	105	4510	3251
Sham Operation	6	HDL_3	26	33	59	1616	1903
Partial Hepatectomy	6	HDL_3	23	32	55	857	1063
Nonoperated Control	6	HDL_3	25	39	64	1102	1092
Sham Operation	6	BFP	29	4823		29159	950
Partial Hepatectomy	6	BFP	40	5336		17794	521
Nonoperated Control	6	BFP	41	5736		15924	435

[a]Total lipid.

[b]Lipoprotein.

[c]Total activity, dpm/ml fraction.

[d]Specific activity, dpm/mg protein.

[e]The high values obtained may in part be due to turbidity encountered in the determination of protein in this fraction.

proteins are required for tissue repair as well as to augment defense potential and therefore their synthesis was increased in sham operated rats.

The total incorporation of leucine into LDL and BFP fractions was slightly higher in the serum from rats with regenerating liver than in the serum of normal rats (Table I). In the case of VLDL, HDL_1 and HDL_3 from partially hepatectomized rats, a small decrease in the total incorporation of the label was observed as compared to the incorporation in normal rats. However, in the case of HDL_2, the total incorporation decreased to almost a third of the value observed in the case of normal rats and to almost a fourth of the value observed in the case of sham operated animals.

When the total incorporation was corrected

for the weight of the liver, it was observed that the incorporation (dpm/ml/g liver) was higher in all lipoprotein and protein fractions, except HDL_2, from partially hepatectomized rats as compared with both normal and sham operated rats. That the capacity of the regenerating liver to synthesize lipids and proteins is undiminished is well known (1). Therefore the pronounced decrease in the total incorporation (dpm/ml/g liver) of the label into serum HDL_2 of partially hepatectomized rats is difficult to explain. It has been previously suggested that HDL are perhaps utilized in the formation of structural components of cells (1). In order to explain the present data, it may be postulated that not all the newly synthesized HDL_2 is released into circulation, but that a substantial portion of it is utilized in liver regeneration.

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Animal Endogenous Triglycerides: I. Swine Adipose Tissue

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ABSTRACT

The objective of this study was to define the composition of endogenous glycerides of swine adipose tissues as a basis for further studies on the influences of diet and other environmental factors. It was also hoped that the endogenous triglyceride composition might suggest a fresh approach to the problem of the biosynthetic pathway of natural triglyceride mixtures. Swine were fed a fat-free diet from the age of 17 days to 5 months, and the triglycerides of their mesenteric, perirenal and inner and outer back adipose tissues analyzed by silver ion thin layer chromatography and gas liquid chromatography. Four silver ion fractions were found, S_3, S_2M, SM_2 and M_3 (S, saturated; M, monounsaturated fatty acids). Corresponding fractions from different adipose tissues had identical fatty acid composition. The fatty acid compositions of the 2 position of the fractions were also identical. However, the fatty acid composition of the unfractionated triglycerides varied from tissue to tissue. It is concluded therefore, that the various adipose tissues of swine contain the same triglycerides in varying concentrations. Stearic and oleic acids were located mainly in the combined 1 and 3 positions. Myristic, palmitoleic and palmitic were in the 2 position, with almost 90% of the saturated acids being palmitic. This specific distribution of the major fatty acids can explain the marked simplicity of swine endogenous triglycerides.

INTRODUCTION

With rare exceptions (1-3), published analyses of animal adipose tissue triglycerides have been performed on samples obtained from animals which had consumed fat, often of unknown composition. However, it has been known for decades that dietary fats are deposited, and for several years that endogenous fatty acid synthesis is inhibited during exogenous fatty acid ingestion. Therefore, in order to determine the influences of exogenous fat, of temperature, and of other environmental factors on adipose tissue triglyceride composition, one must know the composition of endogenous fat under known environmental conditions.

This series of papers reports the composition of the endogenous triglycerides of swine, rat and chicken adipose tissues and livers. These animals were selected because they represent groups purported to produce different types of triglyceride mixtures, and because of their availability.

EXPERIMENTAL PROCEDURES

Two male, uncastrated, Yorkshire-Hampshire cross pigs of the same litter were raised from 2 1/2 weeks to five months of age on a fat-free diet (4, diet No. 16). Although the animals developed certain characteristics ascribed to essential fatty acid deficiency (5-8), coarse hair, a waxy exudate in the ear and neck region, and eventually black liquid stools alternating with hard gray stools, their growth was comparable to that of littermates fed conventional swine diets (6). In this laboratory analogous symptoms (except the exudate) have been observed in swine fed the same fat-free diet plus a marine oil supplement.

At slaughter, samples were taken of subcutaneous adipose tissue at various positions along the back and the morphologically distinct inner and outer back fats were separated. Portions of mesenteric and perirenal fat and liver tissue were also removed. Lipids were extracted by the method of Folch et al. (9). After isolation by magnesium silicate (Florisil) column chromatography (10), adipose tissue triglycerides were fractionated by silver ion thin layer chromatography (Ag^+ TLC) on 200 X 400 X 0.5 mm layers containing 20 g silver nitrate per 100 g of silica gel (Adsorbosil-1, Applied Science Labs, State College, Pa.) using the sandwich technique of Brenner and Niederwiesser (11) as modified by deVries and Jurriens (12). Thiophene-free benzene, containing 0.2-0.3% water, was used as the developing solvent. The plates were then sprayed with a methanol solution of 2',7'-dichlorofluorescein and viewed under ultraviolet light. Four bands corresponding to trisaturated (S_3), monounsaturated (S_2M), diunsaturated

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TABLE I

Fatty Acid Composition of the Triglycerides of Four Swine Adipose Tissues

Fatty acid	Adipose tissues			
	Mesenteric	Perirenal	Inner back	Outer back
	mole %			
14:0	1.8	1.4	1.3	1.4
16:0	31.8	30.0	28.4	27.4
16:1	3.5	3.5	3.4	3.9
17:0	0.4	0.6	0.6	0.6
17:1	0.4	0.8	0.8	0.9
18:0	18.7	16.6	15.0	13.6
18:1	42.5	45.7	49.0	50.9
18:2	0.1	0.3	0.2	0.3
20:0	0.2	0.3	0.3	0.2
20:1	0.6	0.8	1.0	1.0

(SM_2) and triunsaturated (M_3) triglycerides were visible. All unsaturated acids were monoenes. These fractions will be called silver ion fractions in the text. The triglycerides from each band were eluted with diethyl ether and stored in ethereal solution under nitrogen at -20 C.

Quantification of the various silver ion fractions was achieved by gas liquid chromatographic (GLC) analysis of the triglyceride fatty acid methyl esters, using methyl pentadecanoate as an internal standard (13).

With the exception of the S_3 fraction, pancreatic lipase hydrolysis was performed in duplicate on 5 mg samples by the method of Luddy et al. (14). For hydrolysis of S_3, methyl oleate was added as a carrier as suggested by Barford et al. (15). Substrate, carrier, bile salts and calcium chloride were warmed to 40 C and shaken for 30 sec prior to the addition of the enzyme solution. After shaking the complete mixture for 5 min at 3,000 strokes per minute, the 2-monoglyceride products of hydrolysis were extracted and isolated by preparative TLC and converted to methyl esters by heating on a steam bath for 20 min in 2 ml of 0.5 N sodium methoxide in methanol.

A Beckman GC-5 gas chromatograph with two flame ionization detectors was used for methyl ester analyses. The column was 6 ft X 1.8 in o.d. stainless steel packed with 10% methyl silicone-ethylene glycol succinate polymer (EGSS-X) on 100-120 mesh Gas Chrom P (Applied Science Labs, State College, Pa.). Analyses were run in triplicate.

Quantitative results with National Heart Institute Fatty Acid Standard D agreed with the stated composition data with a relative error of less than 5% for major components (>10% of total mixture) and less than 33% for minor components (< 10% of total mixture). Weight percentages obtained by GLC were converted to mole percentages.

RESULTS AND DISCUSSION

Fatty Acid Composition

The fatty acid compositions of endogenous swine adipose tissues given in Table I show that palmitic, stearic and oleic acids comprise more than 90% of the acids in each tissue. There are small but important differences in the relative amounts of the fatty acids among the different tissues which will be discussed later. The stearic

TABLE II

Swine Adipose Tissue Triglycerides[a]

Adipose tissue	Silver ion fraction					
		S_2M		SM_2		
	S_3	SSM[a]	SMS	SMM	MSM	M_3
	mole %					
Mesenteric	11.2	42.2	1.5	6.4	32.0	6.7
Perirenal	8.5	40.2	1.1	6.7	34.7	8.7
Inner back	6.2	35.3	1.5	7.8	38.5	10.7
Outer back	4.7	32.3	1.8	7.1	43.0	11.1

[a]S, saturated; M, monounsaturated fatty acid.

TABLE III

Composition of the Diunsaturated (SM_2) Triglycerides of Swine Adipose Tissues[a,b]

Fatty acid	SM_2 triglycerides				2-Monoglycerides after pancreatic lipase hydrolysis of SM_2[c]			
	Mes.	Peri.	IB	OB	Mes.	Peri.	IB	OB
	mole %				mole %			
14:0	1.5	1.2	1.3	1.5	4.6	3.1	3.3	3.9
16:0	26.1	26.6	26.1	27.0	75.7	77.1	76.5	78.8
16:1	4.6	3.7	3.7	4.0	4.0	3.8	3.5	3.0
17:0	---	0.1	---	---				
17:1	0.5	0.8	0.8	0.8				
18:0	5.4	5.6	5.9	5.5	3.2	3.0	3.2	2.7
18:1	60.7	60.5	60.5	59.5	12.0	11.9	12.7	10.8
18:2	0.2	0.3	0.3	0.4				
20:1	1.0	1.2	1.3	1.2				

[a]The other three silver ion fractions, S_3, S_2M and M_3 also had similar composition in different tissues. The values in the different tissues were therefore averaged and presented in Table IV.

[b]Mes., mesenteric; Peri., perirenal; IB, inner back; OB, outer back.

[c]Lypolysis by *pancreatic* lipase. Results for fatty acids present in more than 1% in the original triglycerides are reported.

to oleic acid ratio is higher than that in adipose tissue of other nonruminant animals (16), being comparable to subcutaneous adipose tissues in the reminant (17). Unpublished data from this laboratory show a direct relation between the stearic and oleic acid ratio and levels of stearoyl desaturase activity, in sheep, swine, chicken and rat. This suggests that the level of activity of this enzyme may be the determining factor in the stearic-oleic acid composition of the adipose tissues of these animals.

Triglyceride Composition

Ideally a triglyceride analysis of a natural fat should include the percentage of the various triglycerides, including stereoisomers. Since the methodology for this has not yet been perfected, the present discussion will be limited to the relative amounts of the fractions obtained by Ag^+ TLC and the distribution of the constituent fatty acids between the 2 position and the combined 1 and 3 positions of each fraction.

In confirmation of much older reports (17), the data in Table I show that the deeper the tissue the higher the level of saturated fatty acids. Examination of the data in Table II reveals that this is due to high concentrations of trisaturated and disaturated triglycerides in the deeper tissues.

Within the limits of experimental error, the fatty acid composition of each silver ion fraction was found to be identical in all adipose tissues. This is demonstrated in Table II, in which the SM_2 fraction is taken as an example. The table shows further that the fatty acids of the 2 position of the various fractions also have identical composition. Thus, within these criteria the triglycerides of the various adipose tissues are identical, and the variation in fatty acid composition of the adipose tissues are due to variations in the relative amounts of identical triglycerides.

Since the composition of each silver ion fraction is constant, regardless of tissue (Table II), the values for all tissues were pooled and the averages of the composition of each silver ion fraction, of the 2 position and of the combined 1 and 3 positions are presented in Table IV. Myristic and palmitic acids are highly concentrated in position 2, whereas stearic and oleic acids are in the end positions. Interestingly, palmitoleic is distributed differently in the various triglycerides. The high degree of concentration of most major acids in specific positions, as well as the small number of fatty acids present can explain the similarity of the triglycerides in the different adipose tissues, since these conditions reduce the possible number of dominant triglycerides. Supporting this interpretation are the results of a preliminary stereospecific analysis of the mesenteric S_2M fraction in this laboratory which shows that stearic acid is highly concentrated in the 1 position and oleic in the 3 position.

However, the similarity of the triglycerides of the various adipose tissues requires a more basic mechanistic explanation. It could be the result of a common biosynthetic pattern, a common remodeling system, or both. If there is a single biosynthetic pattern, the question arises as to whether the triglycerides of the various tissues have a common origin or utilize similar enzyme systems. If there is a common source, it cannot be the liver, since the hepatic triglycerides of swine are markedly different from

TABLE IV

Fatty Acid Composition of Swine Adipose Tissue Triglycerides[a]

Fatty acid	S_3			S_2M			SM_2			M_3		
	TG	C_2[b]	$C_{1,3}$[c]	TG	C_2	$C_{1,3}$	TG	C_2	$C_{1,3}$	TG	C_2	$C_{1,3}$
	mole %											
14:0	2.7	3.9	2.1	1.9	4.0	0.9	1.4	3.7	0.3	---	---	---
16:0	46.7	88.1	26.0	37.5	88.2	12.2	26.5	77.0	1.3	---	---	---
16:1	---	---	---	1.5	0.9	1.8	4.0	3.6	4.2	13.0	27.1	6.0
17:0	0.5	2.3	---	0.6	0.4	0.7	---	0.3	---	---	---	---
17:1	---	---	---	---	0.2	---	0.7	0.3	0.9	2.0	4.7	0.7
18:0	47.2	5.9	67.9	26.8	3.6	38.4	5.6	3.0	6.9	---	---	---
18:1	1.4	---	2.1	31.0	2.1	45.0	60.3	11.9	84.5	83.3	68.2	90.0
18:2	---	---	---	---	---	---	0.3	---	0.5	---	---	---
19:0	0.9	---	1.4	---	---	---	---	---	---	---	---	---
20:0	0.9	---	1.4	0.4	---	0.6	---	---	---	---	---	---
20:1	---	---	---	0.5	---	0.8	1.2	---	1.8	1.6	---	2.4

[a]Mesenteric, perirenal, outer back and inner back adipose tissues. Since differences between tissues were within the limits of the experimental error, the data were pooled and averaged.

[b]Fatty acid composition of the 2-monoglyceride products of pancreatic lipase hydrolysis.

[c]Fatty acid composition of the 1,3 positions calculated by the equation $C_{1,3} = (3TG-MG)/2$ where TG equals proportion of a particular fatty acid in the unfractionated triglycerides; MG equals proportion of that acid in the 2-monoglyceride products of pancreatic lipase hydrolysis.

those of the adipose tissues (18).

The mechanisms which control the synthesis of a triglyceride mixture of constant composition under constant conditions must be a series of interrelated phenomena which involve not only the positioning of fatty acids on the glycerol but also the control of the proportions of fatty acids produced. It is interesting and profitable to speculate on the possibility that the composition of the triglyceride mixture is a quality determined by genetic factors, and that the fatty acid compositions of the triglycerides are the consequences and not the determinants of such mixtures. The triglyceride mixtures, in turn, may be varied by such factors as temperature, a variable which explains differences in saturated triglyceride content of plant and animal tissues exposed to high and low temperatures. Something similar can be said for dietary variations.

At any rate, only the predetermination by some coding system under the control of basic genetic mechanisms of the composition of the endogenous triglyceride mixtures can account for the uniformity of the endogenous triglycerides in any given species.

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Animal Endogenous Triglycerides: II. Rat and Chicken Adipose Tissue

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ABSTRACT

The endogenous adipose tissue triglycerides of rat and chicken differ markedly in composition from those of swine although all three contain the same major fatty acids. The main difference is that the swine triglycerides have saturated fatty acids in the middle position, whereas in rat and chicken that position is preferentially occupied by unsaturated acids. In swine adipose tissue triglycerides the order of preference for the middle position is 16:0>16:1>18:0>18:1, whereas in rat and chicken triglycerides the order is 18:1>16:1>18:0>16:0. Generalizing, in swine the order of preference for the 2 position is chain length over unsaturation, shorter chains over longer chains, and saturation over unsaturation. In rat and chicken, the degree of unsaturation prevails over chain length, longer chains over shorter chains, and unsaturation over saturation.

INTRODUCTION

In the preceding paper (1) it was shown that the mixtures of endogenous triglycerides of swine adipose tissues are quite simple, various tissues differing only in the relative amounts of triglycerides of identical constitution. The small number of major triglycerides appears to be the product of a high affinity of the major fatty acids for specific positions on the glycerol moiety.

Privett et al. (2) have reported the triglyceride composition of rats fed a fat-free diet. However, these authors did not perform fatty acid distributional analyses on the fractionated triglycerides.

To the authors' knowledge no information is available on the composition of chicken endogenous triglycerides.

EXPERIMENTAL PROCEDURES

Rat

Fifteen albino male rats of the Check-Houston strain were raised from weaning to 13 weeks of age on a fat-free diet (3). In spite of slightly less than normal growth, significant amounts of fat were deposited. The usual symptoms of essential fatty acid deficiency in rats were observed. After the animals were killed, portions of their perirenal and epididymal fat pads and of their livers were excised for lipid analysis.

Chicken

Fifteen white Leghorn chickens were fed a fat-free diet (4) from hatching to 9 weeks of age. Ross and Adamson (4) reported 78% mortality in chickens raised for 6 weeks under analogous conditions. In the present study, mortality was 53%. However, weight gain per week was less in the present experiment than in that of the above authors. The chicks that survived after nine weeks had very little depot fat. Only about 10 g of adipose tissue was collected from various carcass locations of the remaining 8 chicks. There is evidence that the degree of lipogenesis in chicken adipose tissue is very limited (5,6).

The methods of lipid extraction, fractionation, and analysis were as described in the first paper of this series (1). Analyses were run in triplicate.

RESULTS AND DISCUSSION

Rat Endogenous Adipose Tissue Triglycerides

The composition and distribution of fatty acids in rat perirenal and epididymal fat pad triglycerides are presented in Tables I and II, respectively. There is a marked similarity in fatty acid composition and intramolecular distribution between the unfractionated triglycerides and the SM_2 fraction of both tissues. Perhaps this should not be surprising since there is an average 33% saturated and 67% unsaturated acids in the unfractionated and the SM_2 fractions of both the perirenal and epididymal tissues.

Whereas the fatty acid composition of any silver ion-thin layer chromatography fraction is the same in all swine adipose tissues (1), in rats the composition of the fractions varies with tissue (Tables I and II). There are several reasons for this difference between the two species. Swine are unique in having the middle position of their triglyceride molecules occupied almost entirely by palmitic acid, position 1 by stearic

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TABLE I

Composition and Distribution of the Fatty Acids From Rat Perirenal Triglycerides[a]

Fatty acid	Unfractionated TG			S_3			S_2M			SM_2			M_3		
	TG	C_2[b]	$C_{1,3}$[c]	TG	C_2	$C_{1,3}$	TG	C_2	$C_{1,3}$	TG	C_2	$C_{1,3}$	TG	C_2	$C_{1,3}$
	mole %														
14:0	1.7	0.7	2.2	4.9	7.1	3.8	3.5	1.5	4.5	2.1	0.8	2.8	---	---	---
14:1	0.4	0.3	0.5	---	---	---	---	---	---	---	---	---	---	0.5	---
16:0 br	---	0.3	---	---	---	---	---	---	---	---	---	---	---	---	---
16:0	25.8	6.2	35.6	74.0	77.5	72.3	52.5	24.1	66.7	26.5	6.6	36.5	0.8	---	1.2
16:1	11.7	12.0	11.6	---	---	---	5.4	7.8	4.2	10.9	9.2	11.8	17.0	11.2	19.9
17:1	0.4	0.5	0.4	---	---	---	---	---	---	0.2	---	0.3	0.4	---	---
18:0	3.8	1.5	5.0	17.3	15.5	18.2	9.1	4.2	11.6	3.9	1.8	5.0	---	---	---
18:1	55.4	78.1	44.1	---	---	---	29.1	61.6	12.9	55.9	80.7	43.5	81.1	86.9	78.2
18:2	0.1	0.3	---	---	---	---	---	0.6	---	---	---	---	---	0.8	---
20:0	0.1	0.2	0.1	3.8	---	5.7	---	---	---	---	---	---	---	---	---
20:1	0.3	0.1	0.4	---	---	---	0.4	---	---	0.7	0.9	0.2	0.6	0.5	0.6
20:2	---	---	---	---	---	---	---	---	---	---	---	---	---	0.2	---
20:3	0.2	Trace	---	---	---	---	---	---	---	---	---	---	---	---	---

[a]TG, triglycerides; S, saturated fatty acids; M, monounsaturated.

[b]C_2, fatty acid composition of the 2-monoglyceride products of pancreatic lipase hydrolysis.

[c]$C_{1,3}$, fatty acid composition of the 1,3 position calculated by the equation $C_{1,3}$ = (3TG-MG)/2, where TG equals proportion of a particular fatty acid in the unfractionated triglycerides; MG equals proportion of that acid in the 2-monoglyceride products of pancreatic lipase hydrolysis.

TABLE II

Composition and Distribution of the Fatty Acids From Rat Epididymal Triglycerides[a]

Fatty acid	Unfractionated TG			S_3			S_2M			SM_2			M_3		
	TG	C_2[b]	$C_{1,3}$[c]	TG	C_2	$C_{1,3}$	TG	C_2	$C_{1,3}$	TG	C_2	$C_{1,3}$	TG	C_2	$C_{1,3}$
								mole %							
14:0	1.2	0.9	1.4	3.6	7.9	1.5	2.4	2.6	2.3	1.3	0.5	1.7	---	---	---
14:1	0.7	0.4	0.9	---	---	---	---	---	---	---	---	---	---	---	---
16:0 br	---	0.3	---	---	---	---	---	---	---	---	---	---	---	---	---
16:0	27.9	8.8	37.5	83.4	80.7	84.8	56.3	27.4	70.8	29.4	6.2	41.0	1.1	2.1	0.6
16:1	15.8	16.9	15.3	---	---	---	7.5	11.2	5.7	15.8	12.5	17.5	24.7	16.8	28.7
17:1	0.7	0.5	0.8	---	---	---	---	0.5	---	---	---	---	0.4	---	0.6
18:0	3.3	1.3	4.3	11.1	11.4	11.0	6.9	3.3	8.7	2.8	1.0	3.7	Trace	---	---
18:1	50.1	70.3	40.0	---	---	---	26.7	54.8	12.6	50.8	79.6	36.4	73.5	79.9	70.3
18:2	0.3	0.4	0.3	---	---	---	---	0.3	---	---	0.2	---	0.4	1.2	
20:0	Trace	0.2	---	2.0	---	3.0	0.2	---	0.3	---	---	---	---	---	---
20:1	Trace	0.1	---	---	---	---	---	---	---	Trace	---	---	Trace	---	---
20:2	---	Trace	---	---	---	---	---	---	---	---	---	---	---	---	---
20:3	---	Trace	---	---	---	---	---	---	---	---	---	---	---	---	---

[a]TG, triglycerides; S, saturated fatty acids; M, monounsaturated.

[b]C_2, fatty acid composition of the 2-monoglyceride products of pancreatic lipase hydrolysis.

[c]$C_{1,3}$, fatty acid composition of the 1,3 position calculated by the equation $C_{1,3}$ = (3TG-MG)/2, where TG equals proportion of a particular fatty acid in the unfractionated triglycerides; MG equals proportion of that acid in the 2-monoglyceride products of pancreatic lipase hydrolysis.

TABLE III

Composition and Distribution of the Fatty Acids From Chicken Adipose Tissue Triglycerides[a]

Fatty acid	Unfractionated TG			S_3			S_2M			SM_2			M_3		
	TG	C_2[b]	$C_{1,3}$[c]	TG	C_2	$C_{1,3}$	TG	C_2	$C_{1,3}$	TG	C_2	$C_{1,3}$	TG	C_2	$C_{1,3}$
								mole %							
14:0	0.9	1.1	0.8	2.4	2.8	2.2	1.7	0.6	2.3	0.9	0.2	1.3	---	---	---
14:1	0.2	0.6	---	---	0.6	---	---	0.1	---	---	0.3	---	---	0.3	---
16:0 br	---	0.6	---	---	---	---	---	---	---	---	---	---	---	---	---
16:0	25.1	8.8	33.3	66.6	58.5	70.7	47.2	17.0	62.3	24.8	6.1	34.2	2.1	1.0	2.7
16:1	6.4	4.8	7.2	---	---	---	2.8	4.2	2.1	6.6	4.6	7.6	9.8	5.1	12.2
17:1	---	---	---	---	---	---	0.6	---	---	0.3	---	---	0.4	0.2	0.5
18:0	8.4	5.7	9.8	27.9	37.0	23.4	16.4	11.3	19.0	8.2	3.9	10.4	0.8	---	---
18:1	58.0	77.1	48.5	1.1	1.3	1.0	30.4	66.0	12.6	57.9	84.0	44.9	84.8	92.5	81.0
18:2	---	0.3	---	---	---	---	---	---	---	---	0.2	---	0.6	0.1	0.9
20:0	---	0.3	---	2.0	---	3.0	0.3	---	---	0.2	---	---	---	---	---
20:1	1.2	0.8	1.4	---	---	---	0.6	0.6	0.6	1.2	0.7	1.5	1.5	0.9	1.8

[a]TG, triglycerides; S, saturated fatty acids; M, monounsaturated.

[b]C_2, fatty acid composition of the 2-monoglyceride products of pancreatic lipase hydrolysis.

[c]$C_{1,3}$, fatty acid composition of the 1,3 position calculated by the equation $C_{1,3}$ = (3TG-MG)/2, where TG equals proportion of a particular fatty acid in the unfractionated triglycerides; MG equals proportion of that acid in the 2-monoglyceride products of pancreatic lipase hydrolysis.

acid and position 3 by oleic acid (7). This results in a relatively simple triglyceride mixture.

The rat has less specific orientation of the fatty acids (7) and therefore a larger number of predominant triglycerides. In addition, swine adipose tissues seem unable to incorporate either partial or intact hepatic triglycerides without previous rearrangement (8) whereas it is known that in rats, partial glycerides can pass through adipose cell walls (9,10). When these partial glycerides are fully reesterified inside the cell, the composition of the resulting triglycerides may or may not resemble that of the triglycerides produced in situ from glycerol precursors. The result is a mixture of triglycerides of various origins and different structural characteristics.

Triglyceride Positional Isomers

The relative amounts of the triglyceride fractions obtained by silver ion-thin layer chromatography were determined. From these values and the lipolysis data the relative amounts of the possible positional isomers were calculated (Table IV).

The data show that the slightly higher saturation of the epididymal tissue over the perirenal tissue (Tables I and II) is due to higher levels of S_2M and S_3 triglycerides in the former. Therefore, the endogenous triglycerides of the various adipose tissues of the rat differ among them not only in the fatty acid composition of their component silver ion fractions as discussed above but also in the relative proportions of those fractions.

Chicken Endogenous Adipose Tissue Triglycerides

Recent reports have shown that hen adipose tissue has limited lipogenic activity (5) even with low fat diets (6). This may explain the very small amounts of adipose tissue produced by the birds in the present experiment since they were fed a fat-free diet from hatching. In contrast, lipogenesis is stimulated in rats receiving a fat-free regimen (11). In spite of these differences between the two animals, there are close similarities in the composition and distribution of fatty acids in rat (Tables I and II) and chick (Table III) adipose tissue triglycerides. Also as in rat tissues, the SM_2 fraction of chickens closely resembles the composition and distribution of acids in the unfractionated triglycerides. The composition of chicken triglycerides (Table IV) is very close to that of rats.

Comparative Aspects

Fatty Acid Composition and Distribution. The distribution of fatty acids in the silver ion

TABLE IV

Triglyceride Composition of Rat and Chicken Adipose Tissues[a,b]

Adipose tissue	S_3	SSM	SMS	SMM	MSM	M_3
Rat perirenal	1.1	6.7	15.9	44.8	4.5	27.0
Rat epididymal	2.7	8.0	15.9	42.5	3.5	27.5
Chicken (pooled tissues)	3.1	6.6	16.5	39.7	4.5	29.7
Swine perirenal[c]	8.5	40.2	1.1	6.7	34.7	8.7

[a]S, saturated fatty acid; M, monounsaturated fatty acid.
[b]Mole %.
[c]Data from first paper of this series (1).

fractions of the adipose tissues of the three species can be better compared if the data are expressed as per cent of each fatty acid present in the 2 position, calculated according to the formula given in Table V. The data show that in each animal the ranking of preference of the fatty acids for position 2 is analogous for all four silver ion fractions. However, upon comparing animals, it is seen that rats and chickens have analogous ranking of preference for position 2, namely 18:1>16:1>18:0>16:0, whereas the order in swine adipose tissue triglycerides is quite different: 16:0>16:1>18:0>18:1. In other words, in rat and chicken adipose tissues the degree of unsaturation is more important than chain length in determining the preference for the middle position. Furthermore, the longer the chain and the higher the unsaturation, the greater the preference for the 2 position. In swine adipose tissues, a reversed situation is observed; chain length prevails over unsaturation, shorter chains over longer chains, and saturated acids are preferred over the unsaturated acids for the 2 position. Other endogenous triglycerides of swine such as those of the intestinal mucosa seem to share these reverse characteristics. On the other hand, swine liver endogenous triglycerides have positional specificities similar to those of rat and chick adipose tissues and livers (8).

The order of preferences found for the rat and chicken can probably be extended to most other animals, since the unusual characteristics of swine depots are known to be shared only by peccaries (12) and elephants (13). If this is so, the differences in fatty acid composition and distribution found among animals fed conventional diets are mostly a reflection of the lipid composition of their diet and of the effect of the lipid intake on the degree of lipogenesis.

Triglyceride Composition. The data in Table IV show a close analogy in triglyceride composition between rat and chicken adipose tissues. On the other hand, marked differences, both quanitative and qualitative, are apparent when the adipose tissue triglycerides of rat and chicken are compared with those of swine.

TABLE V

Fatty Acid Preference for the 2 Position[a] of the Endogenous Adipose Tissue Triglycerides of Swine, Rat and Chicken

Silver ion TLC fraction	Fatty acid[b]	Swine perirenal, %[c]	Rat perirenal, %	Chicken (pooled tissues), %
S_3	16:0	63	35	29
	18:0	4	30	44
S_2M	16:0	78	15	12
	18:0	4	15	23
	16:1	20	48	50
	18:1	2	71	72
SM_2	16:0	97	9	8
	18:0	18	15	16
	16:1	30	28	23
	18:1	7	48	48
M_3	16:1	69	22	17
	18:1	27	36	36

[a]Calculated from: % in 2 position = (% in MG X 100)/(% in TG X 3) (14).
[b]Data for myristic acid were too low to allow for meaningful calculations.
[c]Order of preference of fatty acids for 2 position in swine triglycerides: 16:0>16:1>18:0>18:1.

Swine have less M_3 and SM_2 than rats and chickens. In rats and chickens SMS>SSM, whereas in swine the relation is reversed. The amounts of SM_2 in all three animals are more or less in the same range; however the MMS>MSM in rats and chickens while the inverse situations exists in swine adipose tissues. It is quite surprising that triglycerides differing so little in total fatty acid composition differ so much in the type of fractions of which they are composed.

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Animal Endogenous Triglycerides: III. Swine, Rat and Chicken Liver: Comparison With Adipose Tissue

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ABSTRACT

The endogenous triglycerides of swine, rat and chicken livers were fractionated by silver ion thin layer chromatography and the resulting fractions were analyzed for their fatty acid composition and distribution. Whereas the endogenous triglycerides of swine adipose tissue differ markedly from those of rat and chicken adipose tissue in the location of the major fatty acids, the liver triglycerides of the three species are quite similar. They also resemble rat and chicken adipose triglycerides.

INTRODUCTION

In the first two papers of this series (1,2) it was shown that the endogenous triglycerides of swine adipose tissue differ markedly from those of rats and chickens. Although the triglycerides of all three species are composed of the same acids, swine adipose tissues have a simpler triglyceride population than the adipose tissues of the other two species of animals. Since adipose tissue triglycerides may arise either by synthesis in situ or by recombination of glycerides derived from the plasma, and since the liver is a prime source of plasma triglycerides, knowledge of the composition of liver triglycerides is important for a complete understanding of the origin and composition of endogenous adipose tissue triglycerides.

In the present study, the endogenous triglycerides of swine, rat and chicken liver were fractionated and the resulting fractions were analyzed for their fatty acid composition and distribution.

EXPERIMENTAL PROCEDURES

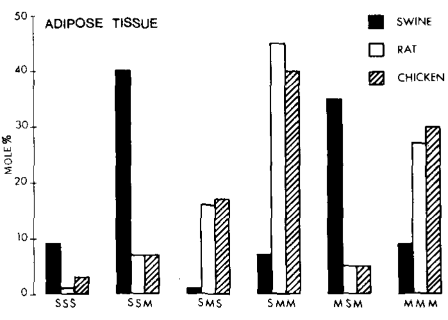
The sources of the tissues and all procedures have been described previously (1,2). Analyses were run in triplicate.

Silver ion thin layer chromatography fractionation of the liver glycerides provided one more band than the four obtained in the adipose tissue fractionation. The fifth band consisted of all the triglycerides of more than three double bonds and lay between the three double bond band and the origin. Due to the presence of dienoic and trienoic acids in the fifth band, it has been labeled >3DB, meaning more than three double bonds.

RESULTS AND DISCUSSION

Fatty Acid Composition

In general, the relative abundance of liver triglyceride fatty acids (column 1, Tables I-III), follows the pattern found in the rat and chick adipose tissue: 18:1>16:0>16:1>18:0 (2). Since the animals were reared on fat-free diets, eicosatrienoic acid, characteristic of essential



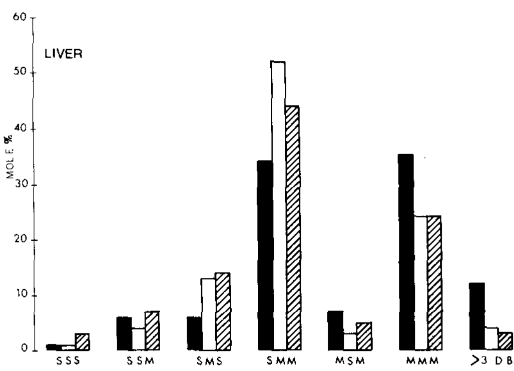


FIG. 1. Composition of adipose tissue and liver triglycerides of swine, rat and chicken. S, saturated fatty acid; M, monounsaturated; >3 DB, silver ion fraction containing triglycerides with more than three double bonds.

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TABLE I

Composition and Distribution of the Fatty Acids From Rat Liver Triglycerides[a]

Fatty acid	Unfractionated TG			S_3			S_2M			SM_2			M_3			>3 Double bonds		
	TG	C_2[b]	$C_{1,3}$	TG	C_2	$C_{1,3}$	TG	C_2	$C_{1,3}$	TG	C_2	$C_{1,3}$	TG	C_2	$C_{1,3}$	TG	C_2	$C_{1,3}$
	mole %																	
14:0	1.1	0.2	1.6	3.1	---	4.6	2.1	4.2	1.1	1.2	1.1	1.3	---	1.3	---	0.8	---	---
14:1	---	0.2	---	---	---	---	---	0.4	---	---	0.5	---	---	0.3	---	---	---	---
16:0	27.7	5.5	38.8	79.9	85.6	77.1	54.8	18.5	73.0	30.2	3.7	43.5	2.9	1.9	3.4	20.1	5.7	27.3
16:1	9.8	10.6	9.4	---	---	---	4.3	8.5	2.2	9.2	9.9	8.9	16.2	10.0	19.3	8.9	7.2	9.8
17:1	0.4	0.2	0.5	---	---	---	0.3	1.7	---	0.1	1.4	--	0.3	1.8	---	0.9	---	---
18:0	2.3	1.0	3.0	15.1	14.5	15.4	6.1	2.4	8.0	1.8	---	2.7	0.2	0.3	0.2	4.3	---	---
18:1	56.0	81.6	43.2	---	---	---	30.1	64.5	12.9	56.9	83.5	43.6	79.8	81.8	78.8	42.7	69.5	29.3
X_1[c]	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	1.4	---	---
18:2	0.5	0.2	0.7	---	---	---	---	---	---	Trace	---	---	0.2	0.9	---	7.7	5.9	8.6
20:0	0.5	0.2	0.7	1.9	---	3.0	1.0	---	1.5	Trace	---	---	---	---	---	0.6	---	---
20:1	0.6	---	0.9	---	---	---	1.3	---	2.0	0.5	---	0.8	0.5	0.6	0.5	0.7	---	---
20:3	1.5	0.4	2.1	---	---	---	---	---	---	---	---	---	---	1.2	---	10.3	11.7	9.6
X_2[c]	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	0.4	---	---
20:4	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	0.4	---	---
20:5	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	0.6	---	---

[a]Since the saturated triglycerides and those with one or two double bonds contain no dienoic acids and those with three double bonds only traces, for all practical purposes they may be considered as S_3, S_2M, SM_2 and M_3 types respectively. (S, saturated fatty acid; M, monounsaturated).

[b]C_2, fatty acid composition of the 2-monoglyceride products of pancreatic lipase hydrolysis.

[c]Unidentified fatty acids.

TABLE II

Composition and Distribution of the Fatty Acids From Chicken Liver Triglycerides[a]

Fatty acid	Unfractionated TG			S_3			S_2M			SM_2			M_3			>3 Double bonds		
	TG	C_2[b]	$C_{1,3}$	TG	C_2	$C_{1,3}$	TG	C_2	$C_{1,3}$	TG	C_2	$C_{1,3}$	TG	C_2	$C_{1,3}$	TG	C_2	$C_{1,3}$
	mole %																	
14:0	0.9	0.1	0.3	2.2	7.4	—	1.6	1.6	1.6	0.9	0.5	1.1	—	—	—	0.4	0.7	0.3
14:1	0.2	0.4	0.1	—	—	—	—	0.3	—	—	0.4	—	—	0.2	—	—	0.7	—
16:0 br	—	0.5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
16:0	25.0	8.9	33.0	62.6	57.3	65.3	45.3	21.0	57.5	24.9	6.0	34.4	1.4	2.2	1.0	12.2	6.5	15.1
16:1	5.8	4.1	6.7	—	—	—	2.5	3.7	1.9	5.3	4.2	5.9	10.3	4.5	13.2	8.0	5.2	9.4
17:1	Trace	—	—	—	—	—	0.5	—	0.8	0.4	—	0.6	0.7	—	1.1	2.9	—	4.4
18:0	8.6	5.3	10.3	34.2	31.8	25.4	18.3	12.1	21.4	6.9	3.3	8.7	0.9	0.9	0.9	8.2	2.7	11.0
18:1	57.9	77.9	47.9	—	—	—	30.8	60.7	15.9	60.3	84.9	48.0	84.0	91.3	80.4	40.7	62.9	29.6
X_1[c]	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.9	—	1.4
18:2	Trace	0.2	—	—	—	—	—	—	—	—	—	—	0.7	—	1.1	3.2	2.6	4.5
20:0	Trace	—	—	1.0	2.9	0.1	0.5	—	0.8	0.2	—	0.3	—	—	—	1.6	0.6	2.1
20:1	1.0	0.7	1.4	—	—	—	0.5	0.8	0.4	1.1	1.0	1.2	1.4	0.9	1.7	1.2	0.9	1.4
20:2	0.2	0.2	0.2	—	—	—	—	—	—	—	—	—	0.5	0.2	0.7	4.1	1.4	5.5
20:3	0.5	1.8	—	—	—	—	—	—	—	—	—	—	—	—	—	9.7	14.1	7.5
X_2[c]	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1.3	—	2.0
20:4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2.7	—	4.1
20:5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2.7	—	4.1

[a]Since the saturated triglycerides and those with one or two double bonds contain no dienoic acids and those *with three* double bonds only traces, for all practical purposes they may be considered as S_3, S_2M, SM_2 and M_3 types, respectively. (S, saturated fatty acid; M, monounsaturated).

[b]C_2, fatty acid composition of the 2-monoglyceride products of *pancreatic lipase* hydrolysis.

[c]Unidentified fatty acids.

TABLE III

Composition and Distribution of the Fatty Acids From Swine Liver Triglycerides[a]

Fatty acid	Unfractionated TG			S_3			S_2M			SM_2			M_3			>3 Double bonds		
	TG	C_2[b]	$C_{1,3}$	TG	C_2	$C_{1,3}$	TG	C_2	$C_{1,3}$	TG	C_2	$C_{1,3}$	TG	C_2	$C_{1,3}$	TG	C_2	$C_{1,3}$
	mole %																	
14:0	1.4	0.9	1.7	5.7	7.1	5.0	3.9	2.4	4.7	2.0	1.0	2.5	0.5	0.5	0.5	1.0	4.7	---
15:0	0.6	1.2	0.3	1.9	4.6	0.6	1.3	1.4	1.3	0.7	0.7	0.7	0.5	0.4	0.6	0.3	1.8	---
16:0	17.7	11.7	20.7	71.8	61.3	77.1	49.1	32.2	57.6	24.2	11.0	30.8	4.3	3.5	4.7	12.2	11.1	12.8
16:1	7.1	5.6	7.9	---	---	---	3.3	3.5	3.2	6.4	6.0	6.6	13.4	7.6	16.3	10.0	5.0	12.5
17:0	2.5	2.3	2.6	5.7	7.9	4.6	5.4	3.5	6.4	3.1	1.1	4.1	---	---	---	0.7	1.1	0.5
17:1	3.5	3.6	3.5	---	---	---	1.3	2.4	0.8	3.2	4.0	2.8	5.2	4.4	5.6	3.5	2.8	3.9
18:0	5.0	3.9	5.6	14.9	19.3	12.7	10.9	8.6	12.1	5.3	2.8	6.6	1.0	1.1	1.0	3.8	3.6	3.9
18:1	56.6	62.9	53.5	---	---	---	24.8	44.7	14.9	53.4	72.3	44.0	73.3	81.1	69.4	46.3	44.0	47.5
18:2	0.7	0.7	0.7	---	---	---	---	1.2	---	1.0	1.4	0.8	1.1	1.4	1.0	1.0	3.8	---
X_1[c]	0.3	0.8	0.1	---	---	---	---	---	---	---	---	---	---	---	---	1.6	---	2.4
18:3	1.7	2.2	1.5	---	---	---	---	---	---	---	---	---	---	---	---	3.7	6.5	2.3
20:1	0.2	---	0.3	---	---	---	---	0.2	---	0.9	0.2	1.3	1.2	0.2	1.7	---	---	---
20:3	2.2	2.3	2.2	---	---	---	---	---	---	---	---	---	---	---	---	11.6	9.8	12.5
20:4	0.2	0.7	---	---	---	---	---	---	---	---	---	---	---	---	---	2.0	3.1	1.5
20:5	0.1	---	0.2	---	---	---	---	---	---	---	---	---	---	---	---	2.1	2.7	1.8
X_2[c]	---	1.1	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

[a]Since the saturated triglycerides and those with one or two double bonds contain no dienoic acids and those with three double bonds only traces, for all practical purposes they may be considered as S_3, S_2M, SM_2 and M_3 types, respectively. (S, saturated fatty acid; M, monounsaturated).

[b]C_2, fatty acid composition of the 2-monoglyceride products of pancreatic lipase hydrolysis.

[c]Unidentified fatty acids.

TABLE IV

Preference of the Major Fatty Acids for the 2 Position[a] of Liver Endogenous Triglycerides of Swine, Rat and Chicken

Silver ion fraction[b]	Fatty acid	Swine, %	Rat, %	Chicken, %
S_3	16:0	28	36	31
	18:0	43	32	31
S_2M	16:0	22	11	15
	18:0	26	13	12
	16:1	35	66	49
	18:1	60	71	66
SM_2	16:0	15	4	8
	18:0	18	0	16
	16:1	31	30	26
	18:1	45	49	47
M_3	16:1	19	21	15
	18:1	37	34	36
>3 DB	16:0	30	9	18
	18:0	32	0	11
	16:1	17	27	22
	18:1	32	54	51
	18:2	127	26	27
	20:3	28	38	48

[a]Calculated by the equation % in 2 position (% in MG x 100)/(% in TG x 3) (5). TG, unfractionated triglycerides; MG, 2-monoglyceride products of pancreatic lipase hydrolysis.

[b]S, saturated fatty acid; M, monounsaturated fatty acid; >3 DB, triglycerides containing more than three double bonds.

fatty acid deficiency, is present in the triglycerides of all three livers. There are some differences between swine on the one hand and rat and chicken on the other. Palmitic acid is much lower, and 18:3 and 20:3 are much higher. Also, the swine liver contains significant amounts of 17:0 and 17:1 acids which are absent or present only in trace amounts in the other two species. The ratio of saturated to unsaturated acids in swine liver is about 1:3 whereas it is about 1:2 in the chicken and rat livers.

Fatty Acid Distribution

The fatty acid composition of the triglycerides derived by silver ion TLC (from now on designated as silver ion fractions) and their pancreatic lipase monoglyceride products is given in Tables I-III along with the calculated values for the fatty acids on the primary positions. Since the saturated triglycerides and those with one or two double bonds contain no linoleic acid, and those with three double bonds only traces, for all practical purposes the triglycerides with none, one, two, or three double bonds may be considered as S_3, S_2M, SM_2 and M_3, respectively, where S is a saturated acid and M is a monoenoic acid.

The determination of the positional distribution of a fatty acid in the unfractionated triglyceride mixture may be a misleading indication of its placement in the constituent fractions. A glance at the data in Tables I-III shows that the ratios of the concentrations of an acid in the 2 position to its concentration in the 1,3 positions is often different in the various silver ion fractions than what it is in the unfractionated triglycerides. A good example of this is palmitoleic acid.

Fatty acid distributions in silver ion fractions from different tissues and animals may be best compared when expressed as per cent in the 2 position, as in Table IV. All liver triglycerides have very similar fatty acid distributions, the order of preference of the major fatty acids for the 2 position being 18:1>16:1>18:0>16:0.

Comparison of swine adipose tissue triglycerides (1) with those of liver shows that they are different. For example, the adipose tissue triglycerides are characterized by a highly specific placement of palmitic acid in the middle position while in liver triglycerides oleic is the acid preferentially in the 2 position. Because of these differences, it is reasonable to assume that swine liver makes little if any contribution of either intact or partial glycerides to the depots, but that the triglycerides of the adipose tissue are produced in situ. One must assume, therefore, that any plasma triglycerides entering the adipose tissue cell are either completely hydrolyzed prior to entry, or are rapidly re-

TABLE V

Composition of the Endogenous Liver Triglycerides of Swine, Rat and Chicken[a,b]

Source	S_3	SSM	SMS	SMM	MSM	M_3	>3 DB
Swine	1.1	5.8	6.2	33.6	6.7	34.7	11.9
Rat	0.7	4.4	13.1	52.1	2.6	23.5	3.5
Chicken	2.9	7.3	13.7	43.7	4.8	24.2	3.4

[a]>3 DB, triglycerides containing more than three double bonds; S, saturated fatty acid; M, monounsaturated fatty acid.
[b]Mole %.

arranged once entry is made. In contrast to swine, rat adipose tissues have been reported to incorporate glycerides directly from plasma, after partial hydrolysis (3,4). Thus, the endogenous triglycerides of the adipose tissues of that animal should be a mixture of those derived from the liver and those synthesized in situ. In the present study, uncomplicated by diet fat, the composition and distribution of the fatty acids in rat liver and adipose tissue triglycerides were found to be quite similar. Thus, the two tissues either produce the same type of triglycerides or there is an interchange between tissues.

The relative amounts of the various silver ion fractions from rat, chicken and swine livers are given in Table V.

Comparison of Species

The similarity of the fatty acid composition of the endogenous triglycerides of swine, rat and chicken adipose tissue (2) applies also to their livers (Table VI). However, swine adipose tissue triglycerides differ in having more stearic than hexadecenoic acid and their livers in having significant amounts of C_{17} acids.

There are also marked differences in the proportions of the triglycerides (Fig. 1). Rat and chicken adipose tissue and rat, chicken and swine liver triglycerides all follow a common pattern. SMM is the most abundant fraction, followed by MMM and SMS, the three constituting more than 75% of the triglycerides. Their common characteristic is an unsaturated fatty acid in the middle position. The predominant fractions in swine adipose tissue, on the other hand, are SSM and MSM, which have a saturated fatty acid in the central position.

The differences in the proportions of the triglycerides of swine adipose tissue can thus be seen as the result of differences in the positional distribution of the saturated and unsaturated fatty acids between the 1,3 and 2 positions, for example, SMM vs. MSM. The SM_2 fraction serves well for comparative purposes since it is the most abundant and because it has saturated and unsaturated fatty acids in approximately the same proportions as in the unfractionated triglycerides. The fatty acid compositions of the SM_2 fractions from the various tissues and animals are given in Figure 2. Except for slight differences in the concentration of hexadecenoic acid, the profiles of the samples studied are quite similar.

The fatty acid distribution in the SM_2 fractions is depicted in Figure 3. Oleic acid constitutes more than 75% of the fatty acids in the middle position of rat and chicken adipose tissues and of the three liver triglycerides. On the other hand, the middle position of swine adipose tissue triglycerides is occupied mainly by palmitic acid.

TABLE VI

Major Fatty Acids of Adipose Tissue and Liver Endogenous Triglycerides of Swine, Rat and Chicken

	Swine		Rat		Chicken	
Fatty acid	Perirenal fat	Liver[a]	Perirenal fat	Liver	Pooled adipose tissue	Liver
			mole %			
14:0	1.4	1.4	1.7	1.1	0.9	0.9
16:0	30.1	17.1	25.8	27.7	25.1	25.0
16:1	3.5	7.1	11.7	9.8	6.4	5.8
18:0	16.6	5.0	3.8	2.3	8.4	8.6
18:1	45.8	56.6	55.4	56.0	58.0	57.9

[a]Swine liver also contains 17:0, 2.5%; 17:1, 3.5%; 18:3, 1.7% and 20:3, 2.2%.

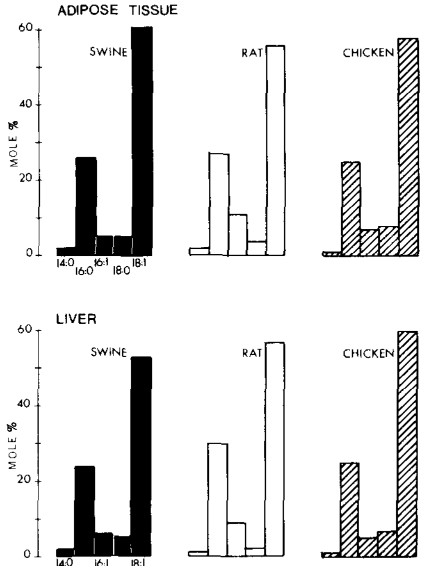

FIG. 2. Major fatty acid composition of the dimonounsaturated triglycerides (SM_2) of swine, rat and chicken adipose tissue and liver.

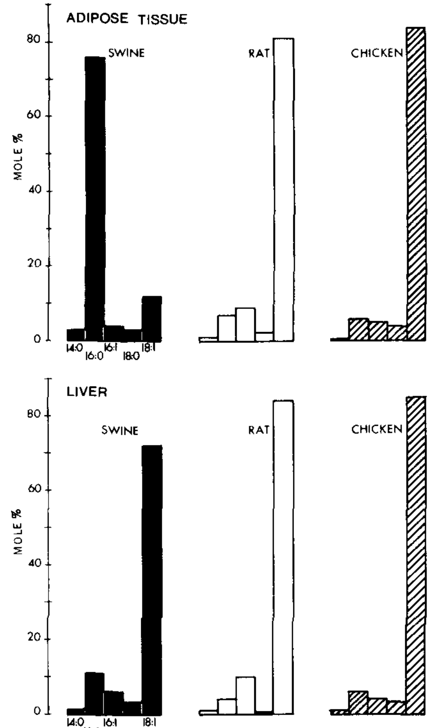

FIG. 3. Major fatty acid composition of the 2-monoglyceride products of pancreatic lipase hydrolysis of SM_2 from swine, rat and chicken adipose tissue and liver.

Figure 4 was designed to compare the degree of preferences of fatty acids for the 2 position calculated according to the formula given on the figure. The various silver ion fractions are depicted on the "x" axis, the major fatty acids on the "z" axis, and the percentages of the latter on the "y" axis. The shape of the peaks is mere idealization, only their heights being relevant. The left part of the figure represents an average of the relative preferences for the 2 position in rat and chicken adipose tissue, and swine, rat and chicken liver triglycerides. The figure on the right describes the situation in swine adipose tissue triglycerides. If one looks along the "z" axis of the left figure one can see that all the silver ion fractions in this group have their fatty acids in equal order of preference for the 2 position: 18:1>16:1>18:0>16:0. In other words, unsaturated acids possess greater preference for the 2 position than saturated acids, and at equal degree of unsaturation longer chain acids prevail over shorter ones. Examination of the figure on the right shows that in swine adipose tissue triglycerides the order of preference for the 2 position is also equal in all silver ion fractions but the order is different from that observed in the left figure, being 16:0>16:1>18:0>18:1. Therefore, in the middle position of swine endogenous adipose tissue triglycerides, shorter chain acids prevail over longer chain ones, and at equal chain length saturated acids predominate over unsaturated ones. What is seen in the right figure is almost the complete reversal of what is observed in the left, thus explaining the unusual characteristics of the endogenous swine adipose tissue triglycerides. These triglycerides appear to be unique since other animals, with the possible exception of peccaries and elephants, have fatty acid populations and distribution characteristics similar to those of the rat and chicken. One must therefore conclude that with those exceptions, there is uniformity in the mechanisms of biosynthesis of endogenous triglycerides whether by different animals or by different organs or tissues of the same animal. This is obviously the result of a rigid homeostatic control. Whether this homeostatic effect results from the existence of similar biosynthetic systems or from similar remodeling effects cannot be determined from the present data.

Comparison of Experimentally Obtained and Theoretically Calculated Triglyceride Compositions

Several distribution hypotheses have been proposed for predicting the composition of

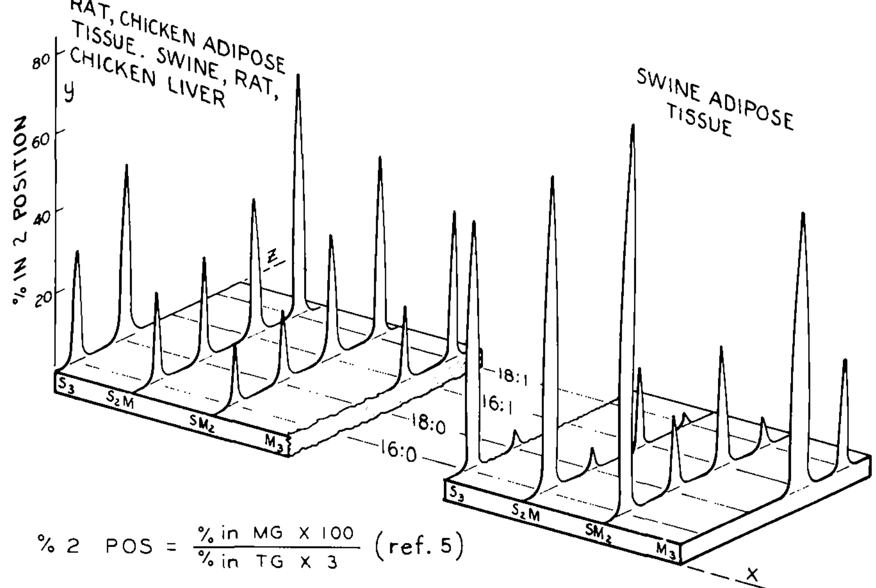

FIG. 4. Degree of preference of the major fatty acids for the 2 position of swine, rat and chicken adipose tissue and liver triglycerides.

natural triglyceride mixtures. The data gathered in the present series of studies provide an opportunity for testing the validity of some of those hypotheses. Therefore, the triglyceride composition data presented in Table III of the first paper in this series (1), in Table IV of the second paper (2), and in Table IV of the present paper were compared with those calculated according to the following distribution hypotheses: (a) Random distribution (6); (b) Restricted random distribution, Kartha (7). The simplified calculations of Hammond and Jones were used (8); (c) Gunstone's hypothesis (9); (d) 1,3-random-2-random distribution, VanderWal (10) and Coleman and Fulton (11).

A Fortran IV computer program analogous

TABLE VII

Experimental and Calculated Values of the Composition of Endogenous Animal Tissues: Adipose Tissue[a]

Hypotheses	S_3	S_2M	SM_2	M_3	SMS	SSM	MSM	MMS
				mole %				
Swine perirenal adipose tissue[b]								
Experimental	8.5	41.3	41.4	8.7	1.1	40.2	34.7	5.7
Random	11.8	36.7	38.2	13.3	12.2	24.5	12.7	25.5
Restricted random	8.5	41.7	38.2	11.7	13.9	27.8	12.7	25.5
Gunstone's	0	54.0	39.0	7.0	54.0	0	0	39.0
1,3-random-2-random	8.7	37.9	45.1	8.3	2.0	35.9	37.0	8.1
Rat perirenal adipose tissue								
Experimental	1.1	22.6	49.3	27.0	15.9	6.7	4.5	44.8
Random	3.1	20.3	44.3	32.3	6.8	13.5	14.8	29.6
Restricted random	1.1	22.6	56.6	30.6	7.5	15.1	15.2	30.4
Gunstone's	0	22.2	49.8	28.0	22.2	0	0	49.8
1,3-random-2-random	1.6	21.1	47.5	29.7	16.8	4.4	2.9	44.6
Chicken adipose tissue (pooled)								
Experimental	3.1	23.1	44.2	27.7	16.5	6.6	4.5	39.2
Random	4.1	23.3	44.4	28.2	7.8	15.5	14.8	29.6
Restricted random	3.1	24.5	44.9	27.5	8.2	16.3	15.0	30.0
Gunstone's	0	26.6	49.9	23.4	26.6	0	0	49.9
1,3-random-2-random	3.2	24.2	46.3	26.3	16.1	8.1	5.2	41.1

[a]S, saturated; M, monounsaturated. U, unsaturated.

[b]Since the composition of the various adipose tissues differed very little, only one is included.

TABLE VIII

Experimental and Calculated Values of the Composition of Endogenous Animal Tissues: Liver[a]

Hypotheses	S_3	S_2M	SM_2	U_3	SMS	SSM	MSM	MMS
				mole %				
Swine liver								
Experimental	1.1	12.0	40.3	46.6	6.2	5.8	6.7	33.6
Random	2.0	16.2	43.2	38.6	5.4	10.8	14.4	28.8
Restricted random	1.1	17.1	44.0	37.7	5.7	11.4	14.7	29.4
Gunstone's	0	16.6	48.3	35.0	16.6	0	0	48.3
1,3-random-2-random	1.9	16.1	43.7	38.3	7.6	8.5	9.6	34.1
Rat liver								
Experimental	0.7	17.5	54.7	27.0	13.1	4.4	2.6	52.1
Random	3.2	20.5	44.4	32.0	6.8	13.7	14.8	29.6
Restricted random	0.7	23.4	45.9	30.0	7.8	15.6	15.3	30.6
Gunstone's	0	22.5	49.9	27.7	22.5	0	0	49.9
1,3-random-2-random	1.3	21.5	48.1	29.1	18.1	3.4	2.2	45.9
Chicken liver								
Experimental	2.9	21.0	48.5	27.7	13.7	7.3	4.8	43.7
Random	4.1	23.4	44.4	28.1	7.8	15.6	14.8	29.6
Restricted random	2.9	24.9	45.0	27.2	8.3	16.6	15.0	30.0
Gunstone's	0	26.8	49.9	23.3	26.8	0	0	49.9
1,3-random-2-random	2.9	24.3	46.6	26.1	16.9	7.3	4.5	42.1

[a]S, saturated; M, monounsaturated; U, unsaturated.

to that of Perkins and Hanson (12) was used for the calculations.

The results (Table VII) show that of the hypotheses tested, the 1,3-random-2-random distribution values compare most closely with those obtained experimentally. However, the similarities may be fortuitous. The 1,3-random-2-random hypothesis assumes equal distribution between the 1- and 3-positions, but stereospecific analyses of unfractionated swine depot (13) and rat liver (14) indicate that saturated fatty acids are preferred at the 1 position and unsaturated acids at the 3 position.

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The Effect of Temperature on the in vitro Formation of Methyl Esters in Mouse Liver

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ABSTRACT

The appearance of methyl esters of fatty acids in lipid extracts was studied. Experimental evidence indicates that these esters are both natural components of tissues as well as products of technical manipulation. Effect of temperature on the latter process was studied. The results of these experiments show that the postmortem increase of fatty acid methyl esters could best be explained by postulating an enzymatic rather than a chemical process.

INTRODUCTION

In recent years the nature of tissue fatty acid methyl esters has been raised. Some have regarded these esters as artifacts of tissue extraction (19,20,29,30) or extract manipulation (10,11). Others have considered the esters as natural components of the tissue (3-5,15,17,21,27).

The issues regarding the presence of fatty acid methyl esters have not all been resolved. The present paper investigates the effect of temperature on in vitro methyl ester formation. The postmortem increase of methyl esters was considered to arise chemically, enzymatically, or both.

MATERIALS AND METHODS

All chemicals in these studies were reagent grade and were used without further purification. Reagent blanks were tested for the presence of spurious spots or peaks by thin layer chromatography (TLC) and gas liquid chromatography (GLC). The absolute methanol, ethyl ether and heptane were purchased from Baker and Adamson. Fischer petroleum ether (bp 34.8-53.6 C) and Merck hexane were also used.

Methyl esters standards were obtained from Applied Science Laboratory and found to be pure by both TLC and GLC. Multibore columns (Med-Chem Laboratories, Detroit, Mich.) were used for all fractionation studies (8). The adsorbent Florisil (60/100 mesh, Floriden Co., Tallahassee, Fla.) was hydrated to contain 7% water.

TLC was carried out on frosted glass plates (14) with silicic acid (Camag Kieselgel G, Arthur H. Thomas Co.) as the adsorbent.

Methyl esters were analyzed on a Packard (No. 7800) dual column chromatograph equipped with hydrogen flame detectors. Argon was used as the carrier gas. Coiled glass columns (6 X 4 mm i.d.) were packed with silane treated Gas Chrom P coated with 14.5% EGSS-X (ethylene glycol succinate with a silicone) or the less polar SE-30 (1%) on Gas Chrom Z.

The lyophilizer used in these experiments consisted of an all glass apparatus attached to a two stage vacuum pump (10 u/in.^{2}).

Extractions

Nine to ten-week old normal white male mice were fed Purina Mouse Chow and water ad lib. Mice were killed by ether anesthesia and livers surgically removed, washed in distilled water, blotted dry and quick-frozen in a dry ice-acetone mixture. Tissues were stored at -30 C.

Before extraction, the frozen tissues were thawed at room temperature and immediately homogenized in distilled water. The aqueous solutions were rapidly extracted with chloroform-methanol (2:1). The ratio of solvent to tissue was approximately 10:1.

Methanol Detection and Measurement

As a check of instrument sensitivity for methanol, serial dilutions of the alcohol in water were measured by injection of these solutions into a gas chromatograph. Both SE-30 and EGSS-X liquid phase columns were run isothermally at 100 C; flame detectors, being water insensitive, were employed. Methanol could be detected at a level as low as .05 μg per sample. Sensitivity level for tissue samples were set at this limit. Aqueous extracts of liver as well as organic solvents used in the study were all examined for their alcohol content.

Effect of Lyophilization

Tissue samples (1.0 g) were homogenized with 10 ml methanol and deliberately incubated for 24 hr at room temperature, so as to induce an increase of artifactual esters. At the termination of incubation, 2-3 ml of water were added and the sample tubes frozen in a dry ice-acetone mixture. Control samples were represented by liver tissue extracted immediately

with chloroform-methanol (2:1) to avoid artifact formation (9).

Aliquots of samples were analyzed for methyl esters before and after lyophilization. Methyl stearate was added to specific specimens when losses during sample manipulation were being measured. Both residue and condensate were checked during these procedures for possible loss of fatty acids during lyophilization.

Effect of Temperature on Methyl Ester Formation

Livers from 10 mice were homogenized in distilled water in ratio of 10 ml/gm tissue. Distilled water was used instead of buffer since our results were more relevant to lipids than enzyme chemistry objectives. The total volume was divided into 22 5 ml aliquots. Three samples were incubated for 24 hr at each temperature interval: -30 C, -5 C, +5 C, 25 C, 37 C, 50 C and 100 C. Several control samples were set up as a check on loss through volatilization during reflux and for destruction of esters already present in the tissue. Some samples were refluxed with a known amount of a standard ester and checked for percentage recovery. Other samples were brought to 100 C immediately and refluxed prior to incubation at the optimum temperature (37 C). After 24 hr incubation, each sample was extracted rapidly with chloroform-methanol (2:1). Extracts were evaporated under nitrogen and crude extracts dissolved in hexane for chromatography.

Chromatography

Column. Cholesterol esters were separated from crude lipid extracts by placing the crude lipid dissolved in hexane on a Florisil multibore column. The hydrocarbon fraction was removed with additional hexane and the cholesterol ester fraction was eluted with hexane-ether (95:5). The latter fraction contained sterol esters as well as esters of other alcohols.

Thin Layer. Cholesterol ester was subfractionated further by TLC. Frosted glass plates covered with Kieselgel were activated for 2 hr at 110 C. Plates were developed by petroleum ether-ether (95:5) or heptane-ethyl ether-petroleum ether-acetic acid (60:20:20:1). Samples were visualized by water spray. The methyl ester spots were eluted with ether.

Gas liquid. Methyl ester samples were dissolved in 0.04 ml hexane containing methyl heptadecanoate as an internal standard. The instrument was calibrated with known amounts of methyl esters. The injection port was kept at 300 C, the detectors at 250 C, and the column was temperature programmed from 100 to 200 C (3.5 C/min). Samples were routinely run

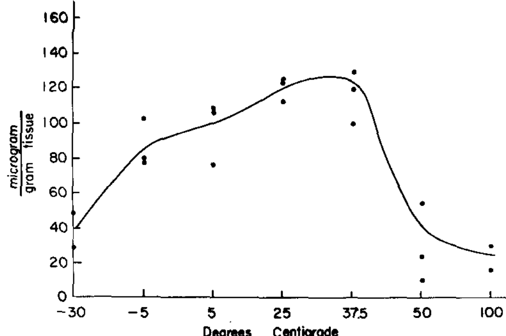

FIG. 1. Mouse liver homogenates were incubated at different temperatures for 24 hr. Fatty acid methyl esters were extracted and measured for each incubation temperature period.

by both polar (EGSS-X) and nonpolar (SE-30) columns.

Peaks obtained from biological samples were compared with retention times of standard fatty acid methyl esters. Quantification was accompanied by use of standard and peak height measurements. Response of the chromatograph to known concentration of several standards was determined. Measurements on peaks representing C_8 to C_{22} methyl esters were obtained.

RESULTS

The aqueous solution of extracted tissues was checked as a possible source of free methanol. Under the conditions of our experiments, a minimum of 0.05 μg of methanol per sample could be detected by GLC. In agreement with previous results (7), no extractable methanol from biological material was detected.

In attempts to determine whether the more volatile methyl esters were lost during lyophilization procedures, both residue and condensate were measured for appearance of esters on GLC. There was no difference between amounts of methyl esters found in lyophilized and nonlyophilized tissue residue. Also, as expected from the results of the first part of the experiment, no peaks were found in the condensate of the lyophilized extracts.

The results from homogenates of mouse liver incubated for 24 hr at various temperatures are presented in Figure 1. Increasing amounts of methyl esters were formed as temperatures increased (-30-37.5 C). At temperatures greater than 37.5 C, i.e., 50 C and 100 C, a marked decrease in methyl esters was measured. Again, volatile methyl esters were not lost during reflux since control samples demonstrated 96%

recovery. Aliquots of the tissue denaturated by raising the temperature to 100 C for 1 hr and refluxed immediately show no increase in ester upon incubation at the optimum temperature (37.5 C). However, aliquots of similar samples when not first refluxed at 100 C doubled in amount of ester formation when incubated at 37.5 C.

DISCUSSION

Whether methyl esters of fatty acids are present in tissue as a natural component or as an artifact has been a question in need of clarification. Some researchers have isolated the presumably natural esters from various tissues with both methanolic and nonalcoholic solvents (4,5,15). Large amounts were identified in both the human and dog pancreas (17,27). More recently, lesser amounts were again analyzed in the human pancreas by another laboratory (21).

On the other hand, the presence of carbonate ion in methanol has been reported as a catalytic agent in artifact formation of methyl esters (20,22,29). Other researchers considered the methyl esters as artifacts produced during extraction procedures or extract manipulation (10,11,19,30).

However, both sources of the esters have been cited by yet another researcher, and an enzymatic factor has been implicated (3). Our own laboratory previously reported a residue of natural ester which could be increased by methanolic incubation (7). It is known that ethanol has been detected in some tissues (6,8,23,24) and also enzymatic formation of ethyl esters has been studied (12,23,25). However, the present investigation could again detect no free methanol on the tissue, though an enzyme directed group transfer is a distinct possibility (1,2; also, Axelrod, personal communication). Conflicting evidence for the identification and amounts of methanol found on other tissues has been reported (6,16,18).

Though this investigation was not designed with an enzymatic reaction in mind, our finding gives rise to such consideration and several important points: (a) There is no doubt that methyl esters can be induced in tissue by the presence of exogenous alcohols, aided by such factors as time, temperature, etc. (b) The residual natural esters are not lost through volatilization, even at 100 C. (c) There is some factor in the tissue that is able to increase the amount of ester in the absence of exogenous alcohol. The in vitro formation of methyl esters and rapid drop at 50-100 C seems to implicate an enzymatic type reaction. The temperature control (denaturation at 100 C for 1 hr) prior to incubation excludes a chemical reaction at higher temperatures or destruction of the ester already present.

Obviously a methyl donor source is available to the tissue. While not explaining the actual production of the esters, this study does point to the fact that some methyl esters can occur by enzymatic reaction. It is believed that these considerations are important from an analytical viewpoint. In addition, they raise questions of other metabolic significance.

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Antioxidant Properties of α-Tocopherol Derivatives and Relationship of Antioxidant Activity to Biological Activity

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ABSTRACT

A series of α-tocopherol analogs substituted at the 5-methyl group were evaluated for their ability to protect β-carotene dissolved in hydrogenated, tocopherol-stripped corn oil from air oxidation. These results were compared with the ability of these compounds to prevent the development of dietary necrotic liver degeneration in rats and vitamin E deficiency-induced muscular dystrophy in rabbits. Although a good correlation was seen between the antioxidant activities of some of the compounds and their biological activities, in the case of other compounds the correlation was poor. The importance of the phytyl side chain of the tocopherols and their derivatives for their antioxidant activities was evident from these studies.

INTRODUCTION

The antioxidant activities of the tocopherols and the relationship between their activities as antioxidants and their biological activities has been much discussed. A comparison of the antioxidant activities of α-, γ- and δ-tocopherol was recently made using tocopherol-stripped hydrogenated corn oil as the substrate. It was found that the antioxidant effect of γ-tocopherol was greater than that of δ-tocopherol which was greater than that of α-tocopherol in protecting this material from air oxidation (1). Adding additional amounts of the tocopherol to the corn oil above a certain optimum level resulted in no greater protection from oxidation or in the case of very high concentrations even less protection occurred. In most biological tests, α-tocopherol has greater biological activity than does γ- or δ-tocopherol. This is rather remarkable in view of the slight differences in their structures. For example, γ-tocopherol (which differs from α-tocopherol only in having no methyl group in the 5-position) has approximately one fifth the activity of α-tocopherol in a variety of biological systems such as resorption sterility (2) and respiratory decline in liver homogenates of vitamin E deficient animals (3). In vitamin E deficiency-induced muscular dystrophy in rabbits, γ-tocopherol was reported to be 30% as active as α-tocopherol (4).

Recently a series of 5-substituted analogs of α-tocopherol and of a model of α-tocopherol, 6-hydroxy-2,2,5,7,8-pentamethyl chroman, were synthesized and evaluated in a number of biological test systems (5,6). We now wish to report the evaluation of these 5-methyl-substituted analogs as antioxidants in protecting β-carotene from air oxidation in tocopherol-stripped hydrogenated corn oil.

EXPERIMENTAL PROCEDURES

The procedure used was essentially that of Stern, et al. (7). β-Carotene was added to tocopherol stripped corn oil (1) to give a 900

TABLE I

α-Tocopherol Model Compounds

[Structures: general chroman structure with R and R'; Orthoquinone; Tetracyanoethylene Adduct]

R	R'	A[a] Half-life (hr)	B[b] Per cent Protection
OH	-H	394	24
OH	$-CH_3$	375	82
OH	$-CH_2OH$	244	–
OH	$-CH_2OCH_2-C_6H_5$	179	61
OAc	CH_2Cl	–	82
OH	$-CH_2OCH_2CH_3$	171	67
OH	$-CH_2OCH_3$	169	15
OA	CH_2I	–	24
OH	$-CH_2OCO-C_6H_5$	66	0
OH	-CHO	62	21
-	Orthoquinone	41	0
-	Tetracyanoethylene Adduct	–	0
OH	$-CH_2N$(morpholino)	39	19
OH	$-CH_2N$(piperidino)	38	3
OH	$-CH_2N(CH_3)_2$	35	2
OH	$-CH_2SC(NH)NH_2 \cdot HCl$	27	2
–	-blank oil	12	

[a]Antioxidant activity of compounds in protecting β-carotene in hydrogenated tocopherol stripped corn oil at 50 C.

[b]Preventive effects of compounds on dietary necrotic liver rats evaluated at 10 mg/100 g of diet.

TABLE II

Tocopherol Compounds

R	R'	A[a] Half-life (hr)	B[b] Average day of death	C[c] ED_{50} (mg/100 g) of diet
OH	CH_3	570	—	0.53
Ac	CH_3	—	26	—
OH	CH_2OCH_2-C_6H_5	263	15	No protection
OH	$CH_2OCH_2CH_3$	225	12	12.8
OH	CH_2OCH_3	221	24	7.0
OH	$CH_2SCH_2CH_3$	180	—	—
Ac	$CH_2SCH_2CH_3$	—	14	9.6
OH	CH_2N (morpholino)	58	10	24.0
OH	CH_2N (piperidino)	37	9	No protection
Ac	$CH_2SCNH(NH_2)$	—	—	No protection
OH	$CH_2N(CH_3)_2$	24	16	No protection
Ac	CH_2Cl	—	18	12.3
Tocopherolhydroquinone diacetate		—	12	—
Tocopherolquinone		—	—	No protection
Tocopherolorthoquinone		—	—	No protection
Blank oil		12	—	—
Negative control		—	7	—

[a]Antioxidant activity of compounds in protecting β-carotene in hydrogenated tocopherol free corn oil at 50 C.

[b]Preventive effects of compounds on vitamin E deficiency-induced muscular dystrophy in the rabbit (doses were 50 mg/g i.v. except α-tocopherol acetate was given at 25 mg/g i.v.).

[c]Preventive effects of compounds on dietary necrotic liver degeneration in rats.

μg/ml solution. The tocopherol derivatives and model compounds were dissolved in ethanol at a concentration of 0.1 mg/ml and aliquots were added to 10 ml of the oil to give a final concentration of 0.233 μmole/ml. The alcohol was removed in vacuo and the oil was warmed slowly until it melted. The mixture was stirred in vacuo until completely mixed. Five milliliters of this solution were placed in a tube and air bubbled through it at 50 C ± 0.5 C. The disappearance of β-carotene was followed spectrophotometrically by reduction of absorption at 462 mμ. Readings were made twice daily on controls and daily on samples. The curves were plotted so that the half-life could be determined.

RESULTS AND DISCUSSION

Table I summarizes the results of the antioxidant studies with the model compounds. Half-life values are averages of at least two separate determinations. As in the case of γ-tocopherol, the γ-model is a better antioxidant than is the α-model. However, the α-model is much better as an antioxidant than are any of those analogs where the 5-methyl group is substituted.

Table II summarizes the results of the antioxidant studies using α-tocopherol derivatives. Again α-tocopherol is a much better antioxidant than are any of its derivatives substituted in the 5-methyl position. When one compares the results of Table I (model compounds) and Table II (tocopherol derivatives) the importance of the phytyl side chain of the tocopherols as far as antioxidant activity becomes apparent, e.g., the half-life of α-tocopherol (570 hr) vs. that of the model compound (356 hr). Similar comparisons of the benzyloxy, methoxy and ethoxy derivatives also emphasize the importance of the phytyl side chain. Since at the concentrations used in the oil, all of these derivatives are completely soluble, molecular configuration and orientation in the oil must be involved in the greater protective power of the tocopherol derivatives.

There is a general agreement between the ranking of the various 5-substituted analogs in the two series indicating that structural changes

in the 5-position influence the antioxidant activity in à regular manner. It appears that the introduction of a basic function on the 5-methyl group (morpholine, piperidine, dimethylamino) is detrimental to its antioxidant activity.

Tables I and II summarize results previously found on the preventative effects of tocopherol models and derivatives on dietary necrotic liver degeneration in rats (5,6). A comparison of these data show a certain similarity in rankings of biological activities. Here, as in the antioxidant activities, the importance of the phytyl side chain is apparent. It should be recognized, however, that we are comparing antioxidant activities toward protecting β-carotene in hydrogenated tocopherol stripped corn oil and biological activity in protecting against dietary necrotic liver degeneration.

In all cases, the greatest antioxidant activity and biological activity of these derivatives is with the 5-methyl group intact. However, the γ-model had a better antioxidant but poorer biological effect. Both ethoxy derivatives show good correlation between antioxidant activity and biological activities but the methoxy model compound does not. The benzyloxy model shows a good correlation but the benzyloxy tocopherol analog does not. The basic substituted derivatives of α-tocopherol and of the model compound have both poor antioxidant effects and poor biological activity.

Table II summarizes the biological effects of the tocopherol derivatives in vitamin E deficiency-induced muscular dystrophy in the rabbit. None of the model compounds were active in this biological system (5). Again, α-tocopherol had the greatest biological activity. In addition, the methoxy derivative had both good biological activity and antioxidant activity. However, the dimethylamino derivative showed good biological activity but was a poor antioxidant. The benzyloxy derivative was a good antioxidant but had only fair biological activity.

Bieri and Prival (8) have shown that N-methyl-β-tocopherolamine and N-methyl-γ-tocopherolamine have high tocopherol like biological activity. They also have shown that N-methylation of β- and γ-tocopherolamines greatly improves the compounds deposition and retention in the liver and their biological activities. Gloor, et al. (9) have provided evidence with γ-tocopherol, a compound with low biological activity, that its absorption by tissues is not greatly different from that of α-tocopherol.

While some correlation can be seen between in vitro antioxidant effects of these vitamin E derivatives and certain of their biological activities, in vitro antioxidant activity is only one of several factors to be considered in relating structure to biological activity. It would appear that there are some discrepancies in the correlation of the antioxidant activities of the tocopherol derivatives with their biological activities that require explanation beyond simply uptake and turn-over of the compounds by the tissues and their in vitro antioxidant effects.

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Characterization of the Nonvolatile Compounds Formed During the Thermal Oxidation of 1-Linoleyl-2,3 Distearin: I. The Nonacidic Fraction[1]

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ABSTRACT

A compound isolated from the nonacidic fraction of thermally oxidized 1-linoleyl-2,3-distearin was shown to be 18-pentatriacontanone. A combination of chemical and instrumental analyses was used to determine the structure.

INTRODUCTION

A review of the literature has shown that relatively few nonvolatile nonacidic compounds formed in fats heated in the presence of air have been identified. Sen Gupta reported 9-octadecene as a major decomposition product of methyl oleate heated in an inert atmosphere (1). Reddy et al. (2) characterized hydrocarbons of 18 carbons or less, alcohols of 10 carbons or less, aldehydes of 15 carbons or less, and ketones of 12 carbons or less, which were formed in heated hydrogenated cottonseed oil. Anfinsen identified 1-decyl-2-(dec-6′-enyl)-cyclohexane as a decomposition product of thermally oxidized triolein (3). More recently Nawar (4) has reviewed the current literature dealing with thermally induced reactions occurring under nonoxidative conditions. Mechanisms for the formation of hydrocarbon and ketonic products are discussed. In this report the isolation and characterization of the major nonacidic component (18-pentatriacontanone) formed as a result of the thermal oxidation of 1-linoleyl-2,3-distearin are presented.

EXPERIMENTAL PROCEDURES

Tetrabromostearic acid was prepared according to the method originally described by Rollett (5) and modified by Frankel and Brown (6). 1-Mono-(9,10,12,13-tetrabromo)-stearin was synthesized by the method of Hartman (7) as modified by Anfinsen and Perkins (8). The isopropylidene glycerol was obtained commercially (Aldrich Chemical Company). Stearic acid was purified by distilling the methyl esters of Neo Fat 18-S (courtesy of Armour and Company). The methyl stearate (99.9%) was saponified and the resulting stearic acid was converted to the corresponding acid chloride with oxalyl chloride according to the method of Bauer (9). 1-(9,10,12,13-Tetrabromo)-stearyl-2,3-distearin was synthesized according to the methods of Daubert and Baldwin (10) and Hartman (11). Debromination (12) of the above gave 1-linoleyl-2,3-distearin, mp 32-33 C (lit. mp 32-33 C) (10).

Analysis calculated for $C_{57}H_{106}O_6$: C, 77.14; H, 12.04; Found: C, 77.02; H, 12.06.

The purity of the synthesized triglyceride was further established by the lipase hydrolysis procedure of Ast and Vander Wal (13) using Lipase 448 (Nutritional Biochemical Corporation). Gas liquid chromatographic (GLC) analysis of the methyl esters of the resulting free fatty acids indicated the presence of 50.4% by weight of linoleate and 49.6% stearate in the 1 and 3 positions of the triglyceride. GLC analysis of the methyl esters of the fatty acids resulting from the acidic hydrolysis of the triglyceride indicated the presence of 33.7% linoleate and 66.3% stearate. The percentage of isolated *trans* double bonds in 1-linoleyl-2,3-distearin as quantitatively determined by infrared spectrometry (14,15) using rapeseed oil and trielaidin as standards was 5.1%.

1-Linoleyl-2,3-distearin (147 g) was heated for 24 hr at 200 C in a 250 ml round bottom flask fitted with a thermometer, a fritted glass gas dispersion tube, and a heating mantle. The flow of compressed air, bubbled through the oil, was maintained at a rate of 0.10 ml/min/g of oil. Before entering the oil, the air was passed through a trap containing 5A molecular sieve and anhydrous calcium chloride. During the oxidation, the volatile degradation products were allowed to evaporate from the flask.

GLC was carried out on a Barber-Colman Model 10 instrument equipped with a flame ionization detector. Components were separated on a 6 ft X 4 mm glass column packed with 15% ethylene glycol succinate coated on

[1]This work represents a portion of a thesis presented by L. R. Wantland as partial fulfillment of the requirements for the Ph.D. degree at the University of Illinois.

[2]Present address: Mead Johnson & Co., Evansville, Indiana.

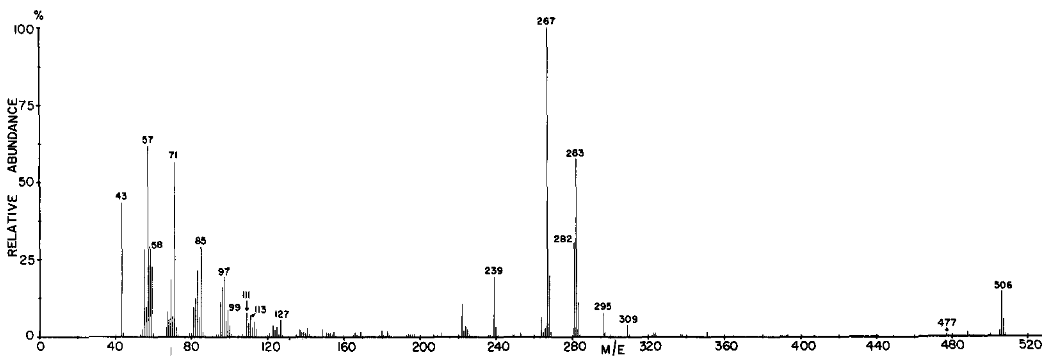


FIG. 1. Mass spectrum of Compound A (22 ev).

60-80 mesh Chromosorb W (AW) (Applied Science Laboratories, State College, Pa.). The column was operated at 185 C; detector 210 C and injector at 225 C. A column flow of 60 cc/min of Argon was used.

Carbon hydrogen analyses were carried out by the Clark Microanalytical Laboratory, Urbana, Illinois. Infrared spectra were determined on a Beckman Model IR-7 spectrophotometer. Thin layer chromatography (TLC) was carried out according to the method of Stahl (16). Mass spectra were obtained on a Hitachi Perkin-Elmer RMU-6E single focusing instrument as previously described (17). A gas chromatographic inlet system was employed for the analysis of mixtures (17).

Trimethylsilyl ethers were prepared by mixing 1 mg of sample with 0.5 ml of bis(trimethylsilyl)acetamide (BSA) and heating the mixture under reflux for 10 min at 110 C. Excess BSA was removed under reduced pressure (0.05mm) at room temperature.

18-Pentatriacontanone (courtesy of L. Metcalf, Armour & Co., Chicago, Ill.) was reduced to the corresponding secondary alcohol by $LiAlH_4$. To a suspension of 2 mg of $LiAlH_4$ in 20 ml of dry diethyl ether in a 100 ml flask, was added dropwise, a solution containing 5 mg of the ketone in 30 ml of dry diethyl ether. The contents of the flask was swirled throughout the addition of the ketone. After allowing the reaction to stand for 20 min, 30 ml of H_2O and 3 ml of dilute sulfuric acid were added. The ether layer was drawn off and the aqueous layer re-extracted with a 20 ml portion of diethyl ether. The combined ether layers were washed free of mineral acid and dried over anhydrous $MgSO_4$. Evaporation of the solvent under reduced pressure yielded the alcohol which was used without further purification.

A modification of the method of Bryant and Smith (18) was employed to prepare the oxime derivative of 18-pentatriacontanone. A 10 mg sample of the ketone and 10 mg of hydroxylamine hydrochloride were added to a solution of 0.1 ml of pyridine and 5 ml of absolute ethanol contained in a 10 ml round bottom flask. The mixture was refluxed on a steam bath for 3 hr. The solvent was then removed under a stream of nitrogen and the residue washed well with water. The oxime derivative was recrystallized from absolute ethanol.

Isolation of Compound A

The oxidized triglyceride was saponified with 2 N KOH in ethanol to release the substituent fatty acids. Extraction of the potassium soaps with redistilled Skellysolve F and evaporation of the petroleum ether resulted in the isolation of 991 mg of a light brown nonacidic residue. It was noted that when attempts to dissolve the residue in solvents varying in polarity from *n*-hexane to methanol at room temperature, a white precipitate persisted. The nonacidic mixture was taken up in diethyl ether, brought to a boil, and the resulting slurry containing the white material was filtered while hot. Crystallization of the white material from a benzene-ethanol (2:1) solution yielded 83 mg of a substance designated as Compound A which represented 8.4% of the nonacidic fraction or 0.067% of the isolated saponification mixture.

RESULTS AND DISCUSSION

Compound A, isolated from the nonacidic fraction of oxidized 1-linoleyl-2,3-distearin was a white crystalline solid, mp 88-89 C. Infrared spectrometry (KBr pellet) showed bands at 1708 cm^{-1} (ketone) and 721, 732 cm^{-1} (tetramethylene). Elemental analysis of A found: C, 83.16; H, 13.78. A mass spectral analysis of A

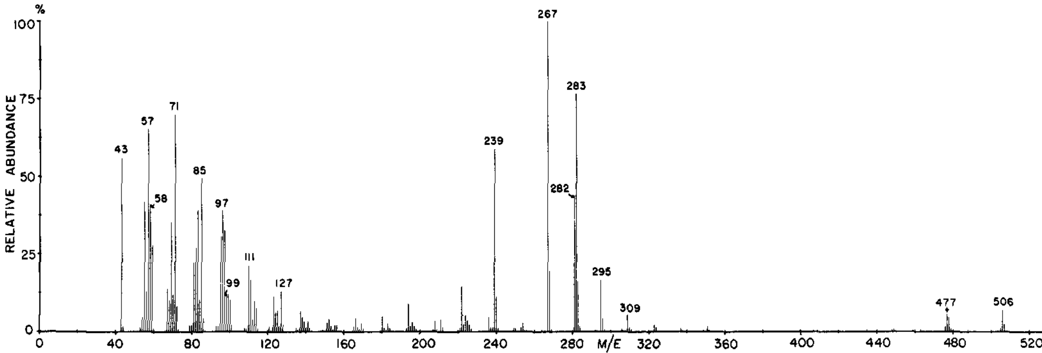


FIG. 2. Mass spectrum of 18-pentatriacontanone (22 ev).

(Fig. 1) indicated a molecular weight of 506. The molecular weight and the elemental analysis are consistent with an empirical formula of $C_{35}H_{70}O$. Additional support for a large number of carbon atoms was derived from the intensity of the mass 507 peak in the mass spectrum. It had an intensity, relative to the mass 506 peak of 38.1%, which corresponds to the presence of 35 carbon atoms (19).

The base peak in the mass spectrum of compound A at m/e 267 was assigned to a $C_{17}H_{35}$-C≡O$^+$ ion fragment and the intense peak at 282 was assigned to a $C_{17}H_{35}$-C($\overset{+}{O}$H)=CH_2 fragment. Compound A was thus tentatively identified as 18-pentatriacontanone (lit. mp 88.4 C) (20). Authentic 18-pentatriacontanone was compared with Compound A as follows; the admixed compounds melted at 88-89 C and could not be resolved by TLC and GLC. The oxime of 18-pentatriacontanone has two reported melting points, 62-63 C and 67 C (21). The oxime of the unknown melted at 67 C and that of the authentic compound at 62-63 C. Recrystallization of both oximes failed to alter their melting points. Finally, the mass spectrum of the standard (Fig. 2) was superimposable upon that of Compound A.

Saturated hydrocarbon ions of the formula C_nH_{2n+1} (n=3-17) and olefinic ions of the formula C_nH_{2n-1} (n=4-17) are observable in both spectra. The intense peak at m/e 283 may be a rearrangement of the m/e 282 fragment. Peaks occur in the mass spectrum (70 ev) of 11-henecosanone at mass 184 (β-cleavage) and at m/e 185 (22). Fragments at mass 58 in Figures 1 and 2 are attributed to $H_2\overset{+}{O}$-C(CH_2)=CH_2 and CH_3C($\overset{+}{O}$H)CH_2• ions (23).

To further compare Compound A with 18-pentatriacontanone, both compounds were reduced with $LiAlH_4$ and then silylated with BSA. The mass spectrum of the TMS derivative of reduced compound A appears in Figure 3 and is superimposible upon the spectrum of the

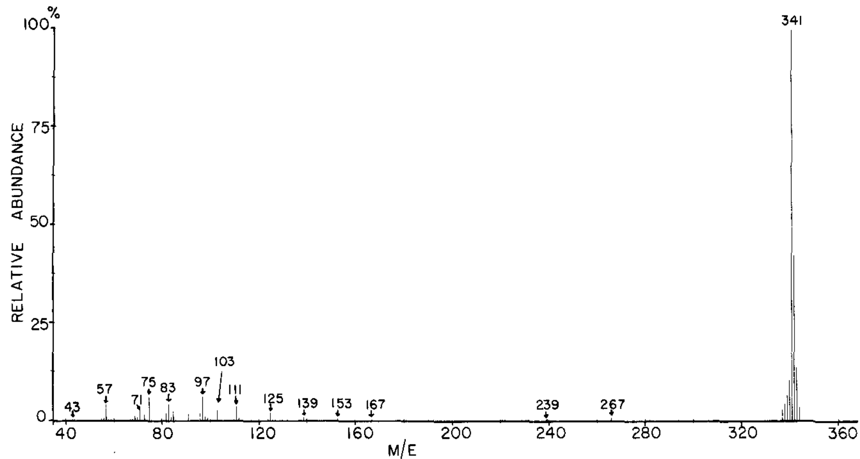
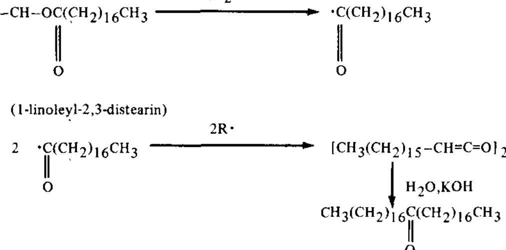


FIG. 3. Mass spectrum of trimethylsilyl ether of reduced Compound A.

TMS derivative of 18-pentatriacontanol. Peaks at m/e 75 and 103 were assigned to $(CH_3)_2Si{=}\overset{+}{O}H$ and $(CH_3)_3Si{=}\overset{+}{O}CH_2$ fragments, respectively, and the intense peak at m/e 341 to a $CH_3(CH_2)_{16}CH{=}\overset{+}{O}Si(CH_3)_3$ fragment. On the basis of the reported evidence, the structure of Compound A was established as 18-pentatriacontanone.

18-Pentatriacontanone has been previously prepared chemically by heating stearic acid with mineral salts (24-29). Since free fatty acids are formed by the thermal oxidation of triglycerides (30), stearic acid exists in the reaction medium of thermally oxidized 1-linoleyl-2,3-distearin. Thermal reaction of this stearic acid with trace amounts of metallic salts may lead to the formation of 18-pentatriacontanone in this manner.

The formation of 18-pentatriacontanone via the oxidation of a triglyceride can be rationalized by the following free radical mechanism.



The $\cdot CO\text{-}(CH_2)_{16}CH_3$ radical presented above, would be stabilized by the location of the unpaired electron on either the carbon or the oxygen atom of the carbonyl group. Abstraction of a proton by a R• species could then lead to a ketene dimer. Reaction of the dimer with water during saponification would give a β-keto-acid, and subsequent further saponification, the ketone.

ACKNOWLEDGMENT

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Characterization of the Nonvolatile Compounds Formed During the Thermal Oxidation of 1-Linoleyl-2,3-Distearin: II. The Acidic Fraction[1]

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ABSTRACT

Four compounds were isolated from the acidic fraction of 1-linoleyl-2,3-distearin which was heated at 200 C for 24 hr with aeration. Following saponification of the oxidation mixture, the fatty acids were partitioned between ethanol and pentane-hexane (Skellysolve F). The ethanol soluble acids were subsequently esterified with methanol and subjected to further separation by preparative thin layer and gas liquid chromatography. A combination of chemical and instrumental analyses as well as a comparison with standard compounds was used to determine the structures. Substances characterized were isomeric C_{18} aromatic esters, isomeric methyl keto-octadecanoates, isomeric methyl keto-octadecenoates and dimethyl undecane-1,11-dioate.

INTRODUCTION

Many workers have characterized the volatile degradation products of thermally oxidized fats, however, few have studied the thermal oxidation products of synthetic triglycerides (1-3). In this report, the characterization of several compounds which were isolated from the nonvolatile acidic fraction of a synthetic triglyceride, 1-linoleyl-2,3-distearin, is presented.

EXPERIMENTAL PROCEDURES

Synthetic 1-linoleyl-2,3-distearin was thermally oxidized at 200 C for 24 hr in the presence of air (0.10 ml/min/g) (4). The resulting material was saponified and the nonacidic compounds removed (4).

Fractionation of the fatty acids of the saponifiable material was carried out as summarized in Figure 1. A 20 g portion of the oxidized fatty acids was partitioned between equilibrated 80% ethanol and redistilled Skellysolve F (5).

Esterification of the resulting polar and nonpolar fatty acid fraction with 2% sulfuric acid in methanol yielded the corresponding methyl esters.

The oxidized methyl ester fraction was subjected to further separation by preparative thin layer chromatography (TLC) according to the method of Stahl (6). The plates were developed in a solvent system of Skellysolve F-diethyl ether (90:10). After developing and upon viewing the plates under ultraviolet light, four bands were observed which were scraped from each glass plate and pooled into four fractions (Fractions 1-4, Fig. 1), prior to extraction of the absorbed material with moist ether. An analytical TLC plate of this fractionation is presented in Figure 2.

Preparative GLC was carried out on a Barber Colman Model 5000 gas chromatograph equipped with a thermal conductivity detector. Two different glass columns were employed: a 6 ft X 14.5 mm column packed with 3% OV-17 coated on 80-100 mesh Chromosorb W (AW-DCMS) (Supelco, Inc.), and a 2.5 ft X 14.5 mm column packed with 3% OV-101 coated on 80-100 mesh Chromosorb W (AW-DCMS) (Supelco, Inc.). Helium flow rate was 60 cc/min; samples varying in weight from 2-100 mg were injected directly onto the columns and fractions were collected in glass tubes, using the apparatus described by Wood and Reiser (7). The collection apparatus operated at approximately 90% efficiency.

Analytical gas liquid chromatograms were obtained on a Barber Colman Model 10 gas chromatograph equipped with a flame ionization detector. Glass columns, 4 mm in diameter, contained one of three packings: (a) 15% HI-EFF-2BP (an ethylene glycol succinate polyester) coated on 60-80 mesh Chromosorb W (AW) (Applied Science Laboratories), (b) 3% OV-1 coated on 80-100 mesh Chromosorb W (AW-DCMS) (Supelco, Inc.) and (c) 3% OV-17 coated on 80-100 mesh Chromosorb W (AW-DCMS) (Supelco, Inc.). Argon was used as the carrier gas. Ester carbon numbers of the unknowns were determined by the method of Miwa (8).

[1]This work represents a portion of a thesis presented by L. R. Wantland as partial fulfillment of the requirements for the Ph.D. degree at the University of Illinois.

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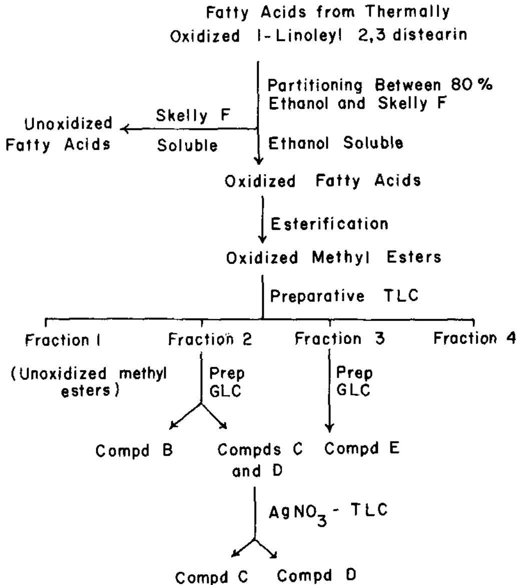
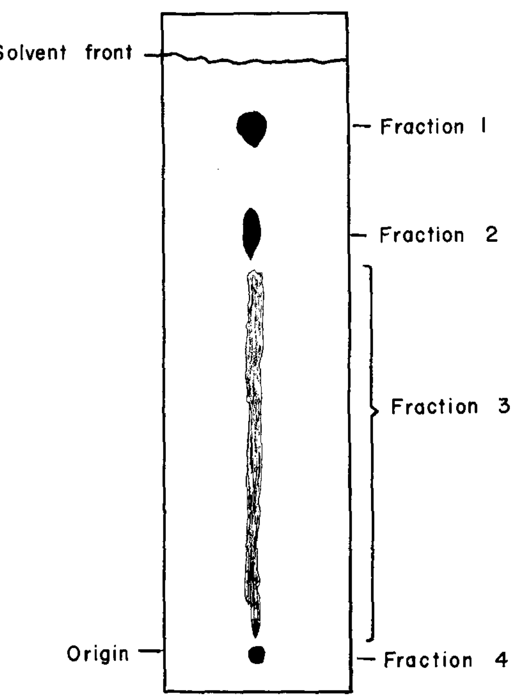


FIG. 1. Fractionation of oxidized fatty acids.



Solvent System -- Skelly F-Diethyl ether (90/10)

FIG. 2. Thin layer chromatogram of methyl esters from thermally oxidized 1-linoleyl 2,3-distearin.

Carbon, hydrogen (CH) analyses, determination of infrared and mass spectra, analytical TLC and preparation of trimethylsilyl ether derivatives were carried out as previously described (4). Ultraviolet (UV) spectra were obtained (ethanol solvent) on a Cary Model 11 M spectrophotometer. Nuclear magnetic resonance (NMR) spectrometry was performed on a Varian Associates Model HA-100 spectrometer (courtesy of Sherwin-Williams Research Center, Chicago, Ill.). Quantitative hydrogenation of unsaturated fatty esters (10^{-5} moles) was accomplished using a Brown Micro Hydro-Analyzer (Delmar Scientific Laboratories) following the method of Brown (9).

Substituted aromatic compounds (2 mg) were oxidized to the corresponding acids using the procedure of Vogel (10), except that the excess permanganate was removed by bubbling ethylene gas through the oxidation mixture. The method of Argoudelis and Perkins was employed to determine the position of double bonds in unsaturated fatty esters (11).

Determination of C-methyl groups was performed using the Kuhn-Roth oxidation by the Clark Microanalytical Laboratory, Urbana, Illinois. Neutralization equivalents were determined according to the method of Schneider (12).

12-Keto-octadec-9-enoic acid was synthesized by the procedure of Nichols and Schipper (13).

RESULTS AND DISCUSSION

Examination of the nonpolar fatty acids (Fig. 1) by TLC and GLC indicated that the acids consisted of approximately 95% unaltered fatty acids. These acids were 97% stearic acid and 3% linoleic acid. Fraction 1 (Fig. 1,2), upon analysis by both GLC and TLC, proved to be unaltered methyl esters which were not completely removed by the solvent partitioning process. These esters had the composition found for the nonpolar fatty acid fraction.

Aromatic Esters

Preparatory GLC of Fraction 2 from the oxidized triglyceride methyl ester (Fig. 1) yielded Compound B, which on OV-1, OV-17 and EGS columns had carbon numbers of 18.7, 19.5 and 23.7, respectively. Infrared spectrometric analysis showed bands at 1180, 1745 cm^{-1} (ester); 1495, 1590 cm^{-1} (aromatic); and 760 cm^{-1} (ortho-disubstituted benzene) (14). Ultraviolet spectrometric (UV) analysis showed the following λ_{max} (log ϵ): 263 mμ (2.36), 272

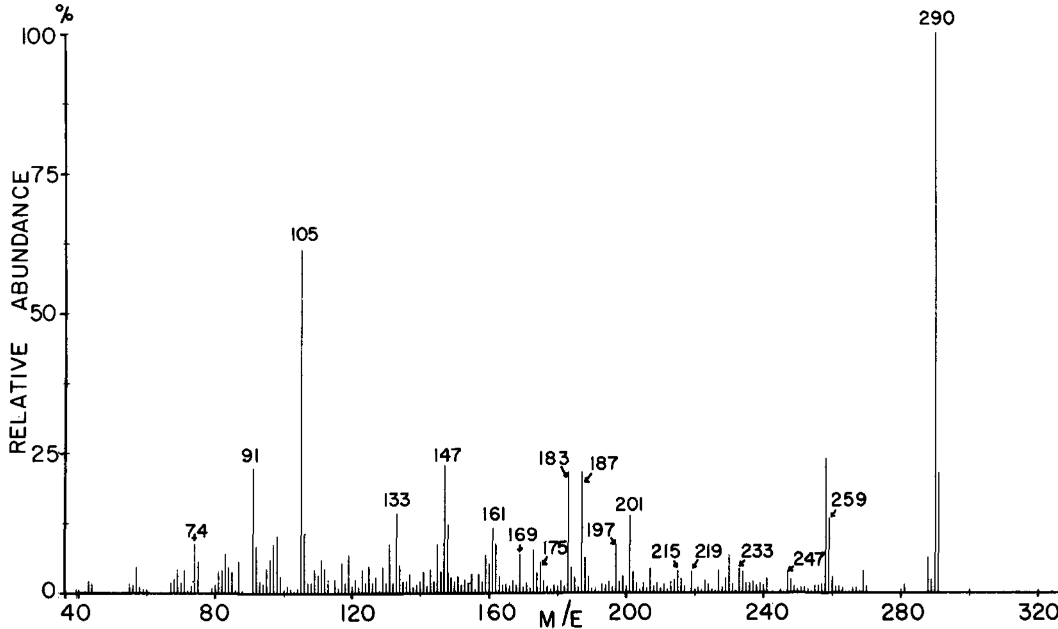


FIG. 3. Mass spectrum of Compound B.

mμ (2.36), and 278 mμ (1.02). These bands are characteristic of an ortho-disubstituted benzene (15,16). Oxidation of the unknown with $KMnO_4$ yielded a product whose UV spectrum exhibited λ_{max} at 274 and 281 mμ. This spectrum was superimposable upon that from a phthalic acid standard. Esterification of the oxidation products with diazomethane gave a compound which could not be resolved by GLC from dimethyl phthalate.

Mass spectral analysis (20 eV) of Compound B (Fig. 3) indicated that a base peak at m/e 290 was the molecular ion. The intensity of the (M+1) peak relative to the molecular ion was 21.0 which related to the presence of 19 carbon atoms. A series of prominent peaks was

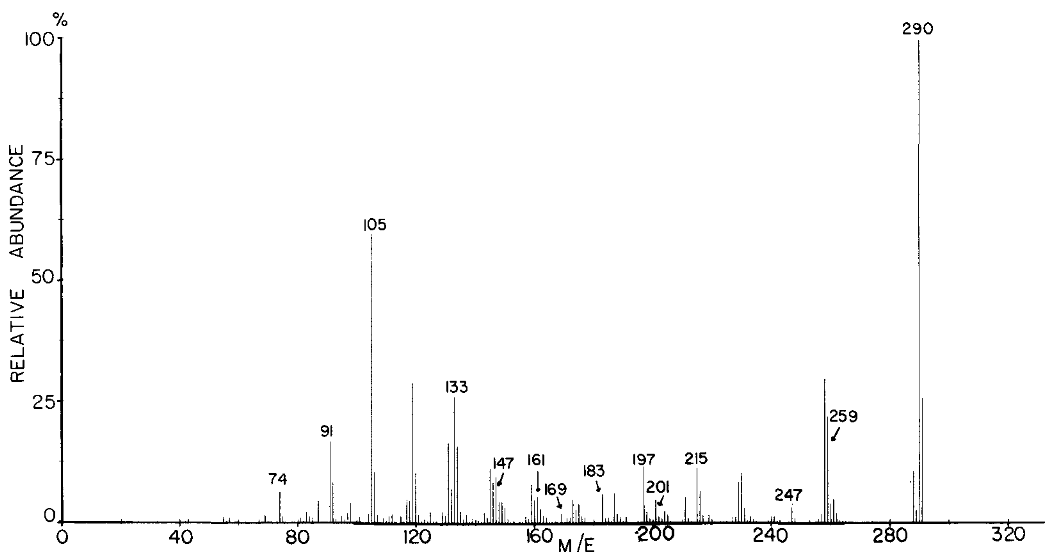


FIG. 4. Mass spectrum of saturated, cyclic monomer from linoleic acid.

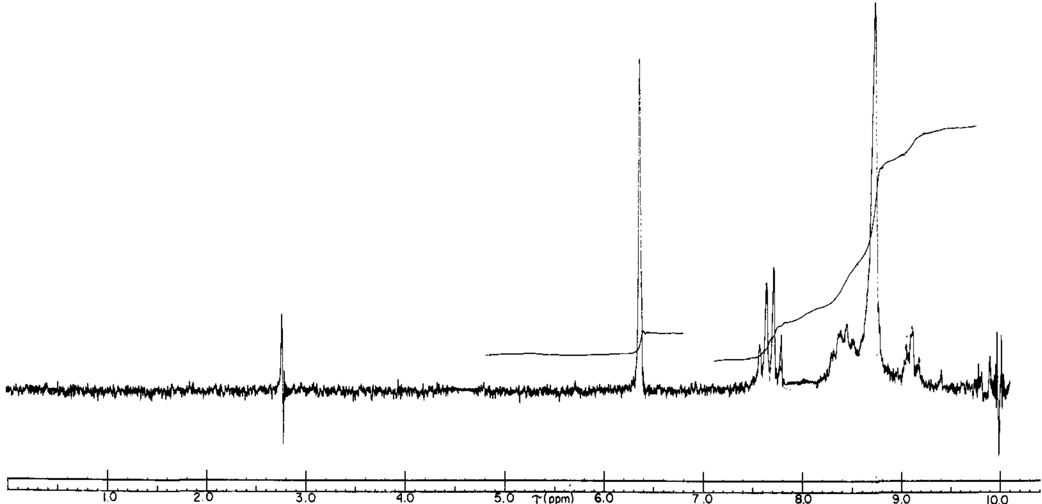


FIG. 5. Nuclear Magnetic Resonance spectrum of Compound C.

observed which corresponded to the formula, $C_6H_5C_nH_{2n}$ (17). Peaks at m/e = 91, 105, 133 and 147 (n = 1,2,4 and 5) were especially intense. The spectrum closely resembled that observed by Michael (18) for an aromatic ester isolated from anaerobically heated linoleate of structure I:

$(CH_2)_xCH_3$

$(CH_2)_yCO_2CH_3$ (x+y=10)

I

A standard containing a mixture of aromatic esters corresponding to the above formula (courtesy of J. P. Friedrick, Northern Regional Research Laboratory, Peoria, Ill.) was compared to Compound B. The mass spectrum of the standard (Fig. 4) closely resembled that of Compound B. From this comparison, Compound B was found to contain aromatic esters corresponding to the above structure whenever x = 2 - 6 and y = 4 - 8. In addition, the IR and UV spectra of the two compounds were superimposable. Also, the admixed compounds could not be resolved by either TLC or GLC. Thus, it was established that Compound B was represented by the structure I.

Keto-Octadecanoates

Preparative GLC of Fraction 2 (Fig. 1) on OV-17 yielded a fraction whose ester carbon number was 21.0. However, analytical GLC analysis on an EGS column revealed the presence of two components with carbon numbers of 24.8 and 25.2. Preparative TLC on glass plates coated with $AgNO_3$ impregnated silica gel (19), using an elution solvent of Skellysolve F-diethyl ether (90:10), resulted in the isolation of two components, Compounds C (saturated) and D (unsaturated). Analysis of Compound C on an EGS-GLC column indicated that one component was present (carbon number 24.8). This represented 0.011% of the isolated methyl esters.

Infrared spectrometry of the unknown compound showed bands at 1743, 1175 cm^{-1} (ester); 1718 cm^{-1} (ketone); and 727 cm^{-1} (tetramethylene). The presence of a ketonic functional group was confirmed by an NMR spectrum, reproduced in Figure 5. Overlapping triplets, centered at 7.62 and 7.72τ were attributed to 6 protons on carbon atoms α to carbonyl and carboxyl groups, respectively. A set of poorly resolved peaks centered at 8.45τ was attributed to 6 protons on carbon atoms β to carbonyl and carboxyl functions. In addition, chemical shifts were observed at 6.34τ (methoxy 3H), 8.72τ (methylene 18H) and 9.10τ (terminal methyl 3H).

The compound was then analyzed by combined GLC-MS (20 eV) (Fig. 6). The mass spectrum exhibited a parent ion of mass 312. This molecular weight was supported by a neutralization equivalent of 304 (determined on the fatty acid of the unknown). A Kuhn-Roth oxidation of the unknown gave 4.11% of terminal methyl groups, corresponding to one methyl group per molecule. The areas of the NMR peaks were then normalized on the basis of three terminal methyl protons. The above information and the results of an elemental analysis are consistent with an empirical

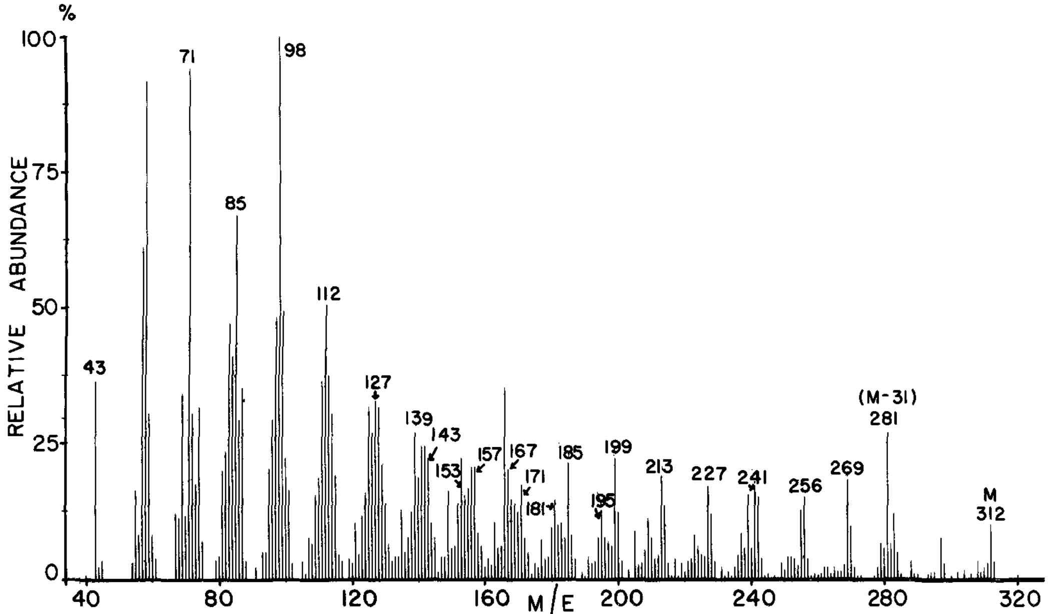


FIG. 6. Mass spectrum of Compound C.

formula of $C_{19}H_{36}O_3$. Compound C was thus tentatively assigned the following structure:

$$CH_3(CH_2)_x\overset{O}{\overset{\|}{C}}(CH_2)_yCO_2CH_3 \quad (x + y = 15)$$

A mixture of the unknown and methyl 10 and 12-keto-octadecanoate could not be resolved by TLC or GLC. Elemental analysis of Compound C found: C, 72.95; H, 12.12; calculated for $C_{19}H_{36}O_3$: C, 73.08; H, 11.54.

To locate the position of the carbonyl function, the mass spectrum of Compound C was compared with the spectra of methyl 10-keto- and 12-keto-octadecanoate, all of which had been obtained under identical conditions. In addition, the mass spectral fragmentation pattern of the compound was compared with those patterns established by Stenhagen for various isomeric methyl keto-octadecanoates (20).

The base peak in the mass spectrum of Compound C occurred at m/e = 98. A mass 98 peak was also prominent in the 20 eV spectra of the standard keto-esters: 10-keto-C_{18}, 54%; 12 keto-C_{18}, 84% relative abundance. These m/e 98 peaks and other peaks at m/e 84 and 112 could possibly be represented by the cycloalkanone ions postulated in the spectra of long chain saturated dibasic esters (21).

A series of intense peaks in the spectrum of Compound C was observed at m/e 129 + 14 n, (n = 0 to 11). The most prominent peaks in the series were at masses 185, 199, 213, 227, 241, 255 and 269. Unfortunately, this series can represent two ion fragment types (20):

$$[O{=}C(CH_2)_yCO_2CH_3]^+ \text{ and } [(CH_2)_{y\text{-}1}CO_2CH_3]^+$$

Both types were equally abundant in the 20 eV spectra of methyl 10- and 12-keto-octadecanoate. Thus, the prominent peaks in this series indicate that the ketonic group lies in the 12 through 17 positions or in the 9 through 15 positions of the C_{18} methyl ester or both.

Intense peaks observed at masses 111, 125, 139, 153, 167, 181 and 195 could represent $[O{=}C{=}CH(CH_2)_{y\text{-}2}]^+$ fragments, which would indicate the presence of methyl 8, 9, 10, 11, 12, 13, 14 and 15-keto-octadecanoates, respectively. In addition, peaks at 242 and 256 $[(CH_2(C{=}O)(CH_2)_yCO_2CH_3{+}H]^+$ can be assigned to methyl 12- and 13-keto-octadecanoates, respectively, and peaks at 155, 141, 127 and 113 $[CH_3(CH_2)_xC{=}O]^+$can be assigned to methyl 9, 10, 11 and 12-keto-octadecanoates, respectively. Finally, peaks at 156, 142, 128 and 114 $[CH_3(CH_2)_x(C{=}O)CH_2{+}H]^+$ can be attributed to methyl 10, 11, 12 and 13-keto-octadecanoates. Elimination of a CH_3O fragment from the molecular ion was observed at mass 281.

According to Ryhage and Stenhagen, mass spectral fragmentation of 2-keto- and 3-keto-esters represent special cases (22). The strong

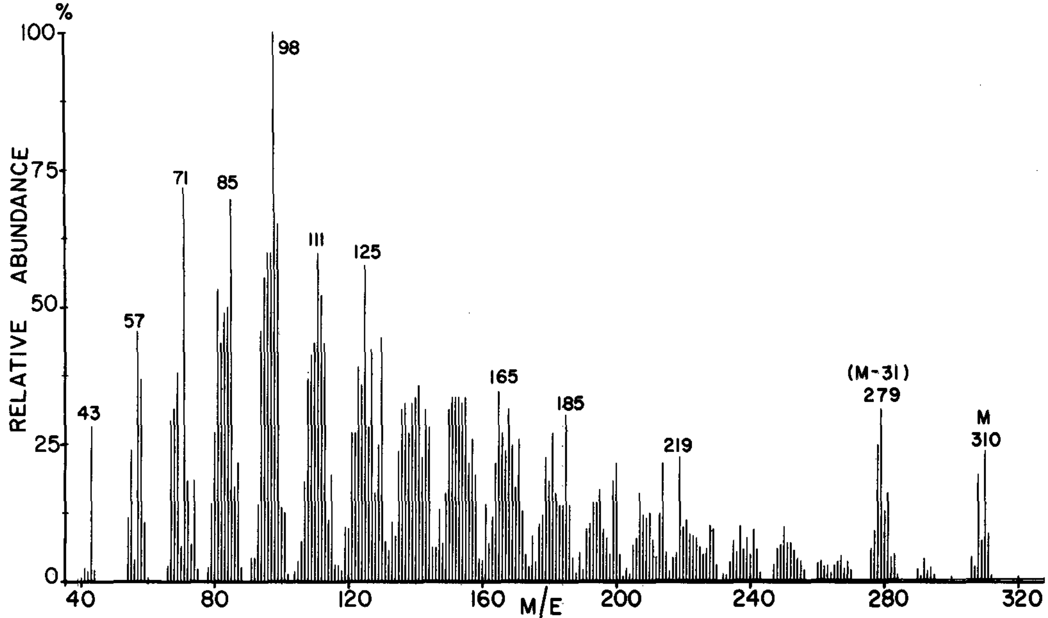


FIG. 7. Mass spectrum of Compound D.

tendency to cleave between adjacent keto-groups leads, in the case of a 2-keto-ester, to the formation of acylium ions of m/e = (M - 59), almost to the exclusion of other ions containing oxygen. Cleavage of a 3-keto-ester results in the appearance in the mass spectrum of a peak at m/e = (M - 58), which is interpreted as due to the ion formed by loss of a molecule of acetone from the molecular ion. The absence of intense peaks at (M - 59) and (M - 58) in the mass spectrum of Compound C leads to the conclusion that 2- and 3-keto-esters are not present in amounts large enough to be detected by mass spectrometry.

On the basis of the reported information, Compound C was identified as a mixture of isomeric methyl keto-octadecanoates, with the ketone group located predominantly in the 9 through 15 positions. The formation of ketones in heated fats has been postulated as a reaction involving the rearrangement of a hydroperoxide (23). The precursor of Compound C was likely the stearyl acyl group. A saturated C_{18} keto-ester was recently isolated from heated soybean oil (24), but the ketonic positions were not determined.

The compound which was resolved from Compound C by preparative TLC was designated as Compound D (representing 0.002% of the isolated methyl esters).

The infrared spectrometric analysis (KBr pellet) showed bands at 1743, 1175 cm^{-1} (ester); 1718 cm^{-1} (ketone); 727 cm^{-1} (tetramethylene); and 970 cm^{-1} (*trans*-double bond). The mass spectrum (20 eV) of Compound D (Fig. 7) indicated a molecular weight of 310, formally consistent with a methyl keto-octadecanoate. Loss of CH_3O^+ and $(CH_3O + H)^+$ fragments from the molecular ion were represented by peaks at m/e 279 and 278, respectively. As in the spectrum of Compound C, the base peak in the mass spectrum of Compound D was observed at m/e 98.

When the remainder of the spectrum of Compound D was compared with that of Compound C, many similarities were observed. However, the spectrum of Compound D was more complex. Intense peaks which were discernible only in the spectrum of Compound D were tentatively assigned to olefinic fragment ions. Because of the general indistinguishability of the spectra of double bond positional isomers (25), no conclusions about double bond positions in Compound D could be drawn from these olefinic fragment ions.

Upon quantitative micro-hydrogenation ($NaBH_4$), the unknown absorbed 0.89 moles of hydrogen/mole sample. When the hydrogenated sample was mixed with a methyl 12-keto-octadecanoate standard, the two components could not be resolved by either GLC on an EGS column, or by TLC on glass plates coated with silver nitrate-impregnated Silica Gel G. Mass spectrometric analysis indicated that the hydro-

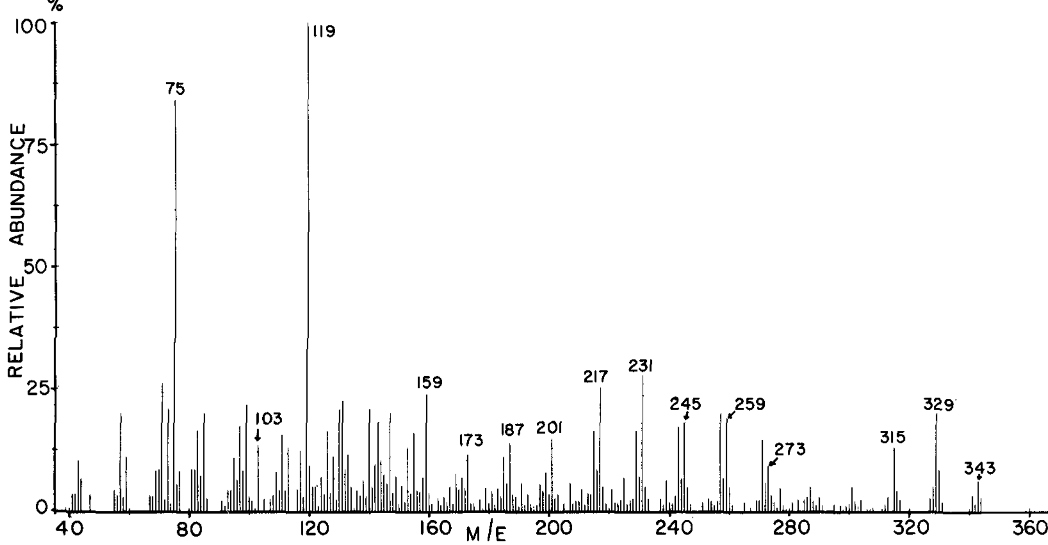


FIG. 8. Mass spectrum of the di-trimethyl silyl ether derivative of hydroxylated Compound D.

genated unknown had a molecular weight of 312, corresponding to a keto-octadecanoate. It was further observed that the spectrum of the hydrogenated unknown was superimposable on that of Compound C.

Thus, it was assumed that the ketonic group in Compound D was located predominantly in positions 9-15 as found in Compound C.

In order to establish the positions of the double bond in Compound D, methyl 12-keto-octadec-9-enoate (II) was first examined. Hydroxylation followed by silylation of (II), gave the ditrimethylsilyl ether derivative. This derivative should fragment between the carbon atoms bearing the substituent groups (11).

The base peak in the mass spectrum (20 eV) of the silyl ether derivative occurring at mass 259 has the structure attributed to the ion containing the ester function:

$$(CH_3)_3\text{-}Si\text{-}\overset{+}{O}\text{=}CH(CH_2)_7CO_2CH_3$$

The fragment corresponding to the remainder of the molecule (m/e 229), is as follows:

$$CH_3(CH_2)_5\text{-}\overset{\overset{\displaystyle O}{\|}}{C}\text{-}CH_2\text{-}CH\text{=}\overset{+}{O}\ Si\ (CH_3)_3$$

However, this ion was not observed, probably due to further decomposition of the carbonyl containing fragment. Other peaks occurred at mass 113, $CH_3(CH_2)_5^+$; 157, $^+(CH_2)_7CO_2CH_3$; 399, $[M - (CH_3)_3\text{-}SiO]^+$; 473, $[M - CH_3]^+$; and 75, $(CH_3)_2Si\text{=}\overset{+}{O}H$.

Unknown D was similarly treated with OsO_4, followed by BSA. The mass spectrum of the di-TMS derivative (Fig. 8) (20 eV) exhibited a mass 75 peak, indicative of silyl ethers. In addition, two homologous series were observed: the first appeared at masses 159, 173, 187, 201, 215, 229, 243, 257 and 271, and the second at masses 217, 231, 245, 259 and 273. Additional peaks in the second series were observed at masses 315, 329 and 343.

For an understanding of the possible origin of the ions represented by these two series, it is necessary to consider that Compound D can be represented by two structures (III) and (IV), which differ only in the relative positions of the double bond and the ketonic groups.

(III)
$$CH_3(CH_2)_l\overset{\overset{\displaystyle O}{\|}}{C}(CH_2)_mCH\text{=}CH(CH_2)_nCO_2CH_3$$
$(l + m + n = 13)$

(IV)
$$CH_3(CH_2)_rCH\text{=}CH(CH_2)_s\overset{\overset{\displaystyle O}{\|}}{C}(CH_2)_tCO_2CH_3$$
$(r + s + t = 13)$

If both types (III) and (IV) are present in Compound D then two corresponding types of di-TMS derivatives, (V), and (VI), would be formed upon hydroxylation and subsequent silylation of Compound D.

(III) (a) OsO_4 (b) BSA →

$$CH_3(CH_2)_l\overset{\overset{\displaystyle O}{\|}}{C}(CH_2)_m\underset{}{\overset{\overset{\displaystyle (CH_3)_3Si}{|}}{\overset{\displaystyle O}{|}}}{CH}\text{-}\overset{\overset{\displaystyle Si(CH_3)_3}{|}}{\overset{\displaystyle O}{|}}{CH}(CH_2)_nCO_2CH_3$$

(V)

TABLE I

Possible Fragment Ions Occurring From the Di-TMS Derivatives of CompoundsD

$CH_3(CH_2)_lC(=O)(CH_2)_mCH(OSi(CH_3)_3)-CH(OSi(CH_3)_3)(CH_2)_nCO_2CH_3$ (V)

(l + m + n = 13)

$CH_3(CH_2)_lC(=O)(CH_2)_mCH{=}O^+Si(CH_3)_3$ + $Si(CH_3)_3O^+{=}CH(CH_2)_nCO_2CH_3$

(VII) (VIII)

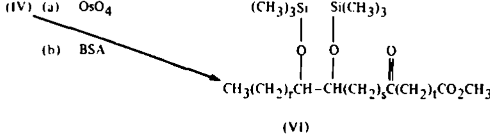
$CH_3(CH_2)_rCH(OSi(CH_3)_3)-CH(OSi(CH_3)_3)(CH_2)_sC(=O)(CH_2)_tCO_2CH_3$ (VI)

(r + s + t = 13)

$CH_3(CH_2)_rCH{=}O^+Si(CH_3)_3$ + $Si(CH_3)_3O^+{=}CH(CH_2)_sC(=O)(CH_2)_tCO_2CH_3$

(IX) (X)

l+m	m/e	Original double bond position	n	m/e	Original double bond position	r	m/e	Original double bond position	s+t	m/e	Original double bond position
1	159	14	4	217	6	3	159	13	2	217	5
2	173	13	5	231	7	4	173	12	3	231	6
3	187	12	6	245	8	5	187	11	4	245	7
4	201	11	7	259	9	6	201	10	5	259	8
5	215	10	8	273	10	7	215	9	6	273	9
6	229	9	11	315	13	8	229	8	9	315	12
7	243	8	12	329	14	9	243	7	10	329	13
8	247	7	13	343	15	10	257	6	11	343	14
9	271	6				11	271	5			



Mass spectral fragmentation of these two di-TMS derivatives between the carbon atoms bearing the silyl ethers, could then produce as many as four ion fragment types, (VII) to (X), all of which are presented in Table I. Each type can be represented by a homologous series. The individual ions that could be present in these four series are also illustrated in Table I. Unfortunately, the series attributed to ion types (VIII) overlaps with the series assigned to ion type (IX), and the series assigned to ion type (VII) overlaps with the series assigned to ion type (X). Thus, from the data in Table I, it can be observed that the series of ions, appearing in the mass spectrum of the di-TMS derivative of Compound D, which ranges in mass from 159 to 271, could be attributed to ion type (VII) or (IX). However, since ions of type (VII) were not observed in the spectrum of the di-TMS model compound (II), the series ranging from 159 to 271 was tentatively attributed to ions of type (IX). If this assumption is correct, it is indicative that the double bond in Compound D is located in positions 5 to 13.

Finally, the series which ranges from mass 217 to 273 and from 315 to 343 can represent ion types (VIII) and (X). It was not determined by a study of any model compound, whether either type predominates in a 20 eV spectrum. From Table I, it can be observed that this second series could indicate that the double bond in Compound D is located in positions 6 to 10 and 13 to 15, or position 5 to 9 and 12 to 14 or both.

α,β- Unsaturated ketones were apparently absent due to the lack of an absorption band at 215-250 mμ in the UV spectrum and at 1665-1685 cm^{-1} in the IR spectrum.

On the basis of the reported information, Compound D was therefore identified as an isomeric mixture of methyl keto-octadecenoates, wherein the double bond is located predominantly in the 5 to 15 position and the ketonic group in the 9 to 15 position.

The formation of Compound D from linoleic acid can be rationalized by considering the formation of an epoxide at one of the double bonds, and the subsequent rearrangement of the epoxide to a ketone (26) to give a monounsaturated keto-ester. The apparent absence of

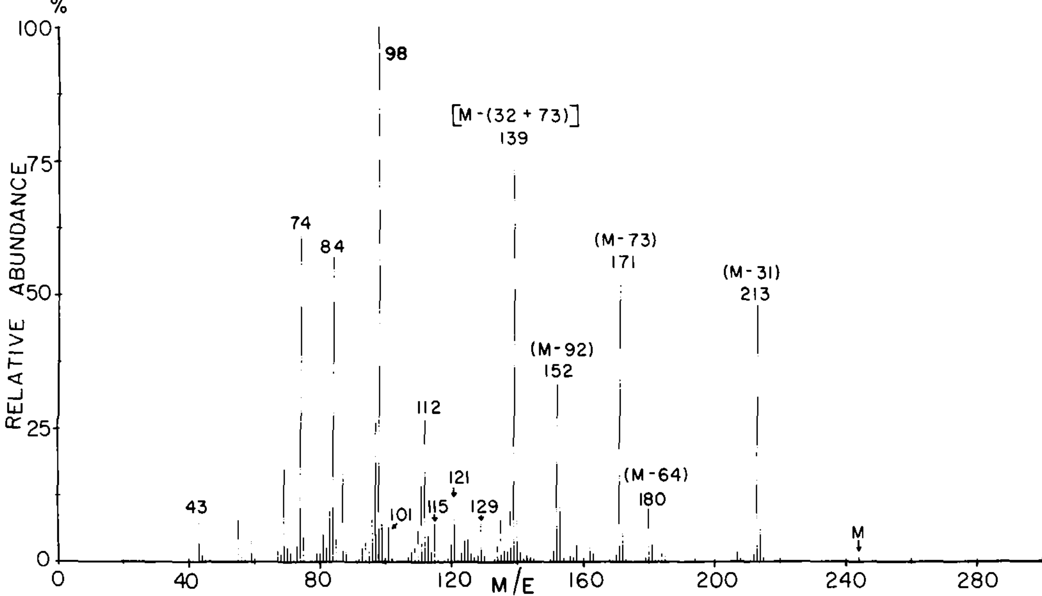


FIG. 9. Mass spectrum of Compound E.

an α- β- unsaturated keto group is evidence against the following mechanism:

$R\text{-}CH_2\text{-}CH{=}CH\text{-}R' \longrightarrow R\text{-}CH(OOH)\text{-}CH{=}CH\text{-}R'$

$\swarrow$

$R\text{-}CH(O\cdot)\text{-}CH{=}CH\text{-}R' \longrightarrow R\text{-}C({=}O)\text{-}CH{=}CH\text{-}R'$

A monounsaturated keto-ester was isolated from thermally oxidized soybean oil (24) but the authors did not determine the position of the double bond and the ketone group.

Dibasic Ester

Compound E (representing 0.005% of the isolated methyl esters) was isolated from Fraction 3 (Fig. 1) by preparative GLC on an OV-101 column.

Infrared spectrometric analysis of the unknown compound indicated bands at 1740, 1175 cm^{-1} (ester); and 727 cm^{-1} (tetramethylene). No absorption was detected in the UV spectrum.

The mass spectrum (20 eV) of the unknown (Fig. 9) exhibited a base peak at m/e 98. This was also the base peak for unknown Compounds C & D, which were identified as keto-esters; but a ketonic absorption band was not visible in the IR spectrum of Compound E. However, as previously mentioned, a strong peak at m/e 98 is characteristic of long chain saturated dibasic esters (21). The mass spectrum of the unknown was, therefore, compared with published spectra of dibasic esters. According to Ryhage and Stenhagen (21), spectra of dibasic esters do not exhibit an intense molecular ion. However, strong peaks at (M - 31) and (M - 73) as well as at masses 98 and 112 should be present. From Figure 9, a strong peak at mass 213 was interpreted as loss of a CH_3O fragment from the small peak at 244. A molecular weight of 244 is consistent with methyl undecane-1,11-dioate. Therefore, the mass spectrum of the unknown was compared with those of methyl decane-1,10-dioate and methyl dodecane-1,12-dioate, all of which were obtained under identical conditions. From this comparison, Compound E was identified as methyl undecane-1,11-dioate.

Supportive evidence for this conclusion was obtained from three additional sources. First, the unknown had an experimental neutralization equivalent of 113 (108 calculated for methyl undecane-1,11-dioate). Second, when the unknown was mixed with methyl dodecane-1,12-dioate, resolution of the two components was not shown by TLC. Lastly, GLC analysis of Compound E indicated ester carbon numbers of 13.7 and 18.4 on OV-1 and EGS columns, respectively, which is consistent with the literature (27). It has been suggested that dibasic acids could be produced by secondary oxidation of unsaturated fatty acids.

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The Stereochemistry of Phytanic Acid in Refsum's Syndrome

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ABSTRACT

Phytanic acid has been isolated from the total lipids of the liver of a human suffering from the disease, Refsum's Syndrome (Heredopathia atactica polyneuritiformis). Optical rotation measurements were made on the acid, and on the ketone 6,10,14-trimethylpentadecan-2-one which resulted from the oxidative degradation of the acid and the reductive ozonolysis of phytol. From a comparison of the optical rotatory dispersion curves of the ketone and from the optical rotation data for the acid it is concluded that the phytanic acid isolated has the configuration 3-DL 7-D 11-D 15 tetramethylhexadecanoic acid with the 3-L diastereoisomer predominating.

INTRODUCTION

The occurrence of phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) in the tissue lipids of humans suffering from Refsum's Syndrome (Heredopathia atactica polyneuritiformis) has been the subject of a number of investigations, among which are studies relating to the stereochemistry of the acid. From evidence obtained by means of gas liquid chromatography (GLC) using butanediol succinate polyester as stationary phase in open tubular columns of high resolving capacity, it has been suggested (1) that phytanic acid from the tissues of Refsum's subjects comprises two isomers (in varying proportions), namely the diastereoisomers 3-DL, 7-D, 11-D (DDD, LDD). However, these findings are somewhat equivocal since it has been pointed out (2) that on liquid phases and column support materials, both of which are optically inactive, GLC does not effect the separation of optical antimers (e.g., DDD from LLL and LDD from DLL).

Isler et al. (3) established the absolute configuration of the centers of asymmetry in the side chain of phylloquinone by comparing the optical rotatory dispersion (ORD) curves of the ketone 6,10,14-trimethylpentadecan-2-one prepared from natural phylloquinone with that of known configuration derived from natural phytol (4,5). This procedure has now been applied to phytanic acid which was isolated, post-mortem, from the tissues of a Refsum's subject (6).

MATERIALS AND METHODS

GLC Conditions

GLC analyses were made with a Pye Argon Chromatograph at 185 C using 4 ft x 4 mm columns with column packings of 5% Apiezon L or 15% polymerized ethylene glycol adipate on acid-washed Celite 545 (Shandon).

TLC Conditions

Thin layers of Kieselgel G (Merck) were prepared on glass plates and chromatography was carried out as described by Malins and Mangold (7).

Infrared Spectroscopy

Samples of the ketone were subjected to infrared analysis as thin films between rock-salt discs by using a twin-beam spectrophotometer model SP 200 (Unicam).

Optical Rotation Measurements

Phytanic acid was examined in chloroform solution in a Peepol 60 (Bellingham and Stanley) fitted with an SP 500 monochromator (Unicam). Samples of the ketone were analyzed in methanol solution in a Polarmatic 62 (Bellingham and Stanley/Bendix Ericsson) automatic recording spectropolarimeter.

Reductive Ozonolysis of Phytol

Phytol (Koch-Light Laboratories Ltd.) was freed of contaminants by column chromatography on silicic acid (Mallinckrodt) from which it was eluted with 3% diethyl ether in petroleum ether (40-60 C). The purified phytol (3 g) was subjected to reductive ozonolysis (8) and the resulting product was chromatographed on a column of silicic acid using 1% diethyl ether in petroleum ether (40-60 C) as eluting solvent. The product (1.8 g) showed a single component on TLC. GLC showed a single component of carbon number 15.1 on Apiezon L and 15.4 on polymerized ethylene glycol adipate.

Isolation of Phytanic Acid From Liver

A sample of liver obtained freshly post-mortem from a Refsum's subject (6) was finely ground and then refluxed for 1½ hr with 0.5 N ethanolic KOH. The total fatty acids were converted to methyl esters which were reacted with mercuric acetate according to Mangold

and Kammereck (9). The saturated fatty acids, as methyl esters, were segregated by chromatography on a silicic acid column from which they were eluted with 1% diethyl ether in petroleum ether (40-60 C). Phytanate was isolated by passing a solution of the saturated fatty acid esters in iso-octane through columns of urea as described by Cason et al. (10).

Identity of the phytanate, which was produced in a yield of 4 g from 130 g liver, was established by co-chromatography with authentic methyl phytanate (11) using GLC on polar and nonpolar phases; in addition, infrared spectroscopy of the ester as a thin film between KBr discs gave a spectrum which was identical with that of synthetic phytanate (11).

Phytanic acid isolated from liver had $[\alpha]_D^{25}$ -1.98° c 10.0 2 d c in $CHCl_3$ and $[\alpha]_{500}^{25}$ -2.80° c 10.0 2 d c in $CHCl_3$.

Oxidation of Phytanic Acid With Permanganate

Phytanic acid (2.9 g) was refluxed with $KMnO_4$ (29 g) in acetone (725 ml) for 30 hr. The ketonic products were isolated according to Murray (12), yielding 0.85 g material of which some 80% was found by GLC to comprise the ketone, 6,10,14-trimethylpentadecan-2-one (11).

The ketonic fraction was subjected to a dry column procedure of chromatography (13) using a column 30 x 2 cm i.d. packed with silicic acid to a height of 25 cm. The mixture of ketones was added in 3 ml 3% diethyl ether in petroleum ether (40-60 C) and the column was developed with this solvent. When the entire column was wetted with solvent, the mixture was changed to 0.5% diethyl ether in petroleum ether (40-60 C) and chromatography was continued until 500 ml solvent had passed through the column. Fractions obtained by ether extraction of 1 cm segments of the silicic acid were examined using TLC and GLC. It was thus found that two of the fractions consisted of a single component; the carbon numbers of the component on Apiezon L and on polymerized ethylene glycol adipate were 15.1 and 15.4, respectively. The ketone in the pooled fractions (450 mg) was found to co-chromatograph precisely on GLC (polar and nonpolar phases) with 6,10,14-trimethylpentadecan-2-one prepared from phytol, and gave an infrared spectrum (14) identical with that of the reference ketone.

RESULTS AND DISCUSSION

The ORD curves were determined for each ketone in methanol solution over the range of wavelength 230-400 mμ. Each curve showed a single positive Cotton effect of similar form which was delineated by the following values: 6,10,14-trimethylpentadecan-2-one, derived from phytol; ORD in methanol (c, 1.2), 25°; $[\phi]_{400}$ 0°, $[\phi]_{300}$ + 40° $[\phi]_{253}$ -57° $[\phi]_{233}$ -46°: ketone, derived from phytanic acid; ORD in methanol (c, 2.0), 25°; $[\phi]_{400}$ + 7.8°, $[\phi]_{300}$ + 35.3°, $[\phi]_{260}$ -55°, $[\phi]_{238}$ -59°.

Since enantiomorphs have ORD curves which are mirror images about the zero ordinate (15), the close correspondence of the ORD curves (amplitudes of +1.0 and +0.9 for the ketones from phytol and phytanic acid, respectively) is indicative that the two ketones have essentially the same configuration, namely 6-D, 10-D. The asymmetric centers at carbons 6 and 10 of the ketone correspond to those at carbons 7 and 11 respectively of phytanic acid, and thus the configuration suggested by the results obtained from ORD measurements is in agreement with that assigned by Eldjarn et al. (1) from GLC investigation.

The ORD curves of the two ketones are of closely similar form but are not perfectly coincident. Though measurement of optical rotation at low wavelengths is known to present some difficulty (15,16) the possibility that isomers other than that of the 6-D, 10-D configuration may also be present, albeit in small amount, cannot be precluded.

The ORD curves obtained by Mayer et al. (3) in their examination of the ketone 6,10,14-trimethylpentadecan-2-one were of the plain positive type. That no Cotton effect was observed is presumably because rotation measurements were made using solutions of the ketone in a nonpolar solvent, namely octane (16), and no measurements were made below 340 mμ.

Kates et al. (14) established unequivocally the configuration of the phytanyl groups in lipids of *Halobacterium cuticubrum*. Included among their results are optical rotation measurements of phytanic acid from which it can be deduced that the phytanic acid ($[\alpha]_D^{25}$ -1.98) from the Refsum's subject has two constituent diastereoisomers (i.e., DDD and LDD) of which the LDD form predominates; the ratio LDD:DDD is about 2. This value for the ratio LDD:DDD is within the range 1.5-3.5 reported by Eldjarn et al. (1) in their investigation (GLC) of phytanic acid from the plasma of a number of cases of Refsum's Syndrome.

It is concluded that the diastereoisomers DDD, LDD of phytanic acid from the liver of the Refsum's patient were derived either directly from phytol [dietary chlorophyll (17-19)] or indirectly from phytol as preformed phytanic acid, e.g., from fish (20), meat

(21,22) and dairy products (23,24). This conclusion accords with the findings of Eldjarn et al. (1).

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Metabolic Control in Isolated Brown Fat Cells[1]

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ABSTRACT

Experiments with brown fat cell preparations from the adult hamsters are described. The mitochondria of brown adipose tissue were shown to have a classical electron transport system. The basal respiration of brown fat cells was demonstrated to be coupled to oxidative phosphorylation. Evidence is presented for partial uncoupling of oxidative phosphorylation as a mechanism for controlling respiration during norepinephrine stimulation. Exogenously added fatty acids were found to mimick the norepinephrine stimulation of respiration. Norepinephrine and cyclic AMP were shown to have no effect on brown fat mitochondria. Experiments with labeled oleate showed that the triglyceride re-esterification cycle does not control respiration in brown adipose tissue.

INTRODUCTION

The general trend in phylogeny has been the development of systems for the conversion of chemically bound energy into physical work with the highest possible efficiency. Thus, oxidative systems have evolved whereby the requirements of the cell for energy regulate the release of chemical energy bound in the substrates. This device has reached its highest degree of efficiency in what we know as mitochondrial oxidative phosphorylation.

In recent years, one of the physiological roles of brown adipose tissue has been demonstrated to be heat production (1,2). In contrast to the usual situation noted above where nature attempts to conserve energy with highest possible efficiency, the production of heat would seem to require a low efficiency system.

It might be inappropriate to implicate the usual mechanisms for the regulation of respiration in brown adipose tissue since this tissue has as its objective the liberation of thermal energy.

Electron micrographs of the brown fat mitochondria show essentially the same features as the mitochondria of other tissues (3). However, brown adipose tissue appears to have several distinctive biochemical characteristics which separate it from other tissues. The experiments below attempt to illustrate some of these unique features. The results are discussed in terms of cellular mechanisms which conserve and dissipate energy.

EXPERIMENTAL PROCEDURES

The following experiments were carried out on brown fat cells isolated from adult hamsters. All of the respiratory measurements were made with a Clark type oxygen electrode equipped with an electronic differentiator in the recording circuit (6; also, Eisenhardt, personal communication, and Liljesvan, personal communication).

As shown in Figures 1 and 2, the basal rate of oxygen consumption can be strongly stimulated by norepinephrine (NE) as well as succinate or α-glycerol phosphate (GP). Other substrates did not increase oxygen consumption measureably. The NE-mediated respiration is pyridine nucleotide linked as seen by its rotenone sensitivity (Fig. 1). The entire system

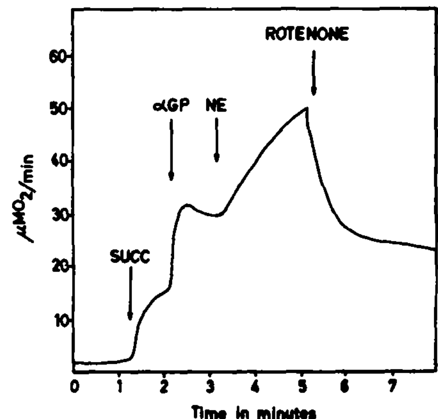

FIG. 1. Stimulation and inhibition of O_2 uptake in brown fat cells incubated in Krebs Ringer phosphate buffer pH 7.4 at 23 C. Final suspension contained 2% cells by volume and 0.4% Pentex Bovine Serum Albumin, Fraction V. Additions: 10 mM succinate, 12 mM GP, 0.04 μg/ml NE, 0.3 μM rotenone.

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[1]One of nine papers to be published from the Symposium "Brown Adipose Tissue," presented at the AOCS-AACC Joint Meeting, Washington, D.C., March 1968.

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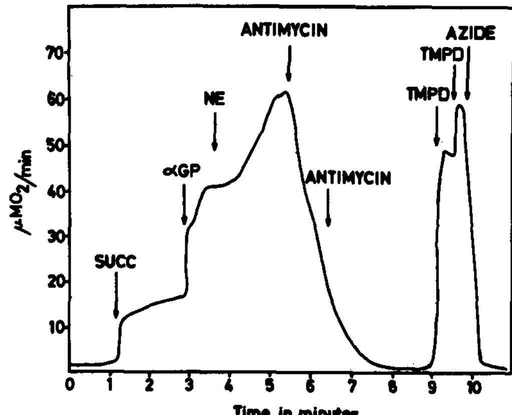


FIG. 2. Conditions as in Figure 1. Additions: 1 μg/ml antimycin, 100 μM TMPD, 1 mM azide.

(Fig. 2) is inhibited by antimycin or azide, and the antimycin block can be bypassed by N,N,N',N', tetramethyl-p-phenylenediamine (TMPD). These experiments strongly suggest that brown adipose tissue has a classical mitochondrial electron transport system as evidenced by its normal response to inhibitors.

The action of NE appears to proceed via the adenyl cyclase system that activates a lipase which then releases free fatty acids from triglyceride stores (2,3,5,7-9). The above demonstrated sensitivity of NE stimulated respiration to rotenone is consistent with this idea. Subsequent experiments in this paper show that NE stimulated respiration can be mimicked by exogenously added fatty acids (5).

The response of isolated cells to NE alone is shown in Figure 3. The initial, marked increase in oxygen consumption appears to be transient. The subsequent decrease in respiration is not caused by a loss of activity of added NE, as further addition has no effect. However it is probably caused by intermediates which accumulate when the cellular metabolism is stimulated by NE.

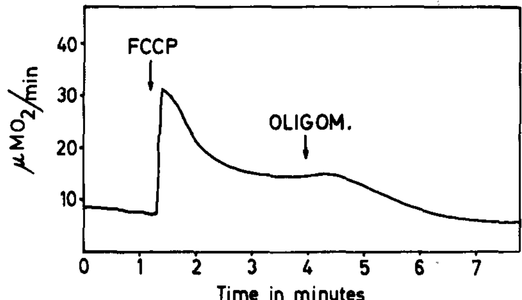


FIG. 4. Release and inhibition of respiration of brown adipose cells by FCCP and oligomycin. Conditions as in Figure 1. Additions: 7 μM FCCP and 4 μg/ml oligomycin.

The basal respiration can be increased by the addition of the uncoupler carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), shown in Figure 4. The increased respiration is, however, of a transient nature and is followed by an inhibition which can be further accentuated by oligomycin.

RESULTS AND DISCUSSION

These results mean that the increased respiration is caused by an uncoupling of oxidative phosphorylation. As fatty acids need to be activated by ATP prior to oxidation, the extra uptake of oxygen elicted by FCCP is presumably equivalent to the amount of activated fatty acids present in the cell before the added FCCP cuts off the energy supply for substrate activation. The effect of oligomycin is also interpreted as an inhibition of the generation of ATP needed for substrate activation. Why the effect of the two agents is additive, however, is not known.

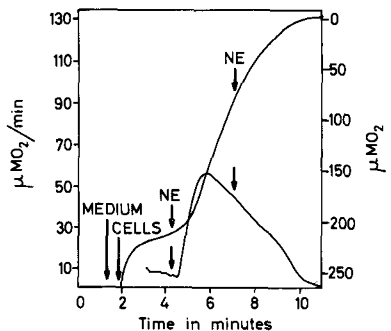


FIG. 3. Stimulation of brown adipose cells with NE. Conditions as in Figure 1.

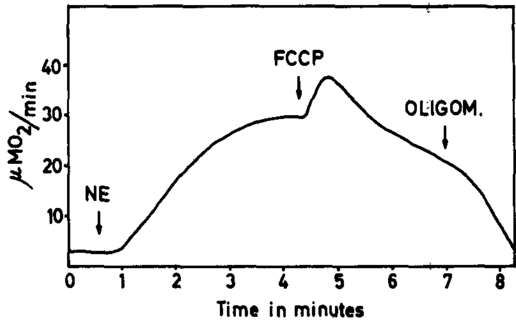


FIG. 5. Effect of FCCP and oligomycin on brown fat cells stimulated with a half maximal dose of NE. Conditions as in Figure 1.

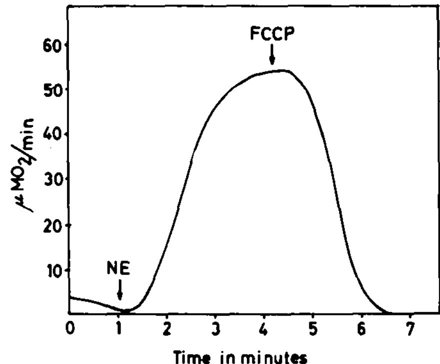


FIG. 6. Effect of FCCP on brown fat cells stimulated with a maximal dose of NE.

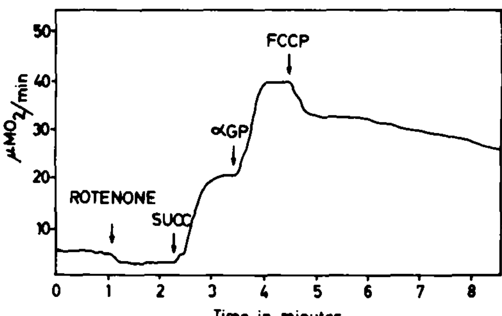


FIG. 8. Effect of FCCP on fp-mediated respiration.

The transient stimulatory effect of FCCP on isolated cell suspensions of brown adipose tissue is gradually decreased as the respiration of the cells is elevated by increasing concentrations of NE. Figure 5 illustrates a study where a half maximal dose of NE was added, and Figure 6 a study where a maximal dose of NE was used to stimulate respiration. Figure 7 shows how the FCCP-peak decreased with increasing amounts of NE being added.

From these experiments it is quite obvious that brown fat cells can carry out electron transport coupled phosphorylation and that the basic respiration of these cells is controlled by phosphorylation according to the generally accepted concept for the respiration of other systems. In the NE stimulated cell, respiration may not be primarily limited by the capacity of the phosphorylating system which, however, is active enough to produce all the ATP needed for the activation of fatty acids.

As the availability of free fatty acids (FFA) increases upon NE stimulation of lipase activity, the rate limitation imposed on the respiratory system by the phosphorylating system continuously decreases. This paradox can be explained in two ways. Either the system consumes more ATP per oxygen taken up as respiration increases, or the cells are equipped with a system that gradually uncouples phosphorylation from respiration as FFA dependent respiration increases. A combination of the two mechanisms is also possible. There may be a partial uncoupling, or loose coupling, of at least the two terminal sites of ATP formation in the electron transport system (Fig. 8). In this experiment, in which only fp-coupled substrates were oxidized because of the presence of rotenone, no release of respiration can be obtained by the addition of FCCP, nor can respiration be inhibited by oligomycin.

The increased respiration evoked by FCCP addition to a succinate and α-glycerophosphate supplemented system without rotenone can probably be accredited to a release of the coupling site in the NAD-fp region (Fig. 9).

Loose coupling as a biological reality has previously been demonstrated by Ernster and Luft (10) in a patient suffering from an

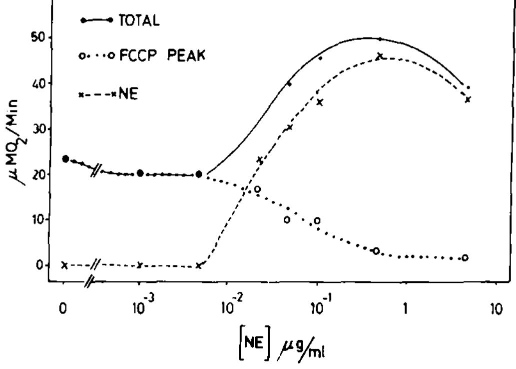


FIG. 7. The inter-relationship between NE and FCCP with respect to respiration. Conditions as in Figure 1.

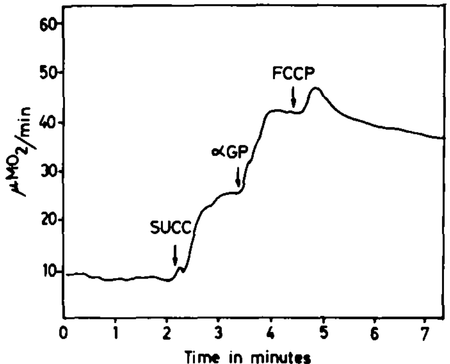


FIG. 9. Effect of FCCP in the presence of succinate and GP induced respiration.

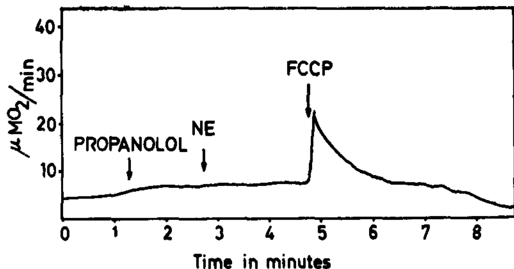


FIG. 10. FCCP stimulation of respiration when NE action is prevented by propanolol.

increased basal metabolic rate of extrathyroidal origin. Thus loose coupling of oxidative phosphorylation is a reasonable postulation for the mechanism by which brown adipose tissue produces heat. Whether or not partial uncoupling is the only aspect of the mechanism is questionable, especially if it is based on the concentration of fatty acids liberated through NE action. To visualize how production of an equivalent amount of ATP needed for FFA activation could be balanced against the gradual uncoupling action of liberated FFA is particularly difficult. A coordinated action of NE on lipase activity and mitochondrial oxidative phosphorylation is one possible explanation; however, we have not been able to demonstrate any effect of NE on isolated mitochondria. Extensive electron microscopic studies of isolated cells elucidated no differences between the structures of the mitochondria before and after norepinephrine addition. The negative finding that NE did not exert a direct effect on mitochondria was also supported by the experiment in Figure 10. In this experiment the effect of NE on the lipase of isolated cells was inhibited by the addition of propranolol, a β-receptor blocking agent (9). The subsequent addition of FCCP released

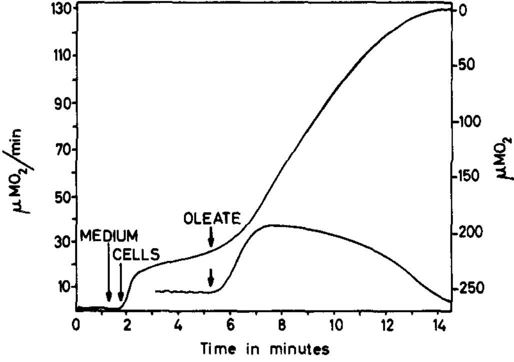


FIG. 11. Oleate mediation of respiration.

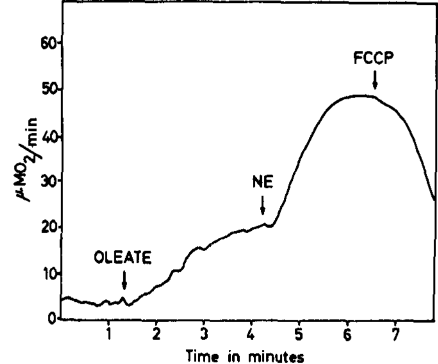


FIG. 12. The action 0.04 μg/ml of NE on a cell suspension stimulated with half maximal dose of oleate.

respiration, indicating a coupled respiratory chain.

Further experimental evidence that neither NE nor cyclic AMP have any effect on mitochondria except that mediated by FFA, is presented in Figures 11-14. Figure 11 shows that the direct addition of fatty acids to the medium can mimic the NE effect. This experiment suggests that the primary action of NE is simply to release fatty acids which are then able to increase respiration. Figure 12 shows that the respiration evoked by a half maximal dose of oleate can be further increased by NE. Figure 13 shows the dose response curve for oleate and demonstrates the relationship between NE and oleate stimulated respiration. Other experiments, not shown here, in which the action of different inhibitors and stimulators of the NE and the oleate systems were compared, were all in accord with the

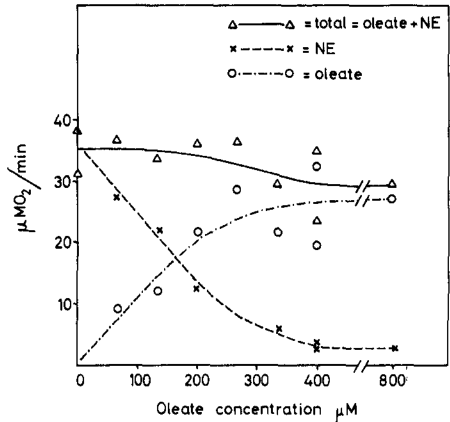


FIG. 13. The relationship between increasing doses of oleate and a maximal dose of NE (0.04 μg/ml) when added as in Figure 12.

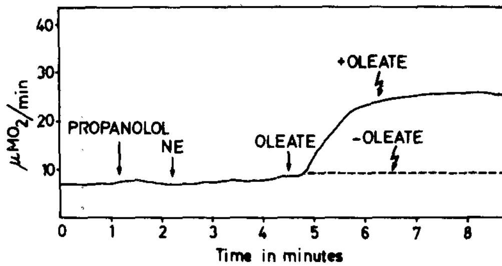
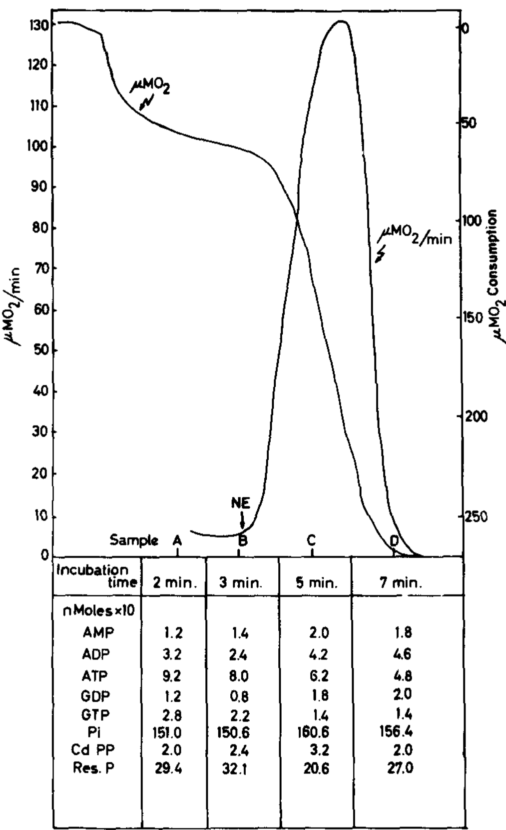


FIG. 14. Oleate-mediated respiration in a system where NE action is blocked by propranolol.

hypothesis that the action of NE can be mimicked by oleate. The only striking difference noted is that oleate can bypass the cyclic AMP system. As demonstrated in Figure 14, the action of NE was blocked by a prior addition of propanolol, but later addition of oleate still evoked increased respiration. The above experiments (Figs. 9-11) demonstrate that the action of NE is to release FFA from triglyceride stores.

Returning to the problem of metabolic regulation of brown fat respiration, two main theories ought to be considered; one based on uncoupling of respiration from phosphorylation and one based on what we would like to call overall adenosine triphosphatase. The latter refers either to an adenosine triphosphatase or a system that rapidly consumes the ATP formed in oxidative phosphorylation by a series of reactions yielding orthophosphatase and ADP or AMP. Of the latter type, the reesterification theory is most clearly defined (2,8,11). It involves a reformation of hydrolyzed triglycerides from activated fatty acids and glycerophosphate with corresponding loss of free energy. The incorporation of 9,10-^{3}H-labeled oleate into glycerides of respiring brown fat cells has been followed. Table I presents the data from one of these experiments. Although a significant amount of ^{3}H is incorporated in the triglyceride fraction, it cannot account for a reaction rapid enough to serve as a trapping system for the theoretical amount of ATP produced if the system were tightly coupled. The ^{3}H-labeled water formed indicated that some of the oleate added had been oxidized. Furthermore, the presence of a potent glycerophosphate dehydrogenase in brown fat, as well as the ability of oleate to mimic NE, make the reesterification theory an unlikely explanation for heat production in this tissue.

The experiments presented here and in a recent communication (12) demonstrate that brown fat cells are at least partially coupled before NE stimulation. Spectrophotometric observations showed that after an initial oxidation of cytochrome b upon NE addition, dicumarol cannot cause a further oxidation of the respiratory chain (Prusiner, Williamson, Lindberg and Chance, unpublished observations). These results suggest that some energy dissipation process, prior to ATP formation, is probably operating after NE has been added. While the main energy dissipation probably takes place prior to phosphorylation, the hydrolase-synthetase cycle we proposed recently might act as a fine control to regulate the ATP level needed for Acyl CoA formation (12). An indication of the low phosphorylation efficiency in brown fat cells following NE addition is presented in Figure 15, where a drop in the ATP and GTP levels occurs while O_2 uptake is rising (13). The concept that partial uncoupling of oxidative phosphorylation occurs upon norepinephrine release of fatty acids from triglycerides is in agreement with the work of Karl Hittelman of our laboratory who has



Incubation time	2 min.	3 min.	5 min.	7 min.
nMoles×10				
AMP	1.2	1.4	2.0	1.8
ADP	3.2	2.4	4.2	4.6
ATP	9.2	8.0	6.2	4.8
GDP	1.2	0.8	1.8	2.0
GTP	2.8	2.2	1.4	1.4
Pi	151.0	150.6	160.6	156.4
Cd PP	2.0	2.4	3.2	2.0
Res. P	29.4	32.1	20.6	27.0

FIG. 15. Nucleotide changes upon NE stimulation of respiration. The nucleotides were measured using a combination of ^{32}P and thin layer chromatography (13).

TABLE I

Incorporation of ^{3}H-Oleate Into Different Components of Brown Fat Cells nMoles of 9, 10^{3}H-Oleate Incorporated

Component	Time, min					
	0	2	3	10	20	30
	(1.2 μMoles ^{3}H-Oleate; 2% cell suspension vol 2.7 ml)			(1.2 μMoles ^{3}H-Oleate + 0.5 μg NE; 2% cell suspension vol 2.7 ml)		
Triglycerides	1	1	1	14	22	23
Diglycerides	2	1	4	0	0	0
Aqueous extract	2	0	0	6	4	4
H_2O	5	14	24	65	96	106
Respiration calculated (μatoms O_2)	255	715	1225	3320	4900	5400

shown the recoupling of isolated brown fat mitochondria by the oxidation of endogenous fatty acids (14). The addition of ATP and carnitine to uncoupled brown fat mitochondria evoked the oxidation of endogenous fatty acid which could completely recouple the mitochondria. The recoupling was also demonstrated by ^{32}P uptake (14).

ACKNOWLEDGMENT

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A Mechanism for Hormonal Activation of Lipolysis and Respiration in Free Brown Fat Cells[1]

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ABSTRACT

Free brown fat cells were obtained by digestion of minced brown adipose tissue with bacterial collagenase. Electron micrographs have shown many mitochondria with tightly packed cristae in free brown fat cells. Lipolysis was activated in free brown or white fat cells; however, only in brown fat cells was lipolysis accompanied by a marked stimulation of respiration. Insulin inhibits the catecholamine-induced respiration in the complete absence of glucose. Catecholamines, theophylline and dibutyryl 3′,5′-AMP all appear to activate lipolysis and respiration by a process dependent upon energy derived from mitochondrial oxidative phosphorylation. Uncoupling agents such as *m*-chlorocarbonyl cyanide phenylhydrazone stimulated respiration. The addition of oligomycin prior to that of catecholamine inhibited the increase in respiration to a much greater extent than if it was added after the catecholamine. The increase in respiration due to lipolytic agents appears to be the result of increased free fatty acid release mediated through activation of lipolysis by cyclic 3′,5′-AMP, since the addition of octanoate or palmitate to brown fat cells mimicked the effects of lipolytic agents on respiration. The activation of energy metabolism may be the result of a large increase in the energy-dependent cyclic transport of K^+, as a result of alterations in the mitochondrial membrane by free fatty acids. This hypothesis is based on the following findings. The activation of respiration by fatty acids or lipolytic agents was dependent upon the presence of K^+ in the medium. The addition of K^+, Rb^+ or Cs^+, but not NH_4^+, to fat cells isolated and incubated in K^+-free buffer restored the ability of lipolytic agents to increase respiration. Valinomycin, an antibiotic which stimulates uptake of K^+ by mitochondria and increases the permeability of lipid membranes to K^+, was a potent stimulator of respiration in free brown fat cells in the presence of K^+. The stimulation of respiration by theophylline or valinomycin was blocked by nigericin which also increases the K^+ permeability of membranes but blocks the uptake of K^+ by mitochondria. The lipolytic action of theophylline was virtually unaffected by K^+ or nigericin.

INTRODUCTION

Free brown fat cells can be obtained by digestion of brown adipose tissue with collagenase (1) and have been found useful as a model system for examining hormonal regulation of brown fat metabolism (1,2). Because of the high rate of respiration by brown fat in the presence of lipolytic agents, free brown fat cells are particularly useful for respiration studies. The present studies are primarily concerned with the elucidation of the mechanism by which lipolytic agents act to increase brown fat thermogenesis. Our studies have led us to favor the hypothesis that the increased respiration due to lipolytic agents is secondary to increased utilization of energy for K^+ uptake by mitochondria to compensate for leakage of mitochondrial K^+ resulting from the increased intracellular concentration of free fatty acids.

EXPERIMENTAL PROCEDURES

Dorsal interscapular brown adipose tissue was obtained from sexually immature female albino rats (Sprague-Dawley) starved for 18 hr prior to being killed. Brown fat cells from male rats were equally as responsive to low concentrations of epinephrine as those from female rats (1). The rats (130-160 g) were maintained on laboratory chow except for those used in the experiments shown in Figure 2 which were fed a high fat diet for 1-2 weeks and weighed 160-190 g. Brown fat cells were isolated by collagenase digestion of brown fat (1,2). The phosphate buffer was made up fresh daily and adjusted to pH 7.4 after addition of 4% bovine fraction V albumin powder (Pentex No. 55). The buffer contained the following: NaCl, 128 mM; $CaCl_2$, 1.4 mM; $MgSO_4$, 1.4 mM; KCl, 5.2

[1]One of nine papers to be published from the Symposium "Brown Adipose Tissue," presented at the AOCS-AACC Joint Meeting, Washington, D.C., March 1968.

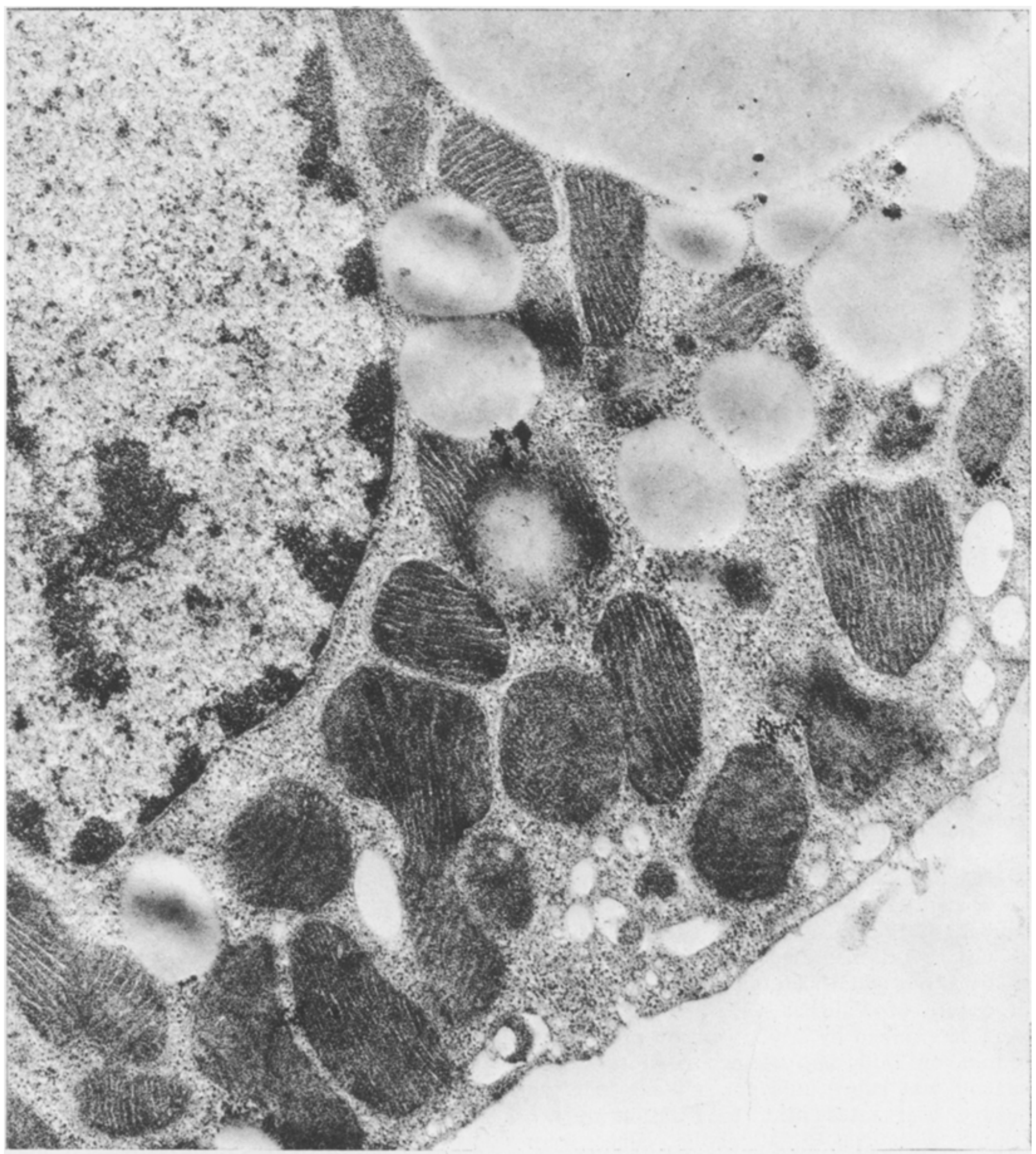

FIG. 1. An electron micrograph of a portion of a multilocular brown fat cell. Part of the nucleus is visible in the upper left hand side. A large lipid droplet is visible in the upper right hand side and several other smaller lipid droplets are present along with numerous mitochondria containing tightly packed cristae. The magnification is 17,500 (reduced approximately 15%).

mM; and Na_2HPO_4, 10 mM (pH adjusted to 7.4 with HCl).

Oxygen uptake was measured polarographically with a collodion coated platinum electrode for short-term studies. The cells were incubated in air-saturated medium at 37 C. Oxygen consumption over a 4 hr period was measured in a respirometer. The Warburg side-arm respirometer flasks were siliconized prior to each experiment; additions were made from the side-arm after incubation of cells for 1 hr. The atmosphere of the respirometer flasks was gassed with 100% O_2 for 3 min, the flasks were then closed and equilibrated for 30 min prior to the start of the experiments which were conducted at 37 C. Carbon dioxide was absorbed by adding 0.2 ml of 10% KOH to the center well.

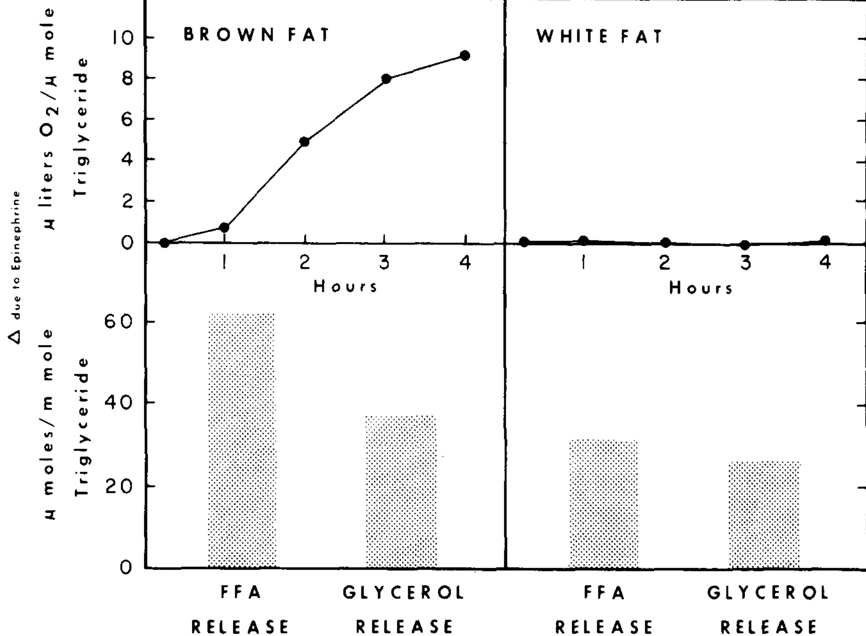

FIG. 2. Comparison of epinephrine effect on respiration and lipolysis in brown and white fat cells. The values are shown as the delta due to epinephrine (3.3 μg/ml) which was added at the end of the first hour of incubation. Fatty acid and glycerol release are expressed as the total for 4 hr incubation. Brown fat cells (35 μmoles triglyceride per flask) and white fat cells (120 μmoles of triglyceride per flask) were incubated in 3 ml of buffer containing 4% albumin and glucose (20 mM). The values are expressed as the means of eight paired replications in which brown and white adipose tissue were obtained from the same animals.

The basal rate of oxygen consumption was linear over the 4 hr period of the respirometer studies. At the end of the incubation period an aliquot of the medium was taken for glycerol analysis by a modification (1) of the enzymatic procedure of Vaughan (3). Free fatty acids were determined by a modification of the procedure of Dole and Meinertz (4) in which hexane was substituted for heptane and the hexane evaporated prior to titration of the sample with NaOH. Metabolic values were standardized to units per triglyceride content based on the total free acid content of the cells (1) as a convenient index of the amount of cells added per flask. The amount of triglyceride per cell was probably greater in cells from animals fed a high fat diet (Fig. 2, Table II) which may account for the lower metabolic activity of these cells per unit of triglyceride.

The sources of the chemicals were as follows: L-epinephrine, crystalline insulin, and oligomycin, Sigma; theophylline, Mallinckrodt; L-norepinephrine bitartrate, Calbiochem. Nigericin was first dissolved in alcohol, then diluted to give a final concentration of 0.45 mg/ml in water containing 4% albumin and 10% alcohol. Valinomycin was also dissolved in alcohol, then diluted to give a final concentration of 0.24 mg/ml in water containing 4% albumin and 5% alcohol. In all experiments with these antibiotics an equal amount of alcohol was added to control flasks.

The procedures for fixing, embedding and staining fat cells for electron microscopy have been previously described (1).

RESULTS AND DISCUSSION

Suspensions of brown fat cells can be prepared by digestion of rat, hamster or gerbil dorsal interscapular brown fat with bacterial collagenase and readily separated from the other cells present in brown fat, since only fat cells float when cell suspensions are centrifuged for a few seconds. It has been estimated that only about 25% of the cells present in brown fat are fat cells (1). The free multilocular brown fat cells are identical in appearance, based on electron microscopy, to cells present in intact tissue except for the absence of extracellular amorphous material (or basement membrane) exterior to the plasma membrane (1). Figure 1

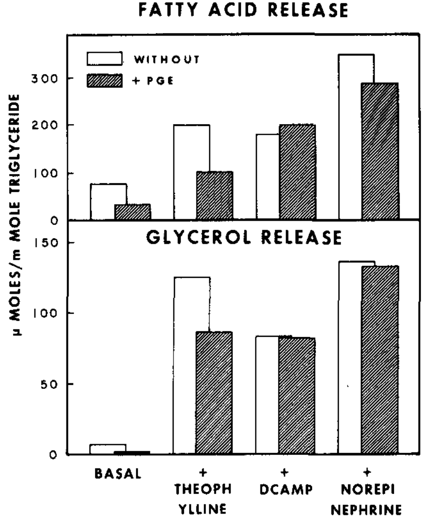

FIG. 3. Effect of prostaglandin E_1 (PGE_1) on brown fat cell lipolysis. Free brown fat cells (3.3 μmoles of triglyceride per flask) were incubated for 4 hr in 1.5 ml of 3% albumin buffer without added glucose. Theophylline, 1 mM, dibutyryl 3',5'-AMP (DCAMP), 0.8 mM, and L-norepinephrine, 2.5 μg/ml were added at the start of the experiments. The values are the means of seven paired experiments and the clear bars represent values in the absence while striped bars represent those in the presence of 0.1 μg/ml of prostaglandin E_1. Prostaglandin E_1 significantly reduced ($P < 0.05$ by paired comparisons) the activation of both glycerol and fatty acid release by theophylline and that of fatty acid release by norepinephrine.

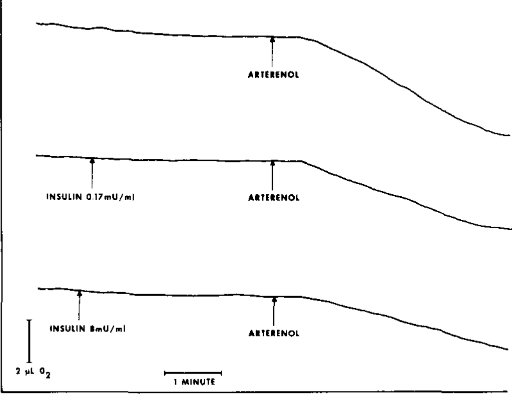

FIG. 4. Inhibitory effect of insulin on stimulation of respiration by catecholamines. Free brown fat cells (5 μmoles of triglyceride per flask in the upper two traces and 3.3 in the bottom trace) were isolated and then incubated in 1.5 ml of 4% albumin buffer without added glucose. L-norepinephrine (Arterenol) at a concentration of 0.12 μg/ml was added as indicated. The curves represent the oxygen electrode tracings from a typical experiment and the mean rate of oxygen consumption per minute after norepinephrine addition was 0.30 μl O_2/μmole triglyceride in three experiments but if cells were preincubated with 0.17 mU/ml of insulin the increase due to catecholamine was only 0.18 μl O_2. The average increase in fatty acid release over the first 5 min after addition of catecholamine was significantly reduced from 62 to 41 μmoles/mmole of triglyceride by prior incubation with insulin. ($P < 0.05$ by paired comparisons).

is an electron micrograph of part of a single multilocular brown fat cell containing many mitochondria with tightly packed cristae surrounding the lipid droplets.

In free brown fat cells (1), as in the intact tissue (5), catecholamines are potent stimulators of lipolysis while ACTH has little effect unless very high concentrations are used. Brown fat cell lipolysis did not respond to growth hormone and glucocorticoid which activate white fat cell lipolysis (1).

Catecholamines markedly stimulate respiration in free brown fat cells in addition to accelerating lipolysis. Figure 2 shows that the addition of epinephrine at a concentration sufficient to maximally stimulate lipolysis in brown (1) or white fat cells (6) produced a slightly greater stimulation of glycerol release per mmole of triglyceride in brown than in white fat cells from the same animals, while epinephrine markedly stimulated respiration only in brown fat cells. It is not surprising that brown fat shows a higher ratio of fatty acid to glycerol release since epinephrine did not markedly stimulate fatty acid re-esterification with α-glycerophosphate derived from glucose metabolism in brown fat cells (1).

In white fat cells it is thought that the hormonal regulation of lipolysis involves activation of the triglyceride lipase by 3',5'-AMP. The concentration of this nucleotide can be increased by catecholamines through activation of adenyl cyclase which catalyzes the conversion of ATP to 3',5'-AMP or inactivation of the phosphodiesterase which converts 3',5'-AMP to AMP (7). Theophylline and other methyl xanthines appear to activate lipolysis by inhibiting the phosphodiesterase (8) while prostaglandin E_1 reduces lipolysis by inhibiting adenyl cyclase (7).

The mechanism for activation of lipolysis in brown fat cells appears to involve activation of lipase by 3',5'-AMP since theophylline and dibutyryl 3',5'-AMP increased fatty acid and glycerol release (Fig. 3). In brown fat cells prostaglandin E_1 inhibited the lipolytic action of theophylline but not that of dibutyryl 3',5'-AMP (Fig. 3). In white fat cells prostaglandin E_1 had no effect on the lipolytic action of cyclic nucleotide (9). The amount of prostaglandin E_1 required to inhibit the lipolytic action of theophylline was quite small and 1

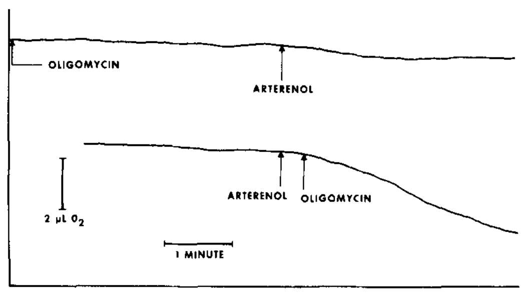

FIG. 5. Oligomycin and catecholamine-induced respiration. Free brown fat cells (3 µmoles of triglyceride per flask in the upper trace and 2.2 in the lower trace) were incubated in 1.5 ml of 4% albumin buffer without added glucose. L-norepinephrine (Arterenol) at a concentration of 0.12 µg/ml and oligomycin, 7.5 µg/ml, were added as indicated. The curves are the actual oxygen electrode tracings from one experiment. In another series of three experiments the rate of oxygen consumption per minute during the first 5 min after addition of norepinephrine was 0.26 µl O_2/µmoles of triglyceride in the absence of added oligomycin (3.75 µg/ml), 0.14 in cells incubated with oligomycin for 1 min prior to catecholamine addition and 0.21 if oligomycin was added 30 sec after the catecholamine.

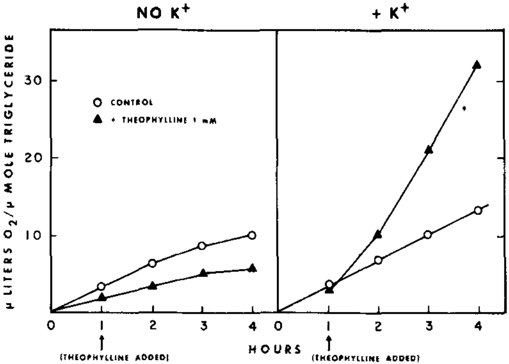

FIG. 6. Failure of theophylline to stimulate respiration in the absence of K^+. Brown fat cells (14 µmoles of triglyceride per flask) were incubated in 3 ml of phosphate buffer containing 4% albumin and 20 mM glucose. The cells in the left part of the figure were isolated and incubated in K^+ free buffer, while those in the right part were isolated and incubated in buffer containing 5.5 mM K^+. Theophylline, 1 mM, was added at the end of the first hour of incubation (triangles). The curves represent the average of five paired experiments. Basal fatty acid release was nil while that due to theophylline was 80 in the absence and 95 in the presence of K^+.

ng/ml of prostaglandin E_1 gave almost 50% inhibition of the lipolytic action of 1 mM theophylline in brown fat cells (9). In brown fat cells prostaglandin E_1 did not effect the increase in glycerol release due to catecholamines but did inhibit fatty acid release (Fig. 3).

Insulin has an antilipolytic effect on lipolysis in free brown fat cells that is independent of its action of glucose metabolism (1). The results indicate that insulin is also able to inhibit the stimulation of respiration due to catecholamine addition to brown fat cells in the complete absence of glucose (Fig. 4). There was no effect of insulin alone on respiration but incubation of cells for only 3 min with insulin markedly reduced the response to catecholamines.

The activation of both respiration and lipolysis by cyclic nucleotide and other lipolytic agents appears to require energy derived from oxidative phosphorylation. The incubation of cells for 1.5 hr with an uncoupler of oxidative phosphorylation, such as *m*-chlorocarbonylcyanide phenylhydrazone, prior to addition of lipolytic agents almost completely prevented the stimulation of lipolysis or respiration by these agents (2). If the uncoupler was added with the lipolytic agents it did not inhibit their action over the next 3 hr (2). Incubation of cells with *m*-chlorocarbonylcyanide phenylhydrazone for as brief a period as 3 min prior to addition of catecholamine markedly inhibited the stimulation of respiration and

TABLE I

Effect of M-Chlorocarbonylcyanide Phenylhydrazone (M-ClCCP) on Catecholamine Action in Brown Fat Cells[a]

	Oxygen consumption µl O_2/µmole of triglyceride	Fatty acid release µmoles/mmole triglyceride
Control	1.29	83
+ M-ClCCP added 3 min before catecholamine	0.44	45
+ M-ClCCP added 30 sec after catecholamine	1.70	78

[a]Free brown fat cells were isolated and then incubated in 1.5 ml of 4% albumin buffer without added glucose. The values are the means of three paired experiments and are shown as the increase over controls during the first 5 min after addition of norepinephrine (0.016 µg/ml). The concentration of M-ClCCP was 6 X 10^{-6}M.

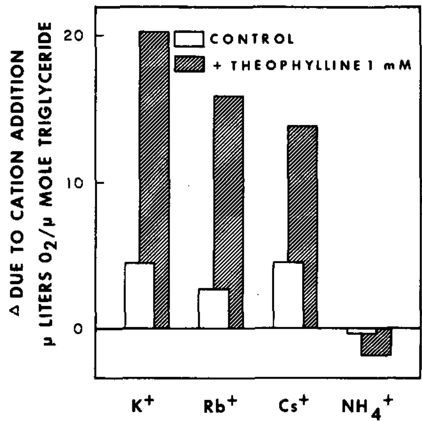

FIG. 7. Effect of adding K^+, Rb^+, Cs^+ and NH_4^+ at 1 hr to cells isolated and then incubated for 1 hr in K^+ free buffer. Brown fat cells (12 μmoles of triglyceride/flask) were incubated for 4 hr in K^+ free phosphate buffer containing 4% albumin and 20 mM glucose. The values are shown as the difference (delta) in respiration during the incubation period due to addition of the various cations over that seen in the absence of added cations. The cations, as their chloride salts, were added at the end of 1 hr incubation to give a concentration of 6 mM. Basal respiration for the incubation period in the absence of cations was 11 without and 8 with theophylline, 1 mM, added at the end of the first hour of incubation. Fatty acid release due to theophylline without added cations was 105 μmoles/mmole of triglyceride and not significantly affected by any of the cations.

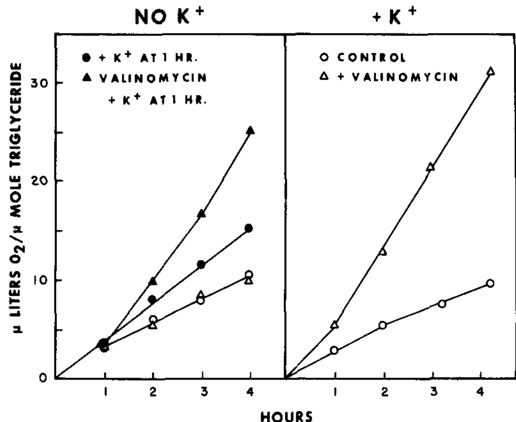

FIG. 8. K^+ requirement for stimulation of respiration by valinomycin. Brown fat cells (13 μmoles of triglyceride/flask) were incubated for 4 hr in phosphate buffer containing 4% albumin and 20 mM glucose. The values in the right part of the figure represent the means of seven experiments with cells incubated in 5.5 mM K^+ in the absence (open circles) and presence of 2 μg/ml of valinomycin (open triangles). The values in the left part of the figure are the means of four experiments with cells isolated and then incubated throughout the incubation period in the absence of K^+ without (open circles) and with 2 μg/ml of valinomycin (open triangles). The filled circles (without valinomycin) and filled triangles (with valinomycin) represent the effect of adding K^+ at 1 hr to give a concentration of 6 mM. Fatty acid release was not significantly different between any of the various experimental groups.

lipolysis by norepinephrine (Table I).

Effects similar to those of uncoupling agents were seen with oligomycin. Incubation of free brown fat cells for 2 min with oligomycin markedly reduced the stimulation of respiration seen with catecholamine (Fig. 5). If oligomycin was added after the catecholamine it had much less effect (Fig. 5).

The above results suggested that under basal conditions respiration is coupled to phosphorylation in intact brown fat cells. This conclusion was supported by the finding that uncoupling agents stimulated respiration in free brown fat cells but the increase was transient and largely occurred during the first 2 hr after their addition to free cells (2). Similar effects of uncouplers have been noted in free brown fat cells from hamsters (10). These results all suggest that in intact brown fat cells as in most other cells respiration is governed by the rate at which the energy produced during electron transport in the form of ATP or high-energy intermediates of oxidative phosphorylation is utilized. If this hypothesis is correct then the increased respiration due to lipolytic agents would be secondary to activation of some process which utilized large amounts of energy. Williamson et al. (11) reported that about 20-40 sec after epinephrine addition to brown fat cells there was an increase in cytochrome-b oxidation and flavoprotein reduction and these changes were accompanied by an increase in the ADP content of brown fat.

Several workers postulated that phosphorylation was uncoupled from respiration in brown fat based on in vitro studies with isolated mitochondria (12,13). Recently it was found that if brown fat mitochondria were incubated in the presence of defatted albumin they exhibited normal P/O ratios (14,15). We have defatted albumin by the procedure of Guillory and Racker (15), but did not find any marked differences in the metabolism of intact cells between this albumin and untreated albumin. Brown fat mitochondria may be readily uncoupled by fatty acids normally present in albumin preparations and thus differ from other mitochondria which are unaffected by albumin-bound fatty acids.

The available data suggest that respiration is coupled to phosphorylation under basal conditions. A major question is how to dispose of the energy derived from the large stimulation of respiration by catecholamines. If the increased

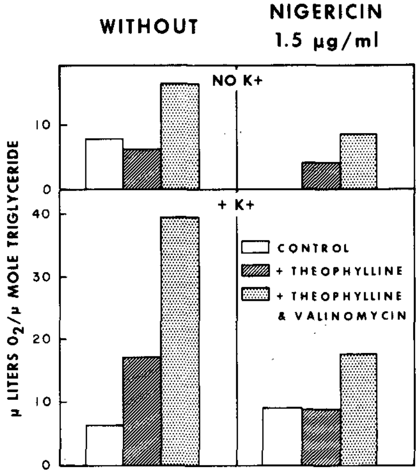

FIG. 9. Inhibitory effect of nigericin on stimulation of respiration by theophylline and valinomycin plus theophylline. Brown fat cells (10 μmoles of triglyceride/flask) were incubated for 4 hr in phosphate buffer containing 4% albumin and 20 mM glucose. The values in the upper half of the figure are for cells isolated and incubated without K^+ and are the means of three paired experiments. The values in the lower part of the figure are for cells incubated in 5.5 mM K^+, and represent the means of five paired experiments. Nigericin, 1.5 μg/ml, was added at the start of the incubation period and these values are shown in the right part of the figure. Valinomycin 2 μg/ml, was also added at the start of the incubation period while theophylline, 1 mM, was added at the end of the first hour of incubation. Respiration is shown as the amount of oxygen consumed over the 3 hr period from the second through fourth hours of incubation. The increase in fatty acid release due to theophylline was approximately 100 μmoles/mmole of triglyceride in the absence of K^+ and 60 in the presence of K^+ and was not significantly affected by either nigericin or valinomycin. Nigericin significantly inhibited ($P < 0.05$ by paired comparisons) the stimulation of respiration by theophylline and theophylline plus valinomycin in the presence of K^+ and of theophylline plus valinomycin in the absence of K^+.

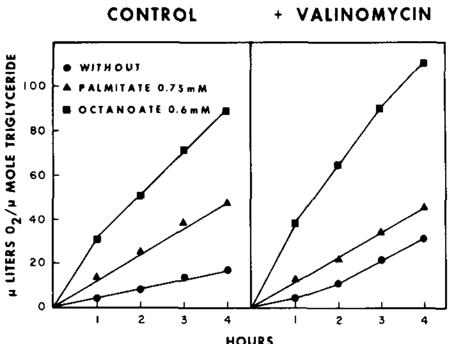

FIG. 10. Stimulation of respiration by palmitate and octanoate. Brown fat cells (10 μmoles of triglyceride per flask) were incubated for 4 hr in phosphate buffer containing 4% albumin and 20 mM glucose. The values in the left side of the figure are for cells incubated without while those in the right side are in the presence of 2 μg/ml of valinomycin and are the means of four paired experiments except for those in which fatty acids were added which are the means of three experiments. Sodium palmitate, 0.75 mM, and sodium octanoate, 0.6 mM, were added to the albumin buffer prior to addition of cells. There was no effect of either fatty acid on glycerol release. The stimulation of respiration by valinomycin alone or by added fatty acids was statistically significant ($P < 0.05$ by paired comparisons).

respiration is also coupled to phosphorylation then tremendous amounts of ATP will result. Ball (16) and Dawkins and Hull (17) originally proposed that in brown fat, as in white fat, this ATP is utilized for re-esterification of fatty acids. However, epinephrine had little effect on the re-esterification of fatty acids with α-glycero-phosphate derived from glucose metabolism (1). There is some direct phosphorylation of glycerol to give α-glycerophosphate, but this process is not affected by glucose or insulin in free brown fat cells (1), or by epinephrine in brown adipose tissue (18).

Prusiner et al. have suggested a system in which the ATP formed is degraded by the acyl CoA hydrolase via the formation of activated fatty acids (10). There is no evidence as yet that such a system could utilize enough ATP to provide enough phosphate acceptor to maintain the high rates of respiration.

A more attractive possibility is that fatty acids produced as a result of activation of triglyceride lipolysis by cyclic nucleotide mediate an uncoupling effect. This could be by a process such as ion transport which by utilizing higher energy intermediates of oxidative phosphorylation produces an apparent uncoupling effect. In the latter case the energy of oxidative phosphorylation is utilized but not in the form of ATP. Energy derived from oxidative phosphorylation appears to be required for activation of respiration and lipolysis by cyclic nucleotide but once the process is started it appears to be independent of ATP formation. This is based on the finding that oligomycin and uncouplers have little inhibitory action if added after the lipolytic agents.

The following section presents data which have led us to the conclusion that fatty acids produced during lipolysis alter the mitochondrial membrane resulting in an acceleration of a K^+ dependent process which utilizes large amounts of energy. This effect could be mediated by either increasing K^+ efflux from mitochondria resulting in a secondary activation of the energy-dependent K^+ transport process or conversion of the activated state of the K^+ transport carrier to an inactive state as

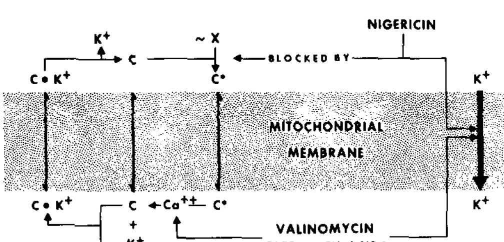

FIG. 11. A model for thermogenesis involving cyclic transport of K^+.

in the model of Lardy et al. (19) for K^+ transport in mitochondria.

The activation of respiration by catecholamines had an absolute dependence upon the presence of K^+ in the medium (Table II). In cells isolated, washed and then incubated in the absence of K^+ there was no stimulation of respiration by norepinephrine but fatty acid release was unaffected by omission of K^+ (Table II). These results indicated that K^+ is required for the activation of respiration by free fatty acids rather than for stimulation of lipolysis by catecholamines.

The lipolytic action of theophylline was the same in cells isolated, washed and then incubated in K^+-free buffer as in cells incubated with K^+. However, there was no stimulation of respiration by theophylline in the absence of K^+ (Fig. 6).

The addition of K^+, Rb^+, or Cs^+ with theophylline to brown fat cells isolated and then incubated for 1.5 hr in K^+ free buffer resulted in a marked increase in respiration (Fig. 7). The addition of NH_4^+ or theophylline alone to cells inhibited respiration. Fatty acid release was approximately the same regardless of whether or not cations had been added with the theophylline.

Valinomycin is a macrocyclic antibiotic which increases both K^+ accumulation by isolated mitochondria (19) and the permeability of artificial and natural lipid membranes to K^+ (20-22). The addition of valinomycin to cells incubated with K^+ (Fig. 8) produced a marked and lasting stimulation of respiration. In the absence of K^+ there was no stimulation of respiration by valinomycin (Fig. 8). However, if K^+ was added to cells isolated and then incubated for 1 hr without K^+ an increase in respiration due to valinomycin was readily detected (Fig. 8).

Nigericin is another antibiotic which increases the K^+ permeability of membranes but it blocks the stimulation of K^+ accumulation seen with valinomycin on isolated mito-

TABLE II

K^+ Requirement for Stimulation of Respiration by Norepinephrine[a]

	$-K^+$	$+K^+$ 5.5mM
Basal respiration in μl O_2/ μmoles triglyceride X 1 min	0.01	0.01
Respiration after addition of 1-norepinephrine, 2.5 μg/ml	0.02	0.50
Fatty acid release in μmoles/ mmole triglyceride X 5 min	77	62

[a]Free brown fat cells (8 μmoles triglyceride per flask) were incubated in 1.5 ml of buffer containing 4% albumin. The values are the means of four paired replications. Respiration was measured with an oxygen electrode and the rate in the presence of catecholamine was that for the second minute after addition of hormone.

chondria (18). Nigericin also blocked the stimulation of respiration by theophylline or valinomycin plus theophylline in intact brown fat cells (Fig. 9). Nigericin did not affect the lipolytic action of theophylline. There was no stimulatory effect of valinomycin alone in K^+ free buffer (Fig. 8) or of theophylline alone (Fig. 6,9) but there was a slight stimulatory effect when both agents were present and this was also blocked by nigericin. This is perhaps due to the possibility that some K^+ was present in the cells even though they had been isolated and washed with K^+ free buffer.

The respiration of cells incubated in albumin buffer containing 0.75 mM palmitate or 0.6 mM octanoate was much higher than in the absence of added fatty acid (Fig. 10). The fatty acids were added to the albumin solution as the sodium salts, but similar results have been seen after addition of the free acids. In all cases the pH of the buffer was adjusted, if necessary, to 7.4 after addition of the fatty acids. All the fatty acids could be readily bound to the primary binding sites on albumin since the molar ratio of fatty acid to albumin was kept between 1 and 2.

The stimulation of respiration by octanoate was dependent upon the presence of K^+ (Table III). We also investigated the effect of omission of divalent cations (Ca^{++} and Mg^{++}) during isolation, washing and incubation of brown fat cells. The omission of Mg^{++} had little effect on the stimulation of respiration due to octanoate but did decrease basal respiration (Table III). The omission of Ca^{++} partly inhibited the stimulation of respiration by octanoate and the effect of omission of both cations was similar to that of buffer from which only Ca^{++} was omitted (Table III).

The results with free brown fat cells suggest

TABLE III

Effect of Cation Omission on Stimulation of Respiration by Octanoate[a]

	Oxygen consumption μl/μmole Triglyceride		Glycerol release μmoles/mmole Triglyceride	
Cation omitted	Control	Difference due to sodium octanoate (0.6 mM)	Control	Difference due to sodium octanoate (0.6 mM)
None	12	+36±2	0	+5±12
Ca^{++} and Mg^{++}	7	+20±3	17	-2+4
Ca^{++}	17	+15±6	26	-18±7
Mg^{++}	4	+36±10	13	+6±3
K^+	6	-3±3	2	-4±2

[a]Brown fat cells (7.9 μmoles of triglyceride per flask) were incubated for 4 hr in buffer containing 4% albumin and 20 mM glucose. The difference due to sodium octanoate is the mean of four paired replications.

that oxidation is coupled to phosphorylation under basal conditions and that energy derived from this source is required for activation of lipolysis by cyclic nucleotide. The thermogenic action of catecholamines appears to be secondary to activation of lipolysis and mediated through an uncoupling action of free fatty acid liberated during hydrolysis of triglycerides. The uncoupling action of fatty acids appears to be due to increased K^+ flux across mitochondrial membranes.

A possible model for such a cyclic energy-dependent transport of K^+ is shown in Fig. 11, which is adapted from the hypothesis of Lardy et al. (19) for ion transport by isolated mitochondria. The symbol C represents a mobile carrier which is freely diffusible across the membrane. Net transport is accomplished by converting C in the matrix space to an active state C* via high energy intermediates ~X of oxidative phosphorylation. The C*, which does not bind potassium, diffuses through the membrane, and when converted to C binds K^+. Lardy et al. (19) have postulated that the rate at which C* is converted to C could be rate-limiting for K^+ uptake and is influenced by Ca^{++}. These investigators (19) suggested that activation of this process by valinomycin could account for its stimulation of mitochondrial K^+ uptake. Nigericin is postulated to block conversion of C to C* (19). In addition, both antibiotics increase the passive permeability of mitochondrial (20,21) and other lipid membranes (21,22) to K^+. The model shown in Fig. 11 postulates that free fatty acids accelerate efflux of K^+ from the mitochondria via a process in which energy is not conserved and which facilitates the conversion of C* to C. The decreased ratio of mitochondrial to cytoplasmic K^+ and the increased availability of carrier (C) results in increased K^+ uptake. The net result is increased utilization of high-energy intermediates of oxidative phosphorylation for K^+ transport.

In the interval since submission of this manuscript we have published more complete reports on the role of K^+ in the stimulation of brown fat cell respiration (23) and the effects of oligomycin on brown fat cell metabolism (24).

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Some Aspects of Fatty Acid Metabolism in Brown Adipose Tissue[1]

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ABSTRACT

The action of catecholamines on white and brown adipose tissue is compared. The available evidence indicates that lipolysis is initiated in both tissues by hormonal action. There are, however, some differences in the behavior of the lipolytic process in the two tissues in their response to theophylline, nicotinic acid and insulin which remain unexplained. It would appear that the release of free fatty acids triggers the stimulation of O_2 consumption in both tissues. In brown adipose tissue no evidence has been obtained to indicate that the increased O_2 consumption is geared to the production of ATP for the purpose of reesterification of the released free fatty acids, as is the case in white adipose tissue.

INTRODUCTION

The addition of catecholamines to white or brown adipose tissue incubated in vitro results in a prompt and marked increase in O_2 consumption (1-3). Our knowledge of the sequence of events that gives rise to this effect in white adipose tissue (WAT) is reasonably complete. In brown adipose tissue (BAT) the situation is less clear. It is the purpose of this paper to examine the process in BAT in the light of what is already known about these processes in WAT.

The action of catecholamines on WAT appears to be brought about by their ability to stimulate the production of 3',5'-cyclic AMP. This substance in turn is believed to convert an inactive triglyceride lipase to an active form (Fig. 1) which results in the breakdown of the tissue triglyceride stores to fatty acids and glycerol. Evidence in support of this chain of events is provided by the finding that after the addition of catecholamines to WAT there is an increased production of free fatty acids (FFA) and glycerol (4,5) and a concomitant rise in the level of cyclic AMP in the tissue (6). A sustained and marked increase in O_2 consumption will also occur if the tissue is able to produce glycerophosphate for the reesterification of the released FFA (7,8).

In considering the action of catecholamines on BAT the first question that may be asked is whether cyclic AMP is involved. To the best of my knowledge no one has as yet measured the levels of cyclic AMP in this tissue before and after the addition of catecholamines. The fact that theophylline, which inhibits the destruction of cyclic AMP (Fig. 1), mimics the action of epinephrine on BAT suggests the involvement of cyclic AMP. As shown in Table I, 10^{-3}M theophylline produces a nearly threefold increase in the O_2 consumption of BAT slices and no further increase occurs upon the addition of epinephrine. By contrast this concentration of theophylline has a very small effect upon the O_2 consumption of WAT (Table II), but the subsequent addition of epinephrine results in a maximum response. Fain and Reed have reported elsewhere in this symposium that both theophylline and the dibutyryl derivative of cyclic AMP mimic the action of catecholamines upon isolated brown adipose tissue cells. The evidence presently available thus suggests that the formation of cyclic AMP may be involved as the initial step in both BAT and WAT.

The available evidence also indicates that in BAT as in WAT the addition of catecholamines in vitro stimulates lipolysis. Joel (2) showed that there was a concomitant rise in O_2 consumption and FFA release in rat BAT slices as the concentration of the catecholamine in the media was increased over the range 0.01 to 1.0 μg/ml. Dawkins and Hull (9) measured both FFA and glycerol release from BAT slices of both adult and newborn rabbits in the presence and absence of norepinephrine (2.5 μg/ml). The addition of the hormone produced a marked increase in the production of both products of lipolysis, the rise in glycerol production being most pronounced in tissue from the newborn. Fain et al. (10) have extended the earlier findings of these workers to include isolated rat BAT cells. In connection with glycerol release from BAT it should be noted that a species difference may exist due to variations in the occurrence of glycerolkinase. Glycerolkinase

[1]One of nine papers to be published from the Symposium "Brown Adipose Tissue," presented at the AOCS-AACC Joint Meeting, Washington, D.C., March 1968.

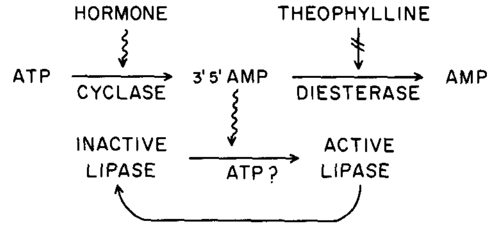

FIG. 1. Schematic representation of the events involved in the hormonal activation of lipolysis in white adipose tissue.

has been reported to be present in rat BAT (11) but absent in BAT of the newborn rabbit (9).

Though lipolysis would appear to be initiated in both WAT and BAT by catecholamines acting through cyclic AMP there is evidence that the processes involved may not be exactly identical in the two tissues. The difference in the action of theophylline, already cited, is one case in point. Another is in the action of agents which display antilipolytic properties on WAT. For example, nicotinic acid, which was demonstrated to have antilipolytic action on WAT by Carlson (12), displays different actions on the O_2 consumption of BAT and WAT. As shown in Table III, nicotinic acid at 10^{-3}M has no effect upon the increase in respiration elicited in BAT by the addition of epinephrine. In WAT, nicotinic acid markedly depresses the respiratory response to epinephrine even at concentrations of 10^{-5}M. As shown in Table II this inhibitory effect of nicotinic acid on WAT may be overcome if theophylline is also present. This suggests, though it does not prove, that the antilipolytic action of nicotinic acid on WAT is a result of lowered levels of cyclic AMP in the tissue. It has been suggested that nicotinic acid exerts its antilipolytic action by enhancing the activity of the phosphodiesterase which catalyses the hydrolysis of cyclic AMP (13). The action of theophylline in counteracting nicotinic acid might thus represent its successful competition with nicotinic acid for a common site on the enzyme.

Insulin is also able to exert an antilipolytic action on WAT when glycose is not present in the medium (14). The lipolytic response of this tissue to 0.1 μg/ml of epinephrine is 90% prevented by insulin. As the epinephrine concentration is increased the antilipolytic action of insulin diminishes and it disappears entirely even at low concentrations of epinephrine if glucose is added to the medium. If the magnitude of the effect of insulin on lipolysis in BAT was the same as in WAT then one might expect insulin to exert an appreciable inhibitory effect on the stimulation of oxygen consumption of BAT, which is elicited by low concentrations of epinephrine in the absence of glucose. As the data presented in Table I show, insulin with or without glucose has no pronounced effect upon the O_2 consumption of rat BAT either in the absence or presence of epinephrine. Dawkins and Hull (9) have reported that insulin has no effect on the unstimulated respiration of newborn rabbit BAT in the presence of glucose. However, they did observe a significant increase in respiration when insulin was added to tissue stimulated by norepinephrine in the presence of glucose.

We may now turn to the question of the cause of the increased respiration elicited in BAT by catecholamines. If the process is similar to that seen in WAT, most of the released FFA should be undergoing reesterification to triglyceride. One way of demonstrating this reesterification in WAT is to measure the incorporation of ^{14}C labeled glucose into the glyceride-glycerol moiety (8). When experiments are run with rat BAT slices in the presence of U-^{14}C-glucose it is found (15) that no increase in the labeling of the glyceride-glycerol fraction

TABLE I

Oxygen Consumption of Rat Brown Adipose Tissue Slices As Influenced By Insulin, Glucose and Theophylline in the Presence and Absence of Epinephrine

Insulin	Glucose	Theophylline	Before[a] μl O_2/100 mg/hr	After[a] μl O_2/100 mg/hr	Increase %
--	--	--	191 ± 24.	812 ± 104.	325
+	--	--	222 ± 27.	828 ± 38.	273
+	+	--	226 ± 21.	865 ± 32.	282
--	--	+	650 ± 82.	589 ± 149.	-9.

[a]Before and after the addition of epinephrine to yield a final concentration of 0.1 μg/ml. Values are the average of 4 experiments ± standard error of the mean. Krebs-Ringer phosphate, pH 7.4; temperature 37.2 C; gas phase O_2; insulin 0.1 unit/ml; glucose 3 mg/ml; theophylline 10^{-3}M. Other experimental details are the same as described by Hayward and Ball (3).

TABLE II

Action of Theophylline and Nicotinic Acid on White Adipose Tissue in the Presence and Absence of Epinephrine

Nicotinic acid	Theo-phylline	O_2 Consumption Before[a] μl/100 mg/hr	O_2 Consumption After[a] μl/100 mg/hr	Terminal tissue FFA μmole/100 mg/hr	Terminal medium glycerol μmole/100 mg/hr
--	--	21	55	0.141	1.33
--	+	25	61	2.56	1.23
+	--	19	23	0.132	0.25
+	+	21	52	1.85	1.15

[a]Before and after addition of epinephrine to yield 0.1 μg/ml. Values for O_2 consumption are the average of three experiments; FFA and glycerol values are for one experiment. Nicotinic acid concentration was 5 x 10^{-4}M; theophylline, 10^{-3}M. All other experimental conditions were as described in Table I.

accompanies the marked oxygen consumption elicited by the addition of epinephrine. Such a result in rat BAT might, however, be attributed to the fact that since this tissue contains glycerolkinase the glyceride-glycerol moiety is derived from the glycerol released during lipolysis. Experiments were therefore performed in which labeled glycerol was added to the medium. Again no significant labeling of the glyceride-glycerol of the tissue occurred as a result of epinephrine addition.

In view of these negative results on intact tissue Kornacker and Ball (15) investigated the behavior of rat BAT homogenates as made with 0.25 M sucrose. The O_2 consumption of such homogenates is low and it is not increased by the addition of catecholamines. A 25-fold increase in O_2 consumption occurs, however, if ATP, carnitine, fumarate and coenzyme A are added to the homogenate. The rate of respiration thus achieved is comparable to that observed after the addition of epinephrine to tissue slices. The addition of any one of these cofactors alone is without effect. The nature of the cofactors and the fact that the R.Q. of the reaction they stimulate is less than 0.7 support the conclusion that fatty acids are being burned.

This conclusion is further supported by the data of Kornacker and Ball (15), which show that when trace amounts of 1-^{14}C- palmitate are added to the homogenate the addition of cofactors markedly stimulates the production of $^{14}CO_2$. Their experiments also indicate that no increase in incorporation of labeled palmitate into triglyceride accompanies the marked increase in respiration produced by addition of cofactors. If α-glycerophosphate is also added to the incubation media then an enhanced incorporation of labeled palmitate into triglyceride may be observed. This enhancement is most pronounced when cofactors are also present, but even then the amount of FFA reesterified is only about 0.3 of the amount converted to CO_2. It may be calculated from the date of Fisher and Ball (8) that in WAT during the increased O_2 consumption produced by catecholamines the amount of FFA reesteri-

TABLE III

Oxygen Consumption of White and Brown Adipose Tissue as Influenced by Epinephrine in the Absence and Presence of Nicotinic Acid[a]

Nicotinic acid conc. molar	White adipose[a] Before[b]	White adipose[a] After[b]	White adipose[a] Increase, %	Brown adipose[a] Before[b]	Brown adipose[a] After	Brown adipose[a] Increase, %
0	25 ± 8.9	72 ± 5.5	188	255 ± 22.	822 ± 13.	222
10^{-3}	27 ± 3.8	34 ± 2.9	26	262 ± 28.	837± 19.	220
10^{-4}	27 ± 3.9	37 ± 5.3	37			
10^{-5}	26 ± 3.2	37 ± 4.3	42			

[a]μl O_2/100 mg/hr.

[b]Before and after addition of epinephrine to yield 0.1 μg/ml. Values are the average of five experiments ± standard error of the mean. Insulin 0.1 unit/ml, and glucose 3 mg/ml present in medium. All other experimental details were as described in Table I.

fied is 46 times the amount of FFA that can be converted to CO_2.

In summary we may conclude that certain similarities exist in the process whereby lipolysis is initiated by catecholamines in WAT and BAT. There are, however, some differences in the behavior of the two tissues in their response to theophylline, nicotinic acid and insulin that remain unexplained. It would appear also that the release of FFA is the event which triggers a stimulation of O_2 consumption in both tissues. In WAT this stimulation reflects primarily the utilization of ATP for the purpose of reesterification of the released FFA. In BAT no evidence has been obtained that a similar demand for ATP triggers O_2 consumption. Failure in this regard, however, does not permit one to categorically rule out the involvement of an active reesterification process in BAT. For example, the possibility exists in BAT that reesterification proceeds mainly at the mono- or diglyceride stage in which case no turnover of the glyceride-glycerol moiety could be detected. Furthermore, it is possible that only a very small pool of the total triglyceride undergoes a rapid cyclic process of lipolysis and resynthesis.

ACKNOWLEDGMENT

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Effects of Insulin and Norepinephrine Added in Vitro on the Metabolism of Brown Adipose Tissue in the Absence of Added Glucose[1]

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ABSTRACT

Rat interscapular brown adipose tissue slices incubated with succinate as substrate can be stimulated by norepinephrine to exhibit a rate of oxygen consumption that appears to exceed by a wide margin that reported for any other rat tissue under any conditions in vitro. This further supports the concept of brown adipose tissue as a specialized site of heat production. Brown adipose tissue slices under maximal stimulation by norepinephrine and incubated in the absence of added substrate consume oxygen linearly and at a high rate for at least 14 hr. This is taken as further evidence that stored lipid can act as the major fuel to support the vigorous oxidative metabolism of which this tissue is capable. This unusually stable preparation appears to be attractive as a system for the study of relatively long-term effects of hormones in vitro. Brown resembles white adipose tissue in that insulin exerts an antilipolytic effect on both types of tissue in Krebs-Ringer phosphate buffer in the absence of added glucose. On the other hand, insulin stimulates the oxygen consumption of brown but not white adipose tissue under these conditions. A procedure is described for the rapid and nearly quantitative removal of the interscapular brown adipose tissue from a rat, virtually free of contamination by muscle or white adipose tissue.

INTRODUCTION

Considerable interest has recently been focused upon brown adipose tissue due to the discovery that it serves a physiological role as a specialized site of heat production (1-3). It has been established that heat production by this tissue is of major quantitative importance in relation to the total body economy in the case of the newborn rabbit (3) and in the case of the ground squirrel during acute arousal from hibernation (2). The present communication represents one of a series of studies from this laboratory in which the metabolic properties of brown adipose tissue have been elucidated and also compared with the more commonly studied white adipose tissue (3,4-6).

Most studies of the metabolism of white adipose tissue in vitro have been carried out in the presence of glucose as the added substrate. It has been shown, however, that white adipose tissue retains many aspects of its high sensitivity to hormones added in vitro even when the incubations are carried out in the absence of added glucose (7). In the present study it is demonstrated that brown adipose tissue responds both to insulin and to catecholamines added in vitro in the absence of added glucose. In certain instances the responses of brown adipose tissue under these conditions are shown to differ radically from those normally observed with white adipose tissue under the same conditions. In addition, it is shown that the rate of oxygen consumption of brown adipose tissue under stimulation by norepinephrine added in vitro with succinate as substrate appears to exceed that reported for any other tissue of the rat under any set of conditions in vitro. Finally, slices of brown adipose tissue under stimulation by norepinephrine in vitro are seen to be a very stable preparation, consuming oxygen in a nearly linear fashion for at least 14 hr, even in the absence of added substrate.

Portions of this work have been described previously in brief form (2,4).

MATERIALS AND METHODS

Male Holtzman rats were maintained at a room temperature of 22-27 C with a uniform daily cycle of artificial lighting and were allowed water and Purina laboratory chow ad libitum. The animals were decapitated by means of a guillotine (Harvard Apparatus Company) with caution taken not to subject the animals to stress beforehand (8). The brown adipose tissue was removed and sliced as rapidly as possible.

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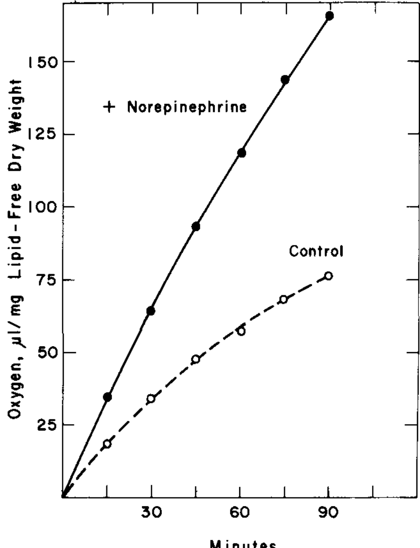

FIG. 1. Oxygen consumption by brown adipose tissue slices with succinate as substrate. The main chamber of the Warburg flasks contained 16-22.5 mg fresh tissue suspended in 0.01 M sodium succinate in Krebs-Ringer phosphate buffer. The vessels were gassed with 100% oxygen for 5 min at 37 C, norepinephrine was then dumped from the sidearm, and readings were begun after an 8 min equilibration period. The final norepinephrine concentration was 0.92 μg/ml. Each curve represents the mean of two incubations. The lipid-free dry weight was determined on portions of the same interscapular brown fat pads as were used in the incubations and was found to have a mean value of 12.2 mg/100 mg fresh tissue in the two 160 g rats used for the experiment shown in this Figure.

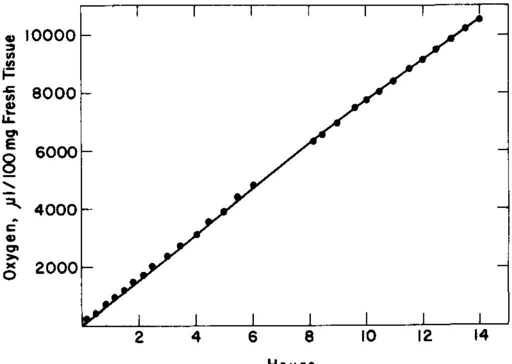

FIG. 2. Incubation of brown adipose tissue slices for 14 hr in the presence of 10 μg norepinephrine per milliliter and in the absence of added substrate. The Warburg vessel contained 39 mg fresh tissue, taken from a 180 g rat. After 9 hr of incubation the vessel was opened, and 0.1 ml saturated aqueous NaOH was pipetted onto the filter paper wick in the center well in order to ensure continued efficient absorption of carbon dioxide. The vessel was then gassed with 100% oxygen and the incubation was continued.

The following dissection procedure was employed for removal of the interscapular brown adipose tissue from the rat. The procedure is designed for the removal of almost the entire interscapular brown fat pad with minimal amounts of elapsed time, mechanical damage to the tissue, and contamination by the surrounding muscle and white adipose tissue. The rat was pinned to the table top with its forelegs crossed beneath the body in order to exert a lateral stretch on the interscapular region. After cutting and taking back the overlying skin, the entire symmetrical brown fat pad was lifted by grasping the overlying white adipose tissue with a pair of forceps, and the brown fat pad was partially separated from the muscle lying directly below by cutting through the appropriate connective tissue with a small pair of scissors. The right and left brown fat pads were separated from one another by cutting through the white adipose tissue, connective tissue and blood vessels between them. The remainder of the dissection was carried out on one side at a time. The overlying white adipose tissue was removed by grasping it with the forceps and cutting it away with repeated small cuts. The brown fat was then separated from the adhering muscle around which it is wrapped, by making repeated small cuts between the muscle and the brown fat while either grasping the muscle firmly or holding the brown adipose tissue gently with the forceps. Finally, any last remaining traces of contaminating muscle or white adipose tissue were trimmed away from the brown adipose tissue sample. The tissue was kept moist during the dissection. As judged by gross inspection, the contamination by muscle and grossly visible white adipose tissue was less than 5%. The recovery of brown adipose tissue was nearly quantitative. The entire dissection of both right and left brown fat pads required approximately 3 min.

Except as otherwise indicated, the weighing of the interscapular brown adipose tissue and the epididymal white adipose tissue, the preparation of brown fat slices, the composition of the incubation media and gas phase, the preparation of hormone solutions, the analytical methods, and other experimental conditions were the same as those previously described (5). The trypsin-treated amorphous pork insulin was assayed by the suppliers at 20 units of insulin/mg and was reported to contain no more than 0.005% glucagon. Sodium pyruvate and sodium succinate were from Calbiochem. Glycerol was determined by a slight modifi-

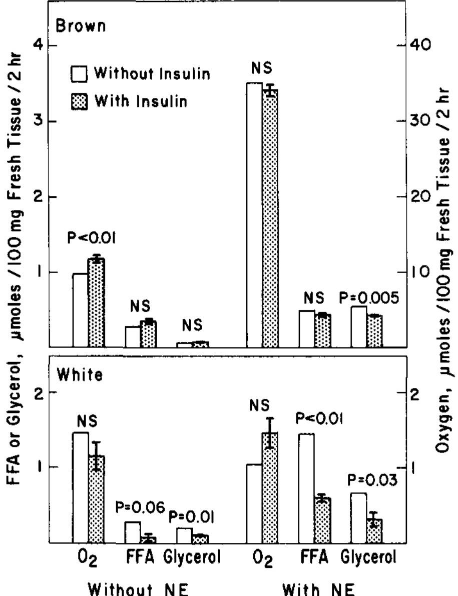

FIG. 3. Effects of insulin and norepinephrine on lipolysis and oxygen consumption in the absence of added glucose. Intact epididymal white adipose tissue pads and slices of brown adipose tissue were used from the same 170-335 g rats. Brown fat slices weighed approximately 2 mg each. After a 5 min gassing period, norepinephrine and insulin or control solutions were dumped together from the sidearm. Then after an 8 min equilibration period readings of oxygen consumption were begun. The final concentration of insulin was 10^{-3} unit per milliliter in the case of white and 10^{-2} in the case of brown adipose tissue. With white adipose tissue, the final concentration of norepinephrine was 0.05 μg/ml in half of the instances and 0.1 in the other half. The final concentration of norepinephrine with brown adipose tissue was always 0.1 μg/ml. There were four replicates of each of the four types of vessels in the case of white adipose tissue. In the case of brown adipose tissue, 12 pairs of vessels were run without norepinephrine and 9 pairs with norepinephrine. Statistical evaluation of insulin effects was by the paired t-test. Standard errors of the differences due to insulin are shown in the Figure. The P values shown are for insulin effects. NS means $P > 0.1$. The P for the effect of insulin on FFA accumulation in the absence of norepinephrine was 0.05 when calculated by groups rather than by pairs. Attention is called to the fact that the scale for oxygen consumption by brown adipose tissue has been compressed by a factor of 10, which further points out the great emphasis on oxidative metabolism that characterizes brown adipose tissue. The open bars represent controls and the darker bars represent vessels containing insulin.

cation of the method of Wieland and Suyter (9), following heating of the aliquots of incubation media in order to destroy the activity of any enzymes that may have leaked from the tissue slices. FFA in incubation media were determined by the method of Dole and Meinertz (10), and FFA in incubated tissues were assayed by a slight modification of the method of Trout, et al. (11).

Due to the high rate of oxygen consumption of brown adipose tissue, it is mandatory to use 100% oxygen or 95% oxygen-5% CO_2 in incubations with slices. For example, the rate of oxygen consumption in the presence of 0.02 M glucose was approximately two times higher in 100% oxygen than in air, and when the tissue was stimulated with epinephrine (2 μg/ml) plus insulin (0.1 unit/ml), the rate was 3.5 times higher in 100% oxygen than in air.

The most satisfactory method of preparing slices of brown adipose tissue was to cut them free hand with a razor blade or scissors. Fragments prepared in this manner, weighing up to 5 mg each, were found to be satisfactory for most purposes. On the other hand, slices of brown adipose tissue prepared by means of a Stadie-Riggs apparatus, set to give a slice thickness of 0.5 mm, yielded relatively low and variable rates of oxygen consumption either in the presence or absence of added catecholamines and proved to be much less satisfactory than those prepared free hand by means of a razor blade or scissors.

The rate of oxygen consumption was negligible when brown adipose tissue that had been heated to 100 C for 30 sec was sliced and incubated under the above conditions either in the absence of added hormones or in the presence of epinephrine at 10 μg/ml.

RESULTS

Oxygen Consumption With Succinate As Substrate

Figure 1 shows the results of incubation of brown adipose tissue slices with succinate as substrate. Even in the absence of added norepinephrine the rate of oxygen consumption with succinate as substrate is much higher than rates we have previously reported for brown adipose tissue under similar conditions using glucose as substrate (4,5). Hook and Barron, using the ground squirrel, long ago reported a marked stimulation of the oxygen consumption of brown adipose tissue slices by succinate (12).

As is shown in Figure 1, the oxygen consumption in the presence of succinate was markedly stimulated by addition of norepinephrine in vitro. The rate of respiration gradually diminished to a small extent throughout the incubation period. In a total of five experiments similar to that illustrated in Figure 1, it was found that the mean rate of oxygen consumption measured over the first 30 min after the equilibration period in the presence of succinate and norepinephrine was

120 ± 6 µl/mg lipid-free dry weight per hour, or 1370 ± 70 µl/100 mg fresh tissue per hour. In our hands, no other conditions have given as high a rate of oxygen consumption by brown adipose tissue slices as those employed for the experiments illustrated in Figure 1. In these experiments, the slices were cut with a razor blade and had an average weight of approximately 1 mg. Use of scissors rather than a razor blade or use of 0.2 mg or 3-5 mg instead of 1 mg slices resulted in slightly but significantly lower respiration rates.

Prolonged Incubations

The question arose as to how long the tissue slices might respire in the absence of added substrate at their characteristically high rate under maximal stimulation by catecholamines, therefore experiments of the type illustrated in Figure 2 were performed.

Slices of brown adipose tissue were incubated in the absence of added substrate but in the presence of norepinephrine at 10 µg/ml. In previous unpublished experiments in this laboratory it had been observed that in the presence of such a high concentration of catecholamine and in the absence of added substrate or bovine serum albumin, the rate of oxygen consumption of brown adipose tissue slices would eventually decline markedly, apparently due to the accumulation of toxic levels of free fatty acids (FFA) within the tissue, reminiscent of a similar situation seen in white adipose tissue, first reported by Hagen and Ball (13). Therefore bovine serum albumin was added at a concentration of 50 mg/ml in order to allow the liberated FFA to leave the tissue slices and not accumulate in toxic concentrations. Penicillin was present in the incubation medium at a concentration of 0.6 mg (1000 units)/ml in order to inhibit bacterial growth.

It is clear from Figure 2 that brown adipose tissue slices under maximal stimulation by norepinephrine under these conditions can consume oxygen at a very high rate which remains stable and nearly undiminished for at least 14 hr. The respiration was in all likelihood due entirely to the brown adipose tissue itself and not to bacterial contamination, since no viable bacterial colonies could be detected when aliquots of the incubation medium taken at the end of the 14 hr period were plated. The linear time course of the respiration is a further indication of the lack of significant bacterial contamination. FFA release was not measured in this experiment, but in another incubation carried out for 9 hr under similar conditions a total of 18 µmoles FFA were released per 100 mg fresh tissue per hour, which is the same rate previously reported by us for much briefer incubations under these conditions (5) and suggests that the FFA release, just as is the case with oxygen consumption, remains stable over a prolonged period in brown adipose tissue slices in vitro under these conditions.

Effects of Insulin With No Added Substrate

It is well known that insulin can promote the conversion of glucose to fatty acids in adipose tissue. Insulin has also been shown to have certain effects on the metabolism of white adipose tissue in vitro in the absence of added glucose. Specifically, Jungas and Ball have shown that when glucose is absent, insulin inhibits lipolysis in white adipose tissue either in the presence or absence of added catecholamines (7). The experiments shown in Figure 3 were carried out to determine whether or not this effect could also be observed in brown adipose tissue. Intact epididymal white adipose tissue pads and slices of interscapular brown adipose tissue from the same rats were incubated simultaneously in a medium composed of Krebs-Ringer phosphate buffer containing no glucose or serum albumin. Since albumin was absent, FFA could not leave the tissues and therefore the FFA measurements represent accumulation of FFA in the tissue and not in the incubation medium. The glycerol measurements represent glycerol in the medium. Except as noted, oxygen consumption was essentially linear throughout the 2 hr incubations, and it appeared that the norepinephrine concentrations employed were low enough and the incubation time short enough to avoid poisoning of respiration due to accumulation of large amounts of FFA within the tissues.

It can be seen from the results shown in Figure 3 that the antilipolytic effect of insulin reported by Jungas and Ball could be repeated, using 0.05 or 0.1µg norepinephrine per milliliter and 10^{-3} units insulin per milliliter, although the inhibition of FFA accumulation due to insulin in the absence of norepinephrine reached only borderline statistical significance in the four pairs of incubations that were performed. On the other hand, in experiments with brown adipose tissue not shown in Figure 3, no antilipolytic effects of insulin could be demonstrated either in the presence or absence of 0.1 µg norepinephrine per milliliter, when insulin was used at this same concentration of 10^{-3} units per milliliter, regardless of whether the insulin was dumped from the sidearm at the same time as norepinephrine or 28 min before addition of norepinephrine.

When the insulin concentration used with

brown adipose tissue was raised to 10^{-2} units per milliliter, the positive results shown in Figure 3 were obtained. No antilipolytic effect of insulin was observed in the absence of added norepinephrine, but in the presence of 0.1 μg norepinephrine per milliliter there was a statistically highly significant suppression of glycerol release by insulin. The simplest interpretation of this inhibition of glycerol release is that it indicates an antilipolytic effect of insulin in brown adipose tissue slices.

Several additional incubations were carried out with brown adipose tissue, using 0.05 rather than 0.1 μg norepinephrine per milliliter. These experiments are not shown in Figure 3. At first glance these experiments appeared to be showing an inhibition of oxygen consumption by 10^{-2} units insulin per milliliter, but inspection of the time-course of the oxygen consumption showed that the difference was due to the fact that the stimulatory effect of 0.05 μg norepinephrine per milliliter on oxygen consumption simply did not last as long as in the presence as in the absence of insulin. Perhaps norepinephrine autoxidizes more rapidly when insulin is present. On the other hand, as indicated above for the experiments illustrated in Figure 3, the oxygen consumption was essentially linear throughout the 2 hr incubation in the presence or absence of 0.1 μg norepinephrine per milliliter.

An unexpected effect of 10^{-2} units insulin per milliliter on the oxygen consumption of brown adipose tissue slices was observed and is illustrated in Figure 3. It can be seen that in the absence of norepinephrine there was a statistically highly significant 20% stimulation of oxygen consumption by insulin. This effect could not be demonstrated with 10^{-3} units insulin per milliliter. This effect was clearly not occurring in white adipose tissue in the same experiments and therefore represents a fundamental difference between brown and white adipose tissue.

In the Krebs-Ringer phosphate buffer used in these experiments, the stimulation of oxygen consumption of brown adipose tissue slices by insulin was seen only in the absence of added glucose. For example, in the presence of 0.01 M glucose, six samples containing 10^{-2} units insulin per milliliter consumed 0.1 ± 0.8 μmoles less oxygen per 100 mg fresh tissue per 2 hr than did their paired controls to which no insulin was added.

DISCUSSION

High Rate of Oxygen Consumption in Brown Adipose Tissue Slices

The rate of oxygen consumption by brown adipose tissue in the type of experiments illustrated in Figure 1 is exceedingly high for a mammalian tissue. The observed rate of 120 μl oxygen per milligram lipid-free dry weight per hour appears to be considerably in excess of those reported from other laboratories for any other rat tissue under any conditions in vitro, including perfused systems (2). Comparable rates of oxygen consumption have been reported for slices of bat brown adipose tissue (14), free-floating isolated brown fat cells (15), and homogenates of brown adipose tissue from the newborn rabbit (16). It appears that brown adipose tissue respires at an even higher rate in vivo (17). This capacity for exceedingly vigorous oxidative metabolism correlates well with the high contents of mitochondria (18-20) and cytochromes (21) that characterize this tissue and adds further support to other lines of evidence for a physiological role of this tissue as a specialized site of heat production of major quantitative importance.

Prolonged Incubations

Since the 14 hr incubation of brown adipose tissue shown in Figure 2 was carried out in the absence of added substrate and since the glycogen content (2) of brown adipose tissue from rats under the dietary conditions employed in this laboratory is at least an order of magnitude too low to account for the total oxygen consumption, it is necessary to conclude that the major fuel supporting the high and sustained rate of oxygen consumption under norepinephrine stimulation must have been lipid. It can readily be calculated that at least 10% of the entire lipid store of the tissue must have been oxidized during the 14 hr incubation period. It can also be calculated that approximately the same number of fatty acids are being oxidized as are being released from the tissue as FFA, and this serves to illustrate further the strong emphasis on oxidative processes in brown as compared to white adipose tissue.

The high rate of fatty acid oxidation seen in brown adipose tissue correlates well with the unusually high content of carnitine reported to be present in this tissue (22) and offers strong support for the postulated role of carnitine in fatty acid oxidation. In this connection, carnitine has recently been shown to be one of several cofactors required for maximal rates of oxygen consumption in brown adipose tissue homogenates (23).

It has been shown that in white adipose tissue, exogenous glucose supplies α-glycerophosphate for the reesterification of FFA, which in turn acts as an ATP-ase to support the accelerated rate of oxygen consumption seen

when this tissue is under stimulation by a combination of catecholamines and insulin (24). On the other hand, it is clear from the experiment with brown adipose tissue shown in Figure 2 that oxygen consumption can proceed at a very high rate in the absence of any exogenous precursor for α-glycerophosphate. Likewise, there is not enough glycogen in brown adipose tissue (2) for an adequate supply of α-glycerophosphate. Therefore it must be concluded that (a) there is another source of α-glycerophosphate such as the action of glycerol kinase on freshly liberated glycerol (since glycerol readily permeates cell membranes and would probably rapidly leak out of the slices into the medium, this seems unlikely as an adequate explanation), or (b) that lipolysis proceeds only to the stage of mono- and di- glycerides, or (c) that the reesterification scheme simply does not operate in brown adipose tissue as the major factor allowing the high rate of oxygen consumption to proceed. Although isolated mitochondria of brown adipose tissue have recently been shown to be capable of coupled oxidative phosphorylation (25-27), it may be that during norepinephrine stimulation of oxygen consumption the mitochondria become uncoupled in order to allow respiration and its concommitant heat production to proceed at a high rate. This matter is discussed in further detail in other papers presented in the present symposium.

The striking stability of the oxygen consumption seen in the 14 hr experiment illustrated in Figure 2 suggests that brown adipose tissue offers an excellent system for the study of relatively long-term effects of hormones in vitro, such as the induction of the biosynthesis of new enzymes. We have not attempted incubation times longer than 14 hr. It has been previously shown that the epididymal white adipose tissue pad is also a very stable system in vitro over a similarly long period of time (28).

Inhibition of Lipolysis by Insulin

Although a clear antilipolytic effect of insulin on brown adipose tissue slices appears to have been demonstrated by the experiments illustrated in Figure 3, it should be pointed out that the concentration of insulin required in the Krebs-Ringer phosphate buffer in order to demonstrate the effect was relatively high and well above the physiological range, namely 10^{-2} units per milliliter. A much lower concentration of insulin was sufficient to demonstrate the stimulation of fatty acid synthesis from added glucose by insulin in brown adipose tissue slices from similar rats, using Krebs-Ringer bicarbonate buffer (6). Likewise, a much lower concentration of insulin will still exhibit an antilipolytic effect in white adipose tissue (7). Following completion of the experiments shown in Figure 3, Fain et al. showed that insulin at very low concentrations can inhibit lipolysis in isolated free-floating brown fat cells (15). It seems reasonable to conclude that brown and white adipose tissue are qualitatively similar insofar as both types of tissue exhibit the antilipolytic effect of insulin.

Stimulation of Oxygen Consumption by Insulin

The stimulation of oxygen consumption in brown adipose tissue slices in the absence of added glucose, shown in Figure 3, has pharmacological interest but questionable physiological significance, not only because of the high concentration of insulin required in order to demonstrate the effect but also because the stimulation is not observed when glucose is present. The effect is not due to contamination by glucagon. The insulin preparation has been assayed for glucagon by the supplier and would have yielded a glucagon concentration in the incubation medium of no more than 2.5×10^{-5} μg/ml, which is much too low to affect oxygen consumption in brown adipose tissue slices (5). Reports of the stimulation of oxygen consumption of tissue preparations by insulin in vitro in the absence of added substrate are rare (29,30).

Whether or not insulin will stimulate or inhibit oxygen consumption in rat brown adipose tissue slices depends upon the nature of the incubation buffer, the presence or absence of glucose, and the previous history of the rat. For example, as noted above, insulin in the absence of added norepinephrine stimulates oxygen consumption by 20% in Krebs-Ringer phosphate buffer in the absence of added glucose. If on the other hand the incubation is carried out in Krebs-Ringer bicarbonate buffer (which contains the bicarbonate required for fatty acid biosynthesis) in the presence of glucose, insulin exerts a strong stimulation of oxygen consumption, to the extent of approximately 50% (6). If norepinephrine is present in the glucose-containing Krebs-Ringer phosphate buffer, insulin exerts a stimulation of oxygen consumption if normal rats are used as the source of the brown adipose tissue slices (4), but if the incubation is carried out under similar conditions using rats that have been cold-adapted for several weeks and then placed at room temperature for 24 hr before killing, then insulin exerts a marked inhibition of oxygen consumption (C. D. Joel, unpublished experiments). The basis for these differences in insulin effects remains to be fully clarified.

ACKNOWLEDGMENTS

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The Composition and Metabolism of Developing Brown and White Fats[1]

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ABSTRACT

This investigation was concerned with analysis of the composition and metabolism of developing adipose tissues in the postnatal hamster. Particular attentions were given to the development of brown fat and the relationship of brown and white fats to each other. Correlations with previous histological studies are stressed. The parameters studied were water content, total lipid content, glycogen content, fatty acid compositions of total lipid extracts, malic dehydrogenase activity as assayed from oxaloacetate to malate, glucose-6-phosphate dehydrogenase activity and aldolase activity.

INTRODUCTION

In recent years much information has been obtained relating to the morphology, chemical composition and metabolism of adipose tissues. There is, however, a significant lack of information concerning the development of these tissues. This has become quite evident following the recognition of an important physiological role for brown fat. This tissue has been assumed by many to be, in some manner, related to the embryonic form of white adipose tissue (1). Others have not accepted this relationship (2). In a recent report (3), we discussed the developmental morphology of brown (multilocular) and white (unilocular) adipose tissues in the hamster. It was shown that the development of brown fat deposits could be separated into several time stages, and these stages were rather unambigously separated in the hamster. This separation indicated that chemical and metabolic studies might be readily correlatable with developmental histology. In addition, the developmental processes of brown and white fats can be readily compared in this species.

In the present report a survey of chemical composition and enzymology of developing hamster brown and white adipose tissues will be presented. Particular attention will be given to correlations of composition and metabolism with previously described histology. Our survey includes examinations of the several parameters listed in the above abstract.

MATERIALS AND METHODS

Animals were obtained and maintained as described previously (3). Water content was determined by drying to constant weight at 105 C. Tissue lipids were extracted with chloroform-methanol (4), and quantitated by the colorimetric method of Dales (5). This was modified extensively for use with small amounts (< 10 mg) of tissue. Adult hamster white fat lipid extracts were used for colorimetric standards. Glycogen was determined by the method of Montgomery (6).

Fatty acid compositions were determined by gas liquid chromatography (GLC) of methyl esters prepared by transesterification using 5% sulfuric acid in absolute methanol. Analyses were made using butanediol succinate or ethylene glycol adipate columns at 205 C, with helium as carrier gas. The linearity of the flame ionization detector was frequently verified by injection of standard mixtures of methyl esters, obtained from the Hormel Foundation. The instrument used was a Varian Aerograph 1520B.

Malic dehydrogenase (1.1.1.37), aldolase (4.1.2.b), and glucose-6-phosphate dehydrogenase (1.1.1.49) activities were determined with a Beckman DB-G spectrophotometer using methods essentially as described by Weber (7). In the malic dehydrogenase assay cyanide was added to final concentration of 0.002 molar to inhibit NADH oxidation in the absence of substrate. Enzyme activities are expressed on the basis of tissue wet weight.

In all instances differences are assumed significant if P is less than 0.05 by Students' t-test.

RESULTS AND DISCUSSION

In a discussion of adipose tissue development in the hamster there are recognized problems of terminology. The adipose deposits of the scapular region will develop into the brown fat of the adult, although they are not brown in color for the first few days of development and the cells are histologically unilocular. For con-

[1]One of nine papers to be published from the Symposium "Brown Adipose Tissue," presented at the AOCS-AACC Joint Meeting, Washington, D.C., March 1968.

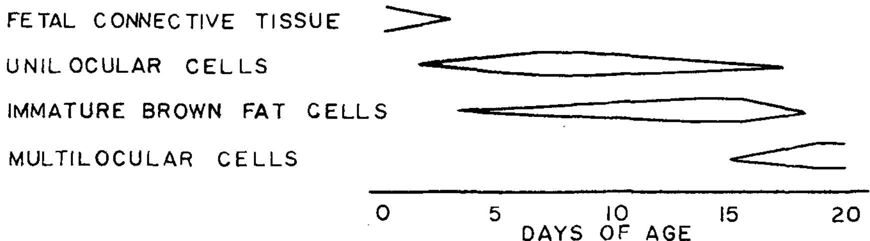

FIG. 1. Sequence of cell types present in developing hamster brown fat. See text for explanation.

venience of presentation we still refer to these deposits as brown fat. Figure 1 is a highly diagramatic presentation of the major cell types present in brown fat deposits of the hamster at various ages. Fetal connective tissue is present at birth, and declines to an insignificant amount at about three days of age. Unilocular cells appear shortly after birth and increase in number, but their relative numbers decline after about eight days. Immature brown fat (IBF) cells appear at about three days and subsequently increase in number. At about 15 days their number is maximal and they are transformed into the multilocular brown fat which persists into adulthood. The development of inguinal white fat in the hamster is essentially as shown in Figure 1, but IBF and multilocular cells do not appear and unilocular cells do not decline in relative numbers.

A more detailed analysis of developmental histology has been presented in a recent report from this laboratory (3).

Water Content

The percentage of water correlated well with histology (Fig. 2). There is, as would be expected, a general inverse relationship between water and total lipid contents.

Total Lipids

At four days of age unilocular cells are the major cytological species present in brown fat deposits, but at 16 days unilocular cells are rarely seen. At this time immature brown fat cells are the major type present (Fig. 1). Lipid contents of the developing tissues correlate well with morphology. In brown fat deposits the levels decline from 50% to 25% of the wet weight as unilocular cells are replaced by immature brown fat cells. A significant increase ($P < 0.01$) in total lipid percentage occurs between days 16 and 20. This corresponds to the time in which the brown fat deposits are becoming multilocular.

In our analysis of tissue lipid, interscapular and subscapular brown fat deposits are analyzed separately, but Figure 3 shows only data for interscapular tissue. For the first 16 days lipid levels of subscapular and interscapular deposits are identical. In the adult the levels in subscapular tissue are approximately 70% of those presented for interscapular brown fat. This difference is well correlated with morphological appearances.

Inguinal white adipose tissue contains little lipid for the first four days. A significant increase occurs at about day five and the level is maintained at about 40% until 16 days, after

FIG. 2. Water contents of hamster interscapular brown fat and inguinal white fat at various ages. Number of animals in each group is indicated on graph. Standard errors are shown.

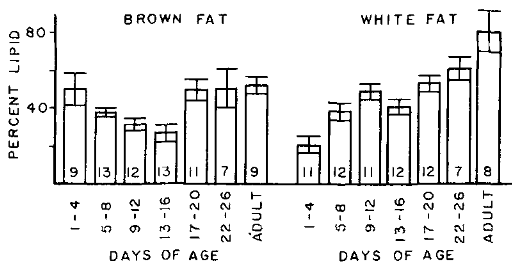

FIG. 3. Lipid content of hamster interscapular brown fat and inguinal white fat at various ages. Lipid content expressed as per cent of tissue wet weight. Number of animals in each group is indicated by numbers on the graph. Standard errors are shown.

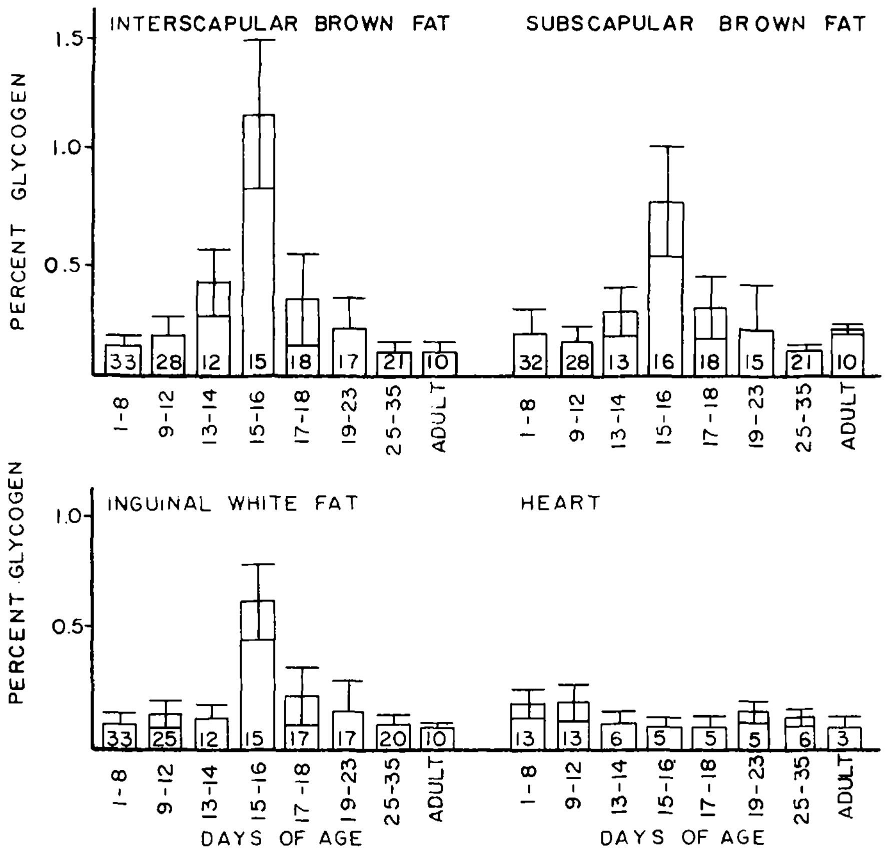

FIG. 4. Glycogen content of hamster adipose tissues and heart at various ages. Glycogen content expressed as per cent of tissue wet weight. Number of animals in each group is indicated by numbers on the graph. Standard errors are shown.

which there is an increase to 80% in the adult. The change in lipid level of white fat at about 16 days is not correlated with readily visible morphological events. It does, however, occur very near the weaning age (15 days) at which time there is an obvious diet change.

Glycogen

Glycogen content of all adipose tissues studied averages about 0.1% of the wet weight for the first 12-14 days of age (Fig. 4). It then increases sharply accounting for as much as 1% of the brown fat, and 0.6% of the white fat wet weight. Comparison of the 9-12 day animals with those 15-16 days of age reveals a six-fold glycogen increase in interscapular brown fat ($P < 0.05$). Similarly significant increases are noted in subscapular brown fat (five-fold increase, $P < 0.05$) and inguinal white fat (four-fold increase, $P < 0.05$). The glycogen increase is closely related to lipid accumulation in subsequent days. Glycogen accumulation prior to lipid storage has been described previously (8-10). Significant variations are not seen in the glycogen content of heart.

Fatty Acid Composition

Palmitic, palmitoleic, stearic, oleic and linoleic are the major fatty acids seen in our studies, accounting for approximately 95% of the 12-20 carbon acids. This has been demonstrated previously (11-13). The percentages of these acids in total lipids at various stages of development are shown in Figure 5. The results are presented only for interscapular brown fat, but no significant differences in fatty acid compositions of interscapular and subscapular deposits were noted at any stage of development. It should be emphasized that in animals older than 18 days our use of the term interscapular brown fat refers only to the brown colored tissue of the interscapular deposits.

In the following discussion each of the previously mentioned acids will be considered

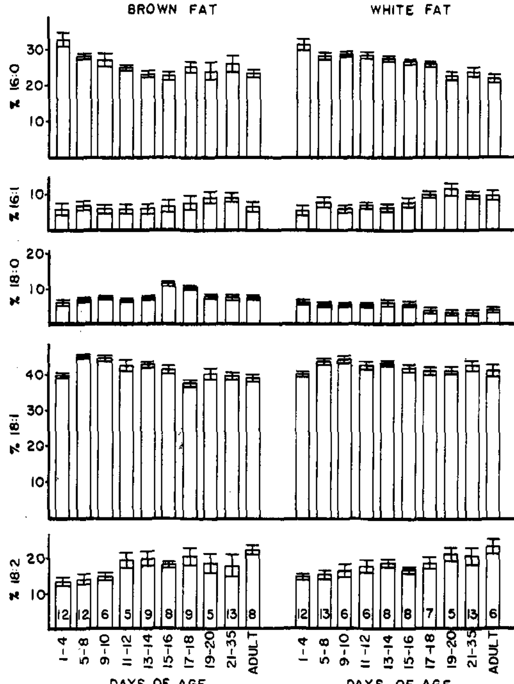

FIG. 5. Fatty acid composition of hamster interscapular brown fat and inguinal white fat at various ages. The age scale is shown only at the bottom of the graph. The 10 bars on the left of figure are brown fat and the 10 bars on the right are white fat. Number of animals used at each age is shown on the 18:2 graph (bottom) and is the same for the other acids. Standard errors are shown.

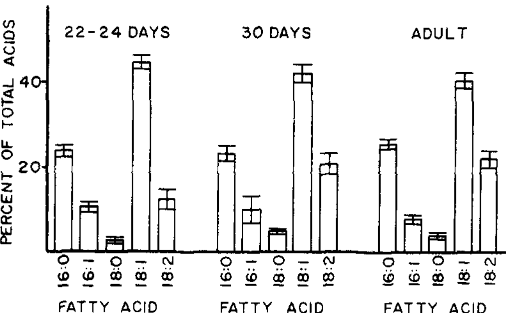

FIG. 6. Major fatty acids of interscapular white adipose tissue. Five animals were used for 22-24 day, two for 30 day and five for adult determinations. Standard errors are shown.

individually, in inguinal white fat, and in interscapular brown fat. All results are shown in Figure 5.

The palmitic acid (16:0) levels decline from about 34% to 28% in both brown and white fats between days four and six. This corresponds to the time period in which adipose cells are replacing the fetal connective tissues. In brown fat the 28% level is maintained during the time period in which unilocular cells are the predominant cellular type. As immature brown fat cells become numerous there is a decline in 16:0 to about 23% ($P < 0.01$, comparing 9-10 with 15-16 days). The 16:0 level does not change significantly after 16 days.

The percentage of 16:0 in inguinal white fat remains quite constant between days 5 and 18, averaging 27%. A minimum of 22% is reached at about 20 days of age ($P < 0.01$, comparing 9-10 with 19-20 days). This is not correlated with readily visible morphological events, lipid levels or other parameters studied.

Palmitoleic acid (16:1) levels of developing brown fat remain constant (about 6%) during days 1-14. An increase to 9% is seen between days 14 and 20 ($P < 0.05$). The decrease to 7% seen in adult brown fat is not significant.

White fat 16:1 levels decline from 8% to 6% between days 8 and 14. Some preliminary data indicate that this decline may be a reflection of the changing composition of hamster milk. The increase in 16:1 between 14 and 20 days is significant ($P < 0.01$).

The stearic acid (18:0) levels of brown fat deposits average about 6% during days 1-14. At about 14 days there is a sharp increase in 18:0 levels to 11% ($P < 0.01$, comparing 13-14 with 15-16 days). This corresponds to the time period just prior to the major lipid accumulation in brown fat (Fig. 1). Our preliminary studies indicate that this increase takes place in both the triglyceride and phospholipid fractions of the tissue, and does not appear to be a reflection of dietary change. The 18:0 level of brown fat drops from 11% to 9% between days 16 and 20 ($P < 0.01$). The 9% level is maintained into adulthood.

The stearic acid levels of inguinal white fat average about 5% for the first 16 days of development, followed by a significant ($P < 0.01$) drop to 3% at 19-20 days. This level is maintained into adulthood.

Oleic acid (18:1) increases from 40% to 45% of the total as adipose cells are deposited in the fetal connective tissues [$P < 0.01$ (BF) and 0.02 (WF)]. As immature brown fat cells are added to brown fat deposits a decrease in 18:1 is noted, reaching a minimum at 17-18 days (39%). This level is maintained with little change to adulthood.

After appearance of unilocular cells in white fat deposits there is no significant change of 18:1 percentage in white fat total lipids.

Linoleic acid (18:2) content of developing brown fat shows no significant change for the

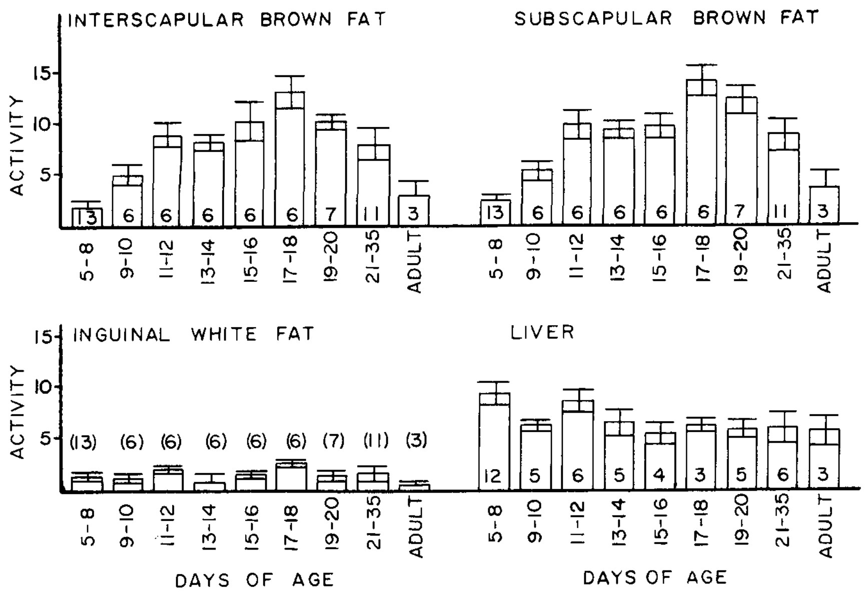

FIG. 7. Malic dehydrogenase activity of hamster adipose tissues and liver at various ages. Activity expressed as (micromoles NADH oxidized per gram wet weight per hour) (10^{-3}). The number of animals used in each group is indicated on the graph. Standard errors are shown.

first 10 days, averaging about 12%. It then increases during days 11-12 to 18% ($P < 0.05$). There is some indication of further increase in the adult, but this is not significant.

In white fat the 18:2 levels increase rather slowly from 14% at 6 days to 22% in the adult.

It is evident from the above presentation that the major changes in fatty acid composition of brown fat deposits occur during proliferation of the immature brown fat cells and subsequent lipid accumulation. At present their significance with regard to cellular metabolism is unknown, as are the controlling factors underlying them. It should be emphasized that our animals are weaned at 15 days of age. Dietary influences are then of possible importance. The changing composition of white fat after 15 days could indicate that diet is an important factor: the changes in white fat are not associated with readily visible morphological events. Preliminary studies of hamster milk composition, however, do not allow firm speculation regarding the influence of diet.

Analysis of the data in terms of differences in fatty acid composition of brown and white fats is of interest. Each of the acids studied has been analyzed in this regard, at each of the ages given in Figure 5. The fatty acid compositions of brown and white fats do not differ from each other in any statistically significant respect for the first 10 days of age. From days 11 to 16, white fat contains significantly more palmitic acid than brown fat. The percentage of palmitoleic acid does not differ in developing tissues or in the adult.

Previous works (11-13) have demonstrated that brown fat contains more stearic acid than white fat. This difference does not become apparent until 15 days of age in the hamster. After this time it is significant at all ages studied ($P < 0.01$ in all cases). Oleic and linoleic acid percentages in the total lipids do not differ in brown and white fats.

White colored adipose tissue surrounds the brown colored tissue discussed above and differs from the brown tissue in fatty acid composition. Preliminary analysis of this interscapular white adipose tissue, which begins to appear at about 18 days of age, is shown in Figure 6. Its fatty acid composition closely parallels that of inguinal white fat (compare Fig. 5), although studies of its origin seem to indicate derivation from multilocular cells of the interscapular brown fat deposits (20).

Enzyme Activity

The foregoing studies have demonstrated major changes in the composition of brown and white fats as the tissues mature. These changes are seen in histological pictures, glycogen, lipid contents and fatty acid composition. The changes are most noticeable from about 12-18 days of age. As a first step toward elucidation of the mechanism responsible, we have assayed

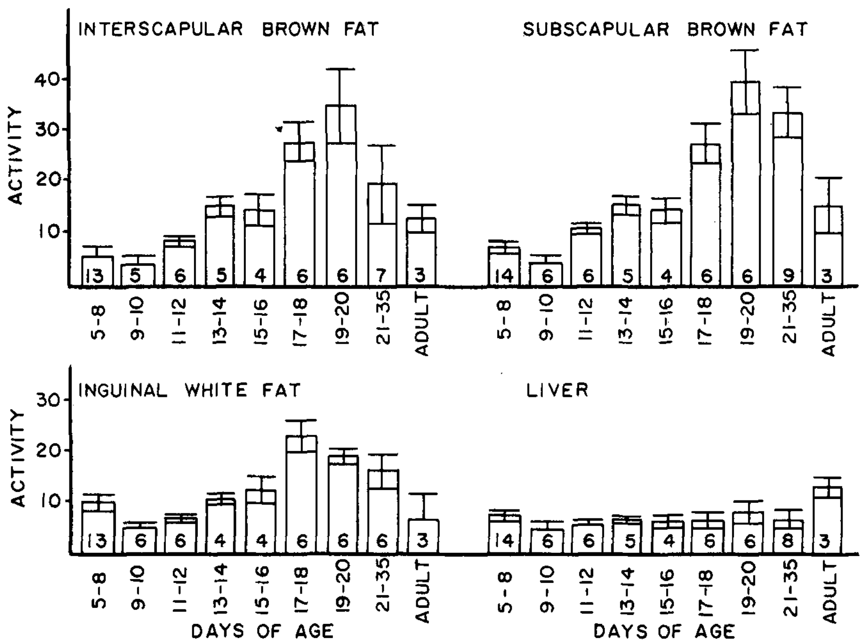

FIG. 8. Glucose-6-phosphate dehydrogenase activity of hamster adipose tissues and liver at various ages. Activity expressed as (micromoles NADP reduced per gram wet weight per hour) (10^{-1}). Number of animals in each group is indicated on graph. Standard errors are shown.

the activity of certain key enzymes in maturing interscapular and subscapular brown fats, inguinal white fat and liver. The enzymes chosed were malic dehydrogenase (1.1.1.37), glucose-6-phosphate dehydrogenase (1.1.1.49) and aldolase (4.1.2.b). Each is important in different metabolic pathways.

The activity of malic dehydrogenase (MDH) increases significantly ($P < 0.05$) in both interscapular and subscapular brown fat deposits between days 7 and 10, and again between days 10 and 12 (Fig. 7). The 12 day level is maintained to 16 days, followed by an increase during days 17-18. The 12 day level is reattained by day 25. Inguinal white fat MDH activity increases by a factor of 2.2 between 14 and 18 days, but drops to its 14 day activity level at 20 days, remaining essentially constant to at least 30 days of age. Liver MDH activity drops to 65% of its 5-8 day level during days 9-10, followed by a significant increase at days 11-12 ($P < 0.05$). The liver activity declines during days 13-14 to reach a level which is maintained to maturity.

At the present time it is felt that attempts at interpretation of the significance of variations in MDH activity are not warrented. Previous work has shown that NADH linked MDH enzymes are found in both the soluble and mitochondrial phases of cells (14-16). In a recent report (17) it has been shown that most of the MDH activity of brown fat is found in the supernate and microsomal fractions of the cell, with relatively little activity in the mitochondria. Our assay system does not differentiate activity in these sites, therefore attempts at interpretation without further studies would be premature.

Glucose-6-phosphate (G-6-P) dehydrogenase activity of both brown fat deposits increases by a factor of about 2.6 between days 16 and 20 ($P < 0.05$, Fig. 8). Significant, but smaller increases are seen in white fat. Activity of this enzyme in liver remains essentially constant for the first 30 days of development. It is probable that the increases in G-6-P dehydrogenase activity of the adipose tissues reflect an increase in the activity of the pentose pathway during the 16-20 day time period. If it is assumed that most of the lipid accumulated in the tissues after 16 days is newly synthesized, then much of the NADPH needed for such synthesis could be supplied through this pathway.

Aldolase activity of subscapular brown fat is significantly higher than that of interscapular brown fat for most of the first 30 days, but the activities of the adult brown fat deposits are at the same very low levels (Fig. 9). The changes in aldolase activity with age are in the same direction for all four tissues studied; that is,

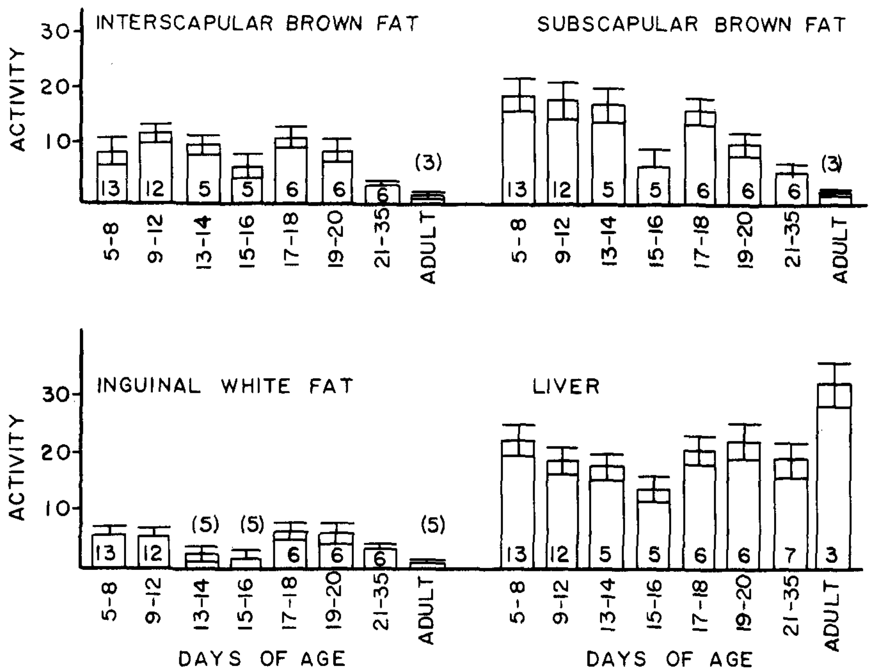

FIG. 9. Aldolase activity of hamster adipose tissues and liver at various ages. Activity expressed as (micromoles NAD reduced per gram wet weight per hour) (10^{-1}). The number of animals in each group is indicated on graph. Standard errors are shown.

there is a minimum at 15-16 days followed by an increase at days 17-18. The increase at 17-18 days is significant in subscapular brown fat ($P < 0.05$) and liver ($P < 0.01$), but not in the other two tissues. The activity decrease at 15-16 days corresponds to the histological time period just prior to the major lipid accumulation in brown fat. The decline in liver activity may indicate that the depression in aldolase activity is not closely related to lipid accumulation in brown fat. It was noted in Figure 4 that the glycogen content of the adipose tissues was maximal at 15-16 days of age. It may be that the glycogen increases seen resulted from the decrease in glycolytic (as measured by aldolase) activity. Glycogen increases prior to lipid accumulation may then be secondary to decreased glycolysis.

The studies reported here indicate that the composition and metabolism of developing brown fat correlate closely with the tissue histology. At present little is known regarding control of lipid storage in brown fat cells of the hamster. The closeness of the time of major lipid accumulation to the weaning age strongly implicates dietary factors in the changes noted. In addition the control of lipid metabolism by numerous hormones suggests that the changes are controlled by the functioning of certain endocrine glands. The ages at which various glands become active in the hamster are not known.

The results presented demonstrate that, in general, the composition and metabolism of unilocular cells in the brown fat and inguinal white fat deposits are similar. There is little difference in lipid content and fatty acid composition during those stages of development in which comparisons of unilocular cells are warranted. The tissues begin to differ when immature brown fat cells become numerous, and particularly when these cells are transformed into mature brown fat cells.

This study has demonstrated that the development of multilocularity in brown fat cells is highly specialized. Therefore, it can no longer be accepted that the brown fat cells as found in the mature animal are identical with the multilocular cells found in developing white adipose tissue deposits of most mammalian species. This does not necessarily imply that the multilocular brown fat cell cannot become transformed into the "signet-ring" cell. Our investigations indicate that the white adipose tissue of the interscapular deposits arises from multilocular cells of the brown fat.

It is not necessary to assume that lack of identity of the brown fat cell with the so-called embryonic fat cell means that the tissues are

entirely unrelated. The similarity of enzymatic responses noted in this report and others (18), indicate a high degree of metabolic relationship between brown and white fats, but do not prove them identical. The question of relationships cannot be answered by morphological, chemical or metabolic studies of the mature animal (19), and must await further and more detailed embryological studies. The hamster should be a good species for such investigations.

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Cuticular Lipids of Insects[1]

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ABSTRACT

Cuticular lipids cover nearly all parts of insects and are the chief agent for restricting water loss. The structure of insect epicuticles is reviewed, with emphasis on the role of cuticular lipids and the metabolism of cuticular lipids. The chemical composition of insect cuticular lipids are discussed with particular emphasis on the types in which hydrocarbons, wax esters and fatty alcohols predominate. What little evidence is available on the biosynthesis of insect cuticular lipids is discussed.

FUNCTION OF INSECT CUTICULAR LIPIDS

Secretion of waxy materials by bees and similar insects has been known since antiquity and is reviewed by Tulloch (this symposium); however, the chemistry of somewhat smaller quantities of waxy materials on the surface of the insect cuticle was not studied until the work of Dusham (1) in 1918. Dusham suggested that the cuticular lipid "probably serves as a protection against moisture," Ramsay (2) was the first to demonstrate that a greasy material on the surface of the cuticle was responsible for waterproofing the cockroach; subsequently, the work of Wigglesworth (3) and Beament (4) produced evidence that a thin layer of wax in the epicuticle covered nearly all parts of the insect and was the chief mechanism for restricting water loss.

The large surface to volume ratio of insects makes it important that excessive evaporation be prevented. Many investigators have measured the transpiration of water from insects. These studies are reviewed by Barton-Browne (5), Beament (6,7), Edney (8), Richards (9), Wigglesworth (10) and Locke (11). The evidence seems to agree that the cuticle of insects in dry air is relatively impermeable to water but is permeable in moist air, e.g., water vapor uptake. However, what passage of water occurs is due to passive diffusion because it has been demonstrated that it is immaterial whether the insect is alive or dead (3,12,13).

Five main lines of evidence support the role of the cuticular lipid layer in the reduction of transpiration:

1. Cuticles from which the surface lipids are removed with chloroform, ether or other organic solvents are relatively permeable to water. Vegetable oils (14), lecithin and a series of wetting agents and detergents show widely different effects on permeability of the cuticle to water (3,4).
2. The waterproofing observed with intact insects is rather closely duplicated when extracted cuticular lipids are deposited on collodion membranes or intact wing membranes (4).
3. The transpiration rate from an insect is found to increase rather abruptly at a temperature that closely corresponds to the transition point or change of phase point of the lipids of the cuticle of the particular species. This transition point is very near the melting range of the extractable lipid (3,4).
4. Rapid desiccation occurs as a result of scratching the outer surface of the cuticle with abrasive dusts. The abrasion need be only deep enough to penetrate the epicuticle. Adsorption of the lipids onto the dust may also play a role to a certain extent, especially on insects where the lipids are soft (3,15).
5. If the abraded insect is kept in a moist atmosphere to prevent desiccation the lipid layer is restored, and along with this the resistance to desiccation returns.

STRUCTURE OF INSECT CUTICULAR LIPID LAYER

With the aid of electron microscopy, M. Locke (11,16) has made some observations on the structure of the epicuticle and his interpretations shed some light on the properties of this membranous structure. The epicuticle is recognized as having distinct sublayers defined from inside to outside as: inner epicuticle, cuticulin, lipid layer and cement layer (11,17-19) (Fig. 1). The thickness is generally about 1μ, or only about 5% of the thickness of the cuticle.

Hurst (20) postulated that the cuticular lipid consists of an innermost layer that orients the overlying orthorhombic crystals of paraffins

[1]One of six papers to be published from the Symposium on Natural Waxes, presented at the AOCS Meeting, San Francisco, April 1969.

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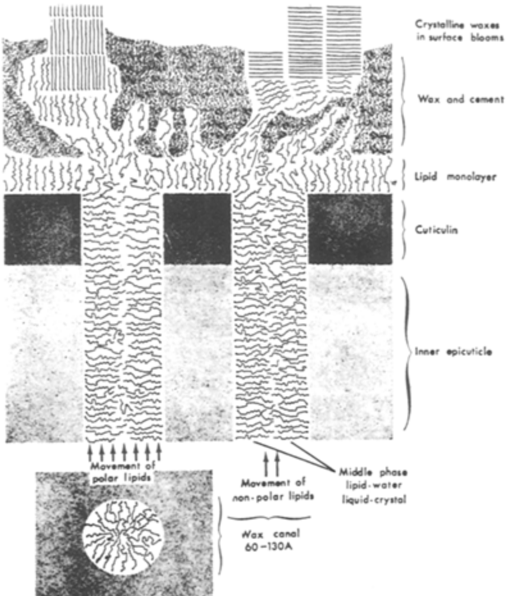

FIG. 1. Diagram of the most probable arrangement of lipid molecules in the epicuticle. The inner epicuticle may extend for about 1μ, that is, about 20 times the thickness shown. Locke, M. (16), reproduced with permission. Copyright 1965 by the American Association for the Advancement of Science.

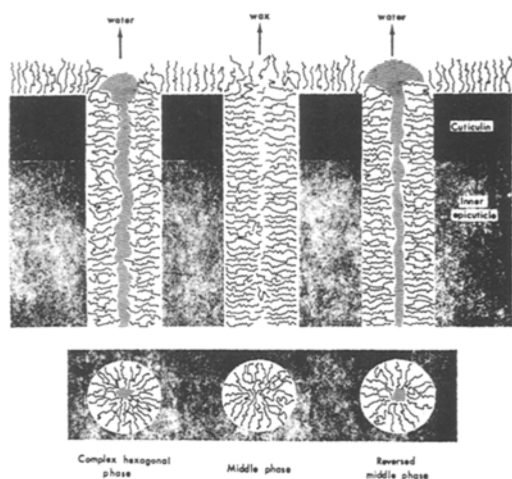

FIG. 2. Three lipid-water liquid-crystalline phases known from in vitro studies. If the middle phase (center) could change to either the complex hexagonal or the reversed middle phase within the epicuticle, there would be an increase in permeability to water. Locke, M. (16), reproduced with permission. Copyright 1965 by the American Association for the Advancement of Science.

which form the main water barrier. The degree of organization of the outermost layer decreases the more removed it is from the organizing effect of the innermost layer. The transition in the temperature-permeability relationship, according to this theory, is an irreversible change from the orthorhombic to a hexagonal crystal system.

Beament (4,6,17,21-23) presented evidence for an oriented monolayer attached to the surface of the cuticle (amounting to about 5% of the grease layer in the case of the cockroach cuticle), which is responsible for approximately 25% of the impermeability to water. The transition phenomenon is explained as a disorganization of the oriented monolayer by thermal agitation of the constituent molecules. The theory postulates that the extreme permeability is due to additional oriented layers of lipid, however, the arrangement is not explained. The impermeability of cuticular lipids is impressively high compared with that of normal hydrocarbon layers (24). Insect cuticular lipids differ either physically or chemically or both from normal paraffinic hydrocarbons.

Locke (25,26) finds that part of the epicuticle is penetrated by pore canals containing numerous lipid filaments. The lipid filaments appear as dense lines of indefinite length in an orderly hexagonal array when fixed with osmium and stained with lead. The lipid filaments and the surface monolayer are probably unsaturated or polar lipids or both since they are fixed by osmium tetroxide (27); they differ from most of the surface lipid, which is unfixed and mainly hydrocarbon (Locke, in preparation). Locke suggests that the most probable arrangements of lipids in the epicuticular canals are middle phase (Fig. 2) with the possibility of some reversed middle phase and complex hexagonal phase [defined by Luzzati and Husson (28) and Stoeckenius (29)]. The following aspects of cuticular permeability are explained by Locke (11) on the basis of the above arrangements of lipids.

The transport of lipid from the site of synthesis across the endocuticle and epicuticle to the surface could be carried out in the hydrophobic center of the pore canal. Movement of polar lipids, on the other hand, might be by displacement of the lipid occupying the pore.

The cuticle is permeable to lipid-soluble molecules (insecticides, pheromones, hormones) which could take up some of the space in the region occupied by the hydrocarbon ends of the pore lipids and eventually find their way into the insect.

The nonabrasive dusts, which apparently function by adsorption of the lipid, could

FIG. 3. Structure of ceroplastol I ($R=CH_2OH$) and ceroplasteric acid ($R=COOH$) (34).

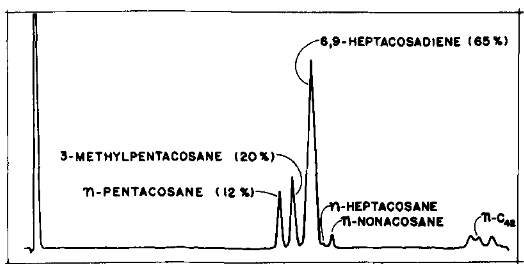

FIG. 4. GLC of *P. americana* hydrocarbons. 6 X 1/4 in. OD, 1% SE-30 on Diatoport W (60-80 mesh). Programmed-120-350 C at 5 C/min. Baker, G. L., et al. (41), reproduced with permission.

seemingly adsorb the lipid from the pore canals and thereby allow water to enter. With the lipid in the pore canals replaced by water, desiccation of the insect is likely to occur.

The possibility of some conversion of middle phase to reversed middle phase could explain the variation in water permeability [e.g., the cockroach is very impermeable to water loss, yet a drop of water on the cuticle rapidly disappeared (6)]. The phase depends on the temperature and the lipid-water ratio, such that a change in environment might change the phase and thereby change the water permeability of the cuticle.

CHEMISTRY OF INSECT CUTICULAR LIPIDS

The dome-shaped covering of lipids over the scale of insects, *Ceroplastes*, is much larger than is usually seen on the epicuticle of insects, but its chief function is still most likely to protect the insect against desiccation (30).

The lipid from *Ceroplastes destructor* (Newst.) has been examined by Hackman (30) and Gilby (31). The lipid consists primarily of wax esters of long chain normal fatty acids and alcohols. There are apparently a group of long chain acids and alcohols of average chain length about C_{27} and another group (average chain length about C_{12}) where the alcohols are unsaturated with at least two double bonds per molecule. Paraffin hydrocarbons were not detected. The composition was not appreciably affected by the age of the insect or the type and locality of the host plant (32).

The scale insect *Ceroplastes albolineatus* secretes a lipid that has been studied in more detail (33,34). Saponification of the lipid gave *n*-alkyl alcohols, ceroplastol I, *n*-alkanoic acids and ceroplasteric acid. The structures of ceroplastol and ceroplasteric acid are shown in Figure 3. Rios and Colunga (33) report the additional presence of albolineol ($C_{13}H_{22}O$) which has not been completely characterized, and ceroplastol II, which is apparently a structural isomer of ceroplastol I. The presence of a hydrocarbon mixture was also observed, but the mixture was not characterized.

The composition of the saponified lipid from *Ceroplastes rusci* is shown in Table I (35). The number of carbon atoms in the chain is the only data presented on the structure of the components.

A white crystalline powder that accumulates superficially on the larval cuticle of eri silkworm [*Samia cynthia ricini* (Jones)] was found to be mostly (92.6%) a mixture of two straight chain saturated alcohols [99.4% *n*-tricontanol ($C_{30}H_{62}O$) and 0.6% *n*-octacosanol ($C_{28}H_{58}O$)] (36). The function of this crystalline powder of aliphatic alcohols on the surface of these larvae is not yet clear. The material does not form a contiguous sheet over the cuticle as do other insect cuticular lipid coverings, but is observed in the form of a fine dust or powder. The study of the orientation of an irregular-shaped, nearly pure alcohol layer would be interesting in view of its possible role in water conservation or possibly some other role.

A summary of the cuticular lipid composition from the Mormon cricket (*Anabrus simplex* Hald.) is shown in Table II (37). Measurable amounts of hydrocarbons, free fatty acids, esters, free cholesterol and acidic resins were characterized. The lipids were isolated and characterized by column chromatography, gas chromatography and infrared spectrophotometry.

The cuticular lipids of cockroaches were investigated as far back as 1918 (1). Dusham indicated that the lipid secreted by the wax glands of the German cockroach (*Blatella germanica*) has saponification and solubility characteristics similar to beeswax. Other than this, further information on the cuticular lipids of German cockroaches has not been reported.

American cockroach (*Periplaneta americana*) cuticular lipids have been studied by Beament

TABLE I

Composition of the Saponified Lipid Secreted by *Ceroplastes rusci* (Per Cent)[a]

	Hydrocarbons	Alcohols	Acids
Lipid composition	11.8	23.6	64.6
Number of carbon atoms in the chain			
14	--	--	--
16	--	--	1.9
18	--	--	4.2
20	--	--	5.7
22	--	--	--
24	--	7.0	1.4
25	1.0	--	--
26	1.0	81.2	1.2
27	58.0	--	--
28	2.7	11.8	0.9
29	26.4	--	--
30	1.7	--	15.2
31	9.2	--	--
32	--	--	56.3
33	--	--	--
34	--	--	13.2
35	--	--	--

[a]From Faurot-Bouchet and Michel, (35), reproduced with permission.

TABLE II

Summary of the Composition of Mormon Cricket Wax[a]

Chemical class	Per cent of wax	Individual component	Per cent of class	Per cent of wax
Hydrocarbons	48-58	C_{12},C_{13},C_{14},C_{15},C_{16}	12.5	6-7
		C_{17},C_{18},C_{19},C_{20},C_{21}	Equally	
		C_{22},C_{23},C_{24},C_{25},C_{26}	distributed	
		C_{27},C_{28}	20.6	10-12
		C_{30},C_{31}	55.3	27-32
		C_{32}	11.4	5-7
Free acids	15-18	C_{12}	0.4	1
		C_{14}	1.7	0.4
		C_{14} 1 double bond	0.5	0.1
		C_{14} 2 double bonds	0.3	0.1
		C_{14} 3 double bonds	0.6	0.1
		C_{16}	7.9	1-1.4
		C_{16} 1 double bond	4.0	0.6
		C_{16} 2 double bonds	0.3	0.1
		C_{16} 3 double bonds	0.7	0.1
		C_{18}	2.4	0.4
		C_{18} 1 double bond	48.4	7-9
		C_{18} 2 double bonds	26.8	4-5
		C_{18} 3 double bonds	6.0	1
		C_{20}	Trace	Trace
Esters	9-11	Saturated	34	3.2-3.9
		Saturated and unsaturated	28	2.7-3.3
		Saturated and/or hydroxy	15	1.4-1.7
		Unsaturated and/or hydroxy	23	2.2-2.7
Free alcohols	2-3	Cholesterol		2-3
Polymers	12-14	Acidic resins of varying molecular size		12-14
Unidentified	2-4			2-4

[a]Baker et al. (37), reproduced with permission.

TABLE III

Hydrocarbon Biosynthesis by Live *P. americana*
(Thorax injected, Incubated 18 hr)

Labeled acid injected	dpm Diene	Saturated-branched	Ratio
^{14}C-1-Acetic acid	3250	2810	1.16:1
^{14}C-1-Malonic acid	6853	6591	1.04:1
^{14}C-1,3-Malonic acid	3267	2411	1:1.02
L-Methionine-CH_3-^{14}C	82	83	--
^{14}C-3-Propionic acid	2267	5705	1:2.52
^{14}C-10-Decanoic acid	3025	3993	1:1.32
^{14}C-1-Palmitic acid	1696	2178	1:1.28
^{14}C-U-Palmitic acid	1560	2800	1:1.8
^{14}C-16-Palmitic acid	897	2433	1:2.71
Palmitic acid-9,10-^{3}H	170	1922	1:11.3
^{14}C-1-Linoleic acid	1348	452	2.98:1

(38) and by Gilby and Cox (39,40). Gilby (40) found that the American cockroach cuticular lipid extract consisted of about 75% hydrocarbons, while fatty acids, esters and aliphatic aldehydes in roughly equal amounts made up the remaining 25%. The major hydrocarbon component was reported to be a 27 carbon unconjugated diene, probably heptacosa-9,18-diene. Baker et al. (41) report that the most abundant hydrocarbon in the whole cockroach and in the cockroach hemolymph is a 27 carbon diene (*cis,cis*-6,9-heptacosadiene). The gas chromatogram of American cockroach hydrocarbons is shown in Figure 4. In yet unpublished work on the cuticular hydrocarbons, we have also observed chromatograms similar to the one in Figure 4, and the presence of hydrocarbons of approximately 41 to 43 carbons.

We have recently investigated the cuticular hydrocarbons of a number of cockroaches. Approximately 90% of the cuticular hydrocarbons of the Madeira cockroach (*Leucophaea maderae*) and the Oriental cockroach (*Blatta orientalis*) consisted of *n*-heptacosane, 11-methylheptacosane, 13-methyheptacosane and 3-methylheptacosane, and the remaining 10% was apparently of a similar series with predominantly 23,25,29 and 31 carbons in the chain (42). This is the first report of similar hydrocarbon compositions in insects of different genera and also the first report of branched hydrocarbons in the cuticular lipids of insects. Leibrand (43), however, indicated the possibility of branched and cyclic hydrocarbons in the cuticular lipids of the Mormon cricket (*Anabrus simplex* Hald.) and Baker et al. (41) found 3-methylpentacosane in the hydrocarbon extract of the whole American cockroach. Even more exciting is the presence of such large quantities of internally methyl branched hydrocarbons that are not of the isoprenoid type. Internally methyl branched hydrocarbons were first discovered in plant waxes (for a review see Kolattukudy, this symposium); however, they were also found in an extract of homogenized house crickets, *Acheta domesticus* (44). It is not certain whether these hydrocarbons were originally on the cuticle or inside the insect; on the other hand, it is very unlikely that appreciable quantities of hydrocarbons will be found inside an insect, even though they have been reported in hemolymph (41,45,46). The internally methylated hydrocarbons found in the house cricket extracts were reported as a homologous series containing 31 (3%), 33 (6.5%), 35 (12.5%), 37 (27%) and 39 (2.5%) carbon atoms where the methyl branch is located on the 13th, 15th and 17th carbon atom in the chain (percentages as per cent of hydrocarbon fraction). Twenty per cent of the hydrocarbon fraction from the house cricket are 2-methyl alkanes where the two most abundant ones have 29 (8%) and 31 (11.5%) carbon atoms. There appears to be an interesting difference between cockroach hydrocarbons (odd-numbered carbon chains with 3 methyl branches) and those of the house cricket (even-numbered carbon chains with 2 methyl branches).

The major cuticular hydrocarbons of the Australian cockroach (*Periplaneta australasiae*), the southern brown cockroach (*Periplaneta brunnae*) and the smoky brown cockroach (*Periplaneta fuliginosa*) are *n*-tricosane, 3-methyltricosane, 11-methyltricosane and 13-methylpentacosane (47). However, there are as yet unexplained quantitative differences between the hydrocarbon composition of males and females of *P. australasiae* and *P. fuliginosa* since *cis*-9-tricosene is an additional major

TABLE IV

Hydrocarbon Biosynthesis by Excised Integument of *P. americana* (Incubated 18 hr)

Labeled acid applied	dpm		
	Diene	Saturated-branched	Ratio
Applied on outside of integument			
^{14}C-1-Acetic acid	387	397	--
Applied on inside of integument			
^{14}C-1-Acetic acid	836	840	--
^{14}C-1-Decanoic acid	887	1488	1:1.68
^{14}C-1-Palmitic acid	236	948	1:4.01
^{14}C-u-Palmitic acid	84	557	1:6.50
Palmitic acid-9,10-^{3}H	777	23,522	1:31.5
^{14}C-16-Palmitic acid	415	13,296	1:32.2
^{14}C-1-Linoleic acid	42	115	--

hydrocarbon in the males only. A complete series of monomethyl branched hydrocarbons ranging in chain length from 23 to 26 carbons with the methyl branch on the 13th carbon from one end was observed.

The surface lipids of the aquatic and terrestrial life forms of the big stonefly, *Pteronarcys californica*, are found to differ in that a larger percentage of hydrocarbons, wax esters, free fatty acids and sterols are found on the terrestrial adult, while the aquatic naiad surface lipid has more triglycerides (48). It appears that this insect's surface lipid composition varies with life stage, depending upon the water conservation mechanism.

BIOSYNTHESIS OF INSECT CUTICULAR LIPIDS

It is generally accepted that acetate units are incorporated into the cuticular lipids of insects, especially hydrocarbons (36,49-56), but beyond that the biosynthesis route to cuticular lipids is unknown. Nelson (57) has recently reported that the integument of the American cockroach can synthesize hydrocarbons from 1-^{14}C-acetate and 1-^{14}C-palmitate. The fat body was not capable of synthesizing hydrocarbons.

We find that whole American cockroaches will incorporate labeled fatty acids into the cuticular hydrocarbons. The cockroaches were injected in the thorax with labeled fatty acids and allowed to incubate for 18 hr. The whole roaches were then extracted with chloroform. The lipid extract was then separated by thin layer chromatography on silica gel and developed in hexane. Three fractions were assayed for radioactivity by liquid scintillation spectrometry. The fractions were: (a) saturated-branched hydrocarbons, mostly pentacosane and 3-methylpentacosane; (b) diene hydrocarbon, mostly *cis,cis*-6,9-heptacosadiene; and (c) mostly fatty acids and glycerides. The results of these incorporation studies are shown in Table III. In whole insects 1-^{14}C-acetate, 1-^{14}C-malonate, 1-^{14}C-decanoate and 1-^{14}C-palmitate are incorporated into the diene and saturated-branched fractions in nearly the same proportions. 1-^{14}C-Linoleate was preferentially incorporated into the 6,9-heptacosadiene, suggesting that linoleate is a direct precursor of the diene hydrocarbon. 3-^{14}C-Propionate led to a disproportionately high activity in the saturated-branched hydrocarbons. L-methionine-CH_3-^{14}C did not give rise to any appreciable amount of labeled hydrocarbon, indicating that most likely the methyl branched hydrocarbon does not come from methionine. Palmitate-9,10-^{3}H gave 10 times the activity in the saturated hydrocarbons than in the diene, suggesting either preferential incorporation into the saturated hydrocarbons, or 9,10-^{3}H loss during diene formation.

We have worked out the conditions that provide an excised integument capable of hydrocarbon biosynthesis. The cockroaches were killed by freezing for 15 min at -29 C. The head, legs and wings were cut off and then the internal organs were removed by making a cut in the ventral side and removing the loose tissues. The dissection was carried out at 4 C. The excised integument was then warmed to room temperature in a chamber saturated with water, after which the labeled acids were inoculated on the inside of the integument (outside in one case). The reaction was allowed to proceed for 18 hr. The data are shown in Table IV. The system incorporated 1-^{14}C-acetate and 1-^{14}C-malonate just as observed with the whole insect (i.e., about equal counts were observed in the saturated-branched hydrocarbons and the

TABLE V

Effect of Trichloroacetic Acid on Incorporation of ^{14}C-1-Acetic Acid by Excised Integuments of *P. americana*

	dpm		
TCA used	Fatty acids	Diene	Saturated-branched
0.0	25,379	836	840
0.5 μmoles	15,826	831	875
2.5 μmoles	3.663	239	86

TABLE VI

Effect of Chloroform Anesthetization on Incorporation of ^{14}C-1-Malonic Acid by Live *P. americana*

P. americana	Diene (dpm)	Saturated-branched (dpm)
Control	13,580	13,112
Treated	2,976	3,528

diene hydrocarbon). Palmitate-9,10-^{3}H and 16-^{14}C-palmitate are preferentially incorporated into the saturated-branched hydrocarbons at a ratio on the order of 30:1 over diene hydrocarbon, which, as with the whole insect, suggests preferential incorporation of palmitate into the saturated-branched hydrocarbons. 1-^{14}C-Palmitate is incorporated at a ratio of 4.0:1, indicating that the carbonyl carbon is not incorporated to the same degree as is the 16 carbon. The data from incorporation of 1-^{14}C-labeled fatty acids (Table III and Table IV) are not easily interpreted, presumably due to some oxidation reactions on 1-^{14}C-fatty acids that compete with hydrocarbon biosynthesis for substrates. The results with 1-^{14}C-fatty acids are comparable to acetate incorporation, leading us to believe that β-oxidation is the principal competing pathway and some of the incorporation observed is from the resulting acetate. 1-^{14}C-Decanoate is incorporated 1.7:1 into saturated-branched hydrocarbons over diene hydrocarbon. These results can be interpreted in at least two possible ways: decanoate is more readily attacked by β-oxidation than is palmitate, or when decanoate and palmitate combine in the condensation pathway of pentacosane biosynthesis, the decanoate is decarboxylated, resulting in less labeling from decanoate than from palmitate.

The results to date, although informative, do not lead to unambiguous answers to the pathway for hydrocarbon biosynthesis. The hydrocarbon biosynthesis enzyme system must be obtained, free of competing enzyme systems.

As a prelude to this development, two possible inhibitors of hydrocarbon biosynthesis have been investigated. Kolattukudy (58) has shown that in *Brassica oderacea* there is an inhibition of incorporation of C_2 to C_{14} acids into hydrocarbons when as little as a fourfold excess of trichloroacetic acid was present. He concluded that the C_{16} chain fatty acid is elongated to a C_{30} unit which on decarboxylation gives rise to the hydrocarbon. Trichloroacetate at up to tenfold excess does not appear to be inhibitory in the case of the American cockroach hydrocarbon biosynthesis, which indicates that either the trichloroacetate cannot penetrate to the enzyme system or that the elongation mechanism of the *Brassica* type is not functioning in the American cockroach hydrocarbon biosynthesis (Table V).

Chloroform has a strong inhibitory action on hydrocarbon biosynthesis by both whole insects and the excised integument systems. If insects were anesthetized with chloroform prior to incubation with labeled fatty acids, there was at least a fourfold decrease in incorporation of label into hydrocarbon, compared to the incorporation by untreated cockroaches (Table VI).

CONCLUSION

It is almost universally accepted that the main function of insect cuticular lipids is to restrict water transpiration from the insect. The structure of the insect cuticular lipid layer is very likely not the same in all insects, but in general there is a lipid monolayer which orders the arrangement of the first layers of cuticular lipid. The outermost cuticular lipid may be in ordered blooms or in microcrystalline form dispersed in a cement. There appears to be considerable diversity in the chemical composition of insect cuticular lipids, the types in which wax ester, fatty alcohol and hydrocarbon predominate are discussed here. Even in the class where hydrocarbons predominate, there is considerable diversity in cuticular lipid components among the insects and at least in one case between sexes. Very little is known about the biosynthesis of insect cuticular lipids, except that the integument in at least some cases is capable of converting fatty acids to hydrocarbons. Now that the function and structure of the insect cuticular lipid layer is fairly well established, we feel that with the modern techniques of analytical organic chemistry and an improved knowledge of possible biosynthesis

pathways, the next few years should see an advance in insect cuticular lipid chemistry and biosynthesis.

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The Composition of Beeswax and Other Waxes Secreted by Insects[1,2]

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ABSTRACT

This review deals with waxes of members of two quite different groups of insects, the bees and the scale insects, which secrete large amounts of wax. The former use wax as a structural material and the latter as a protective material. The compositions of waxes from some of these insects are described and particular attention is paid to the compositions of the unhydrolyzed waxes and to the presence of hydroxy acids. New analyses of beeswax and of wax of a species of bumble bee are reported. The structures of the diesters, hydroxyesters and diols of beeswax are elucidated. The bumble bee wax contains major proportions of saturated and unsaturated hydrocarbons, and of long chain saturated, mono- and diunsaturated esters. The relationship between structure and function of the waxes is discussed.

INTRODUCTION

Insects belong to the class of animals with the following characteristics: the body is divided into head, thorax and abdomen; the head carries a single pair of antennae; and the thorax carries three pairs of legs and usually one or two pairs of wings. These features distinguish them from crabs, lobsters, spiders, mites, millepedes etc.

Nearly a million species of insects have been described and several times this number may actually exist. Insects are divided into about 28 orders; several include a very large number of species but most have only a few thousand. A simple introduction to insect biology has been written by Wigglesworth (1).

The orders which contain some of the best known insects are listed in Table I together with the approximate number of species in the order; for convenience the other, less well known, orders have been omitted. The first 13 orders contain those insects whose young are not too different from the adults and the last seven contain those whose young are radically different from the adults.

Most or all insects are protected from water loss by a thin film of wax in the cuticle; this type of wax is discussed by Jackson and Baker (this symposium). Clearly the study of the composition of insect waxes is an enormous field which has barely been scratched. Jackson and Baker review two different species of cricket, four different species of cockroach, one species of moth and one species of scale insect. The huge orders of flies and beetles do not seem to have been examined at all.

In this review I shall deal with a few members of two orders of insects which secrete much larger amounts of wax than those which produce only a thin waxy cuticle. The most important one to be discussed is the honey bee (genus *Apis*, family Apidae). I shall also deal with the wax of bumble bees (genus *Bombus*, family Apidae). Bees are considered to be among the most highly developed insects.

The other, more primitive, group of insects which secretes large amounts of wax is that comprising the scale insects. They are given this name because, in many species, the female is protected by a scale or shield consisting of a mixture of wax and cast skins. These insects are members of several families of the sub order Homoptera of the order Hemiptera (Bugs, Table I). The scale insects have been investigated because many of them are serious agricultural pests though a few are of commercial value.

The appearance and function of the waxes is discussed later.

The chemistry of waxes secreted by insects has been studied over the last 150 years. Literature prior to 1954 was reviewed by Warth (2), but more prominence was given to early theories of composition than to later, more reliable, results. The present review will deal only with what seem to have been the most important advances, particularly those obtained by modern chromatographic methods, and will report new analyses of beeswax and wax of a species of bumble bee.

Beeswax

At one time the word wax meant only bees-

[1]Issued as National Research Council of Canada No. 11260.

[2]One of six papers to be published from the Symposium on Natural Waxes, presented at the AOCS Meeting, San Francisco, April 1969.

TABLE I

Some Orders of Insects (1)

Order	Approx. No. of Species
Dragonflies (Odonata)	5000
May-flies (Ephemeroptera)	1500
Cockroaches and Mantids (Dictyoptera)	6000
Stone-flies (Plecoptera)	1500
Termites (Isoptera)	1700
Earwigs (Dermaptera)	1100
Stick-insects (Phasmida)	2000
Grasshoppers, Locusts, Crickets (Orthoptera)	10000
Book-lice (Psocoptera)	1100
Bird-lice (Mallophaga)	2600
Sucking-lice (Anoplura)	230
Thrips (Thysanoptera)	3000
Bugs, Aphids, Scale Insects etc. (Hemiptera)	55000
Lacewings etc. (Neuroptera)	5000
Caddis flies (Trichoptera)	5000
Butterflies and Moths (Lepidoptera)	200000
Flies and Mosquitoes (Diptera)	85000
Fleas (Siphonaptera)	1100
Ants, Bees, Wasps etc. (Hymenoptera)	100000
Beetles (Coleoptera)	275000

wax and as the most important insect wax it has attracted the most attention, in fact, Ikuta (3) has remarked that there were about 140 publications dealing with beeswax chemistry between 1848 and 1930. Beeswax generally refers to wax of the European bee, *Apis mellifera*, but Asiatic species *A. dorsata, A. florea* and *A. indica* are sometimes also commercial sources of wax. This wax is known as East Indian beeswax or Ghedda wax. Functional group analysis of Ghedda wax (3-5) indicates only minor qualitative differences between its composition and that of common beeswax. Results of investigations of Ghedda wax will, therefore, be included with those of beeswax. Waxes of wild bees of the genera *Trigona* and *Melipona* (also in family Apidae) have been examined (6), but not by modern methods. Wax of a few species of Bombus has also been investigated (7).

To compare properties of waxes and to consider their biosynthesis it is clearly important to know the composition of the natural unhydrolyzed wax. Some early investigators did try to determine this, but most investigations have been carried out using saponification products. Since wax components are complex mixtures of homologs, it was difficult to make an accurate analysis prior to the application of gas liquid chromatography (GLC). The early investigators, however, were able to distinguish between components of medium chain length, with about 16 carbons, and very long chain components with about 30 carbons. A critical review of investigations of beeswax up to 1962 was made by Callow (8).

In 1848, Brodie (9) reported that the free acids of unhydrolyzed beeswax, obtained by extraction with ethanol, were long chain compounds (C_{27}) and also that part of the remaining wax was a palmitate of a long chain alcohol (10). Later it was gradually established that beeswax was a mixture of hydrocarbons, esters and acids (2).

Further advances were made by Gascard (11) and Damoy (12), who, however, studied only hydrolysis products. They concluded that the hydrocarbons, alcohols and long chain acids were all odd-numbered with 25-31 carbons. Chibnall et al. (13) reinvestigated their results, using x-ray crystallography, and showed that though the hydrocarbons were odd-numbered C_{25}-C_{31} compounds the alcohols and long chain acids were in fact even-numbered with 24-34 carbons.

In 1961, Downing et al. (14) separated the components of hydrolyzed beeswax into hydrocarbons, alcohols, acids, diols and hydroxy acids and reduced them all to hydrocarbons. These were then analyzed by GLC with the results in Table II. The hydrocarbons, 16% of the wax, were mainly C_{25}-C_{33}, the principal alcohols were C_{24}-C_{34}, palmitic acid was the major acid and the long chain acids were C_{24}-C_{34}. These figures not only confirmed the qualitative conclusions of Gascard and Damoy concerning the hydrocarbons and of Chibnall et al. concerning the alcohols and long chain acids, but also Brodie's early isolation of palmitic acid. As the free acids of unhydrolyzed wax were not examined separately, the composition of the acids is that of the total wax acids.

TABLE II

Hydrocarbons Derived From Beeswax Fractions (wt. %)[a]

n-Paraffin hydrocarbon carbon No.	Naturally occurring hydrocarbons		Monohydric alcohols	"Diols"	Acids	Hydroxy acids
	Wax A	Wax B				
12					0.3	0.4
14					0.8	1.5
16					50.5	58.5
17					0.3	0.4
18					8.5[b]	9.8
19	0.5	0.3			---	Trace
20				Trace[c]	0.9[d]	4.1
21	0.8	0.8		---	---	---
22	0.3	0.2		Trace	2.0	1.6
23	3.7	3.7		---	0.3	0.4
24	0.6	0.4	11.9	15.2	17.5	8.2
25	7.5	8.8	---	---	Trace	0.5
26	1.2	1.0	10.1	19.6	4.9	3.7
27	26.8	30.1	Trace	Trace	Trace	0.3
28	2.2	1.3	14.8	39.2	4.3	1.9
29	19.3	16.5	Trace	2.6	Trace	0.5
30	1.6	0.9	31.6	14.8	3.0	0.6[e]
31	20.8	19.0	Trace	2.2	Trace	1.5[e]
32	0.9	1.5	23.5	6.5	4.7	0.4
33	13.8	15.5	---		---	
34			5.4		2.0	
35			---		---	
36			2.7			
Percentage of component in hydrolyzed wax	16		31	3	31	13

[a]Downing et al. (14), with permission.

[b]Consists of 7.8% monounsaturated and 0.7% saturated by examination of the methyl esters.

[c]Trace indicates present but in too small amount (ca. 0.1%) to be estimated satisfactorily.

[d]Includes saturated and unsaturated acids in approximately equal proportions.

[e]A broad peak of the range shown; 3.4% is not absorbed by the Linde Molecular Sieve column.

Although Downing et al. concluded that the hydrocarbons were entirely straight chain and saturated, unsaturated hydrocarbons have been frequently reported (2). In 1966, Streibl et al. (15) showed that about 31% of beeswax hydrocarbons consist of *cis* olefins which were mainly C_{31} and C_{33} compounds, whereas the alkanes are C_{25}-C_{29} compounds; very small amounts of branched chain hydrocarbons (16) and *trans* olefins (17) were also isolated and identified.

Table II contains two other interesting items. First diols (3% of the total), which were isolated for the first time, though without elucidating their structure apart from chain length, and second hydroxy acids (13% of total), which have a longer history.

Beeswax hydroxy acids were first mentioned in 1919 when Lipp and Kovacs (18) reported that the acids of saponified Ghedda wax were mainly C_{17} and hydroxy C_{17} acids. Free acids of this wax were very long chain compounds and different from combined acids (19). In 1933 Ikuta (20), working with Japanese beeswax, which comes from a variety of *A. indica* and is similar to Ghedda wax, showed that the hydroxy acid is a hydroxypalmitic acid and that the major acid is palmitic acid (21). Toyama and Hirai (22), in 1951, reported that Japanese and European beeswaxes contain the same hydroxy acid. After extensive fractionation a portion of the hydroxy acids (representing only about 10% of the original crude hydroxy acids) appeared to be 14-hydroxypalmitic acid. The isolation of tetradecanedioic acid from the products of permanganate oxidation of the mother liquors seemed to support their structure. This is more likely, however, to be evidence for the presence of a 15-hydroxypalmitic acid since nitric acid oxidation of hydroxy acids with penultimate hydroxyl groups results mostly in the loss of 2 carbon atoms (23).

The nuclear magnetic resonance (NMR) spectrum of beeswax hydroxy acids, examined by Horn et al. (24) in 1964, showed conclusively that the principal component is a

15-hydroxypalmitic acid. In connection with this finding it is a remarkable coincidence that an osmophilic yeast of the genus *Torulopsis*, which was isolated from flowers and from bumble bee nests, produces glycosides of several hydroxy acids including 15-hydroxypalmitic acid and also hydroxylates palmitic acid giving a mixture of glycosides of 15- and 16-hydroxypalmitic acids (25). Also 16-hydroxypalmitic acid, as the macrocyclic lactone, is the major constituent of the scent of two species of solitary bee (genus *Halictus*) (26). Presumably this acid is produced by the bee concerned, but I thought that the yeast *Torulopsis* might perhaps be involved in formation of beeswax hydroxy acids.

If these acids had the same optical configuration as hydroxy acid produced by *Torulopsis*, a common origin could be indicated. I have isolated hydroxy acids from beeswax and measured their specific rotation. *Torulopsis* produces 15-L-hydroxypalmitic acid with $[\alpha]_D$ + 4.5, but hydroxy acids from commercial (USP) beeswax had $[\alpha]_D$ + 1.5, suggesting a mixture of racemate and L-isomer. Hydroxy acids from natural sources are usually optically active, but racemic hydroxy fatty acids have sometimes been isolated (27).

Beeswax has been fractionated by column chromatography (28) and by thin layer chromatography (TLC) (29) though the fractions were not clearly identified. Since different optical isomers of 15-hydroxypalmitic acid might be present in different wax fractions, I have investigated the chromatographic separation of the whole wax. Honeycomb cappings were used since commercial wax might have been altered by bleaching and refining. In a TLC chromatogram of beeswax samples, all show the same components; in particular there are several components with R_f's smaller than that of long chain monoester (Fig. 1). Most of the fractions observed by TLC were isolated by silicic acid column chromatography and identified by NMR spectroscopy, GLC and examination of their hydrolysis products (A.P. Tulloch, to be published).

Table III lists the fractions obtained in this way and compares them with a beeswax composition calculated by Findley and Brown (30) from the results of functional group analysis. The percentage of hydrocarbons is similar to that reported before (14). Chromatography on silver nitrate silicic acid (15) gave alkanes and *cis* olefins (26%), and the compositions of these two fractions, determined by GLC, were very similar to those reported by Streibl et al. (15).

Monoesters A (35%) contained 40-50 carbon atoms with C_{46} and C_{48} as major components.

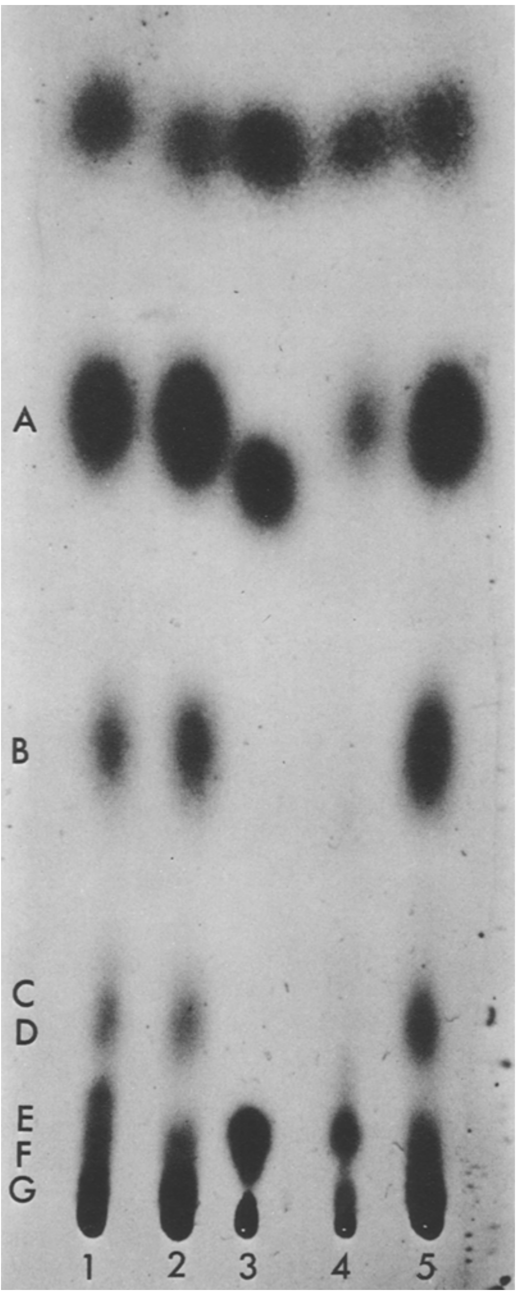

FIG. 1. Thin layer chromatograph of beeswax and bumble bee wax. 1, USP beeswax; 2, beeswax from honeycomb cappings; 3, mixture of triacontane, octadecyl stearate, octacosanol and octacosanoic acid; 4, bumble bee wax; 5, local, unrefined beeswax. The letters A-G refer to ester fractions of beeswax. Plate was Silica Gel G, development solvent was benzene at 32 C, spots were detected by spraying with 50% sulfuric acid and heating with an infrared lamp. Standards were synthesized as previously described (40).

TABLE III

Composition of Unhydrolyzed Beeswax

SiO_2 Column chromatography		Composition[a]	
Hydrocarbons	15		23
Esters A (monoesters)	35		45
Esters B (diesters)	12		6
Esters C (hydroxy esters)	4		9
Esters D (hydroxy esters)	4	Acid esters	5
Esters E (hydroxy esters)	4	Free alcohols	1
Esters F (hydroxy esters)	8		--
Esters G (hydroxy esters)	4		--
Free acids	8		12
Not identified	6		--

[a]Calculated by Findley and Brown (30).

Hydrolysis yielded palmitic acid and only traces of longer chain acids, and C_{24}-C_{34} alcohols, the original esters are thus palmitates of these alcohols. Very recently Holloway (31) has reported similar results for the composition of beeswax monoesters. The presence of 15-hydroxypalmitate in esters B-G was shown by NMR spectroscopy. NMR can also give an estimate of the extent to which the hydroxyl group is acylated, since studies of methyl hydroxystearates (32) and their acetates (A.P. Tulloch, unpublished work) show that the signal due to the terminal CH_3 of a hydroxy acid with the hydroxyl group on the penultimate carbon atom undergoes a downfield displacement of about 0.05 ppm on acylation.

Esters B are C_{56}-C_{64} diesters, mainly with the structure:

$$CH_3(CH_2)_{14}CO_2\overset{CH_3}{\overset{|}{C}H}\;(CH_2)_{13}CO_2CH_2(CH_2)_nCH_3 \quad (n = 22\text{-}30)$$

On hydrolysis they give three groups of components: acids (almost entirely palmitic acid), hydroxy acids together with a minor amount of diols, and C_{24}-C_{34} alcohols (approximately one molar proportion of each group). Diesters of 2-hydroxy acids and of 1,2-diols with chromatographic properties similar to esters B have recently been isolated from the skin surface lipids of rat (33) and other animals (N. Nicolaides, H. C. Fu and M. N. A. Ansari, this symposium).

Esters C and D consist partly, and esters E almost entirely, of C_{40}-C_{50} esters with a free OH group. These hydroxy esters are mainly composed of C_{24}-C_{34} alcohols esterified with 15-hydroxypalmitic acid but monoesters (mostly palmitates and lignocerates) of diols are probably also present. Hydrolysis of esters F and G gave higher proportions of hydroxy acids and diols than the other ester fractions indicating the presence of hydroxy diesters and triesters.

Palmitic acid was almost the only nonhydroxy acid obtained from esters A, B, C and F, but D and E gave lignoceric acid as well, and G gave C_{24}-C_{34} acids only; the free acids were C_{24}-C_{34} and contained no palmitic acid, in agreement with Brodie's conclusions (9). Hydroxypalmitic acid formed at least 80% of the hydroxy acids from B-G and the remainder was an assortment of longer chain hydroxy acids. GLC examination of the acetylated methyl hydroxypalmitates (34) showed that they consisted of mixtures of about 85% 15-acetoxypalmitate and 15% 14-acetoxypalmitate except for those from esters D, which had about 50% of each. None of the hydroxy acid samples were optically pure, most having $[\alpha]_D$ ~+ 2.0°. Thus there seems to be no evidence so far for the involvement of the yeast *Torulopsis* in the formation of the hydroxy acids.

Alcohols (C_{24}-C_{34}) were obtained from each ester fraction with only minor variations in the relative amounts of each alcohol. Diols from B to E were C_{24}-C_{28} with C_{24} the major component, but F and G gave C_{24}-C_{30} diols with C_{28} the major component. Esters with free carboxyl groups and free alcohols, suggested by Findley and Brown (30) were not detected in this investigation. Free alcohols are very minor components of unhydrolyzed beeswax (8).

The diols were shown to have the structure:

$$CH_3\underset{OH}{\underset{|}{C}H}(CH_2)_n\;CH_2CH_2OH \quad (n = 20\text{-}26)$$

by examination of their NMR spectrum and that of their acetates and by comparison of their GLC retention times with those of synthetic model compounds. With one primary

TABLE IV

Composition of Bumble Bee Wax[a]

Carbon[b] number	Hydrocarbons[c]		Esters[c,d]		
	Saturated[e]	Monoenoic[e]	Saturated[f]	Monoenoic[f]	Dienoic[f]
23	9	2	--	--	--
24	1	1	--	--	--
25	59	47	--	--	--
26	1	1	--	--	--
27	12	25	--	--	--
28	1	1	--	--	--
29	12	17	--	--	--
30	1	1	--	--	--
31	4	5	--	--	--
34	--	--	0.5	--	1
36	--	--	0.5	--	3
38	--	--	1.5	1	2
40	--	--	12	2	6
42	--	--	18	17	19
44	--	--	11	38	30
46	--	--	6	9	8
48	--	--	40	20	18
50	--	--	8	11	11
52	--	--	2	2	2
54	--	--	0.5	--	--
56	--	--	0.5	--	--
Per cent of[g] total wax	28	9	6	19	4

[a]Brood cells and honeypots from nests of *Bombus rufocinctus* supplied by G. A. Hobbs, Canada Department of Agriculture, Lethbridge, Alberta, were extracted with chloroform. The reddish orange wax formed 30% of the original weight; the residue consisted of insect debris and the paperlike walls of the cells. The wax has mp 35-45 C.

[b]Carbon numbers measured as before (40). GLC performed with an F & M model 402 gas chromatograph with flame ionization detectors. Column was ¼ in. x 3 ft glass column packed with 20-30 mesh glass beads coated with 0.3% silicone SE 30, He 45 ml/min, temperature programmed at 3 °/min from temperatures between 100-200 C to 325 C depending on sample. Other columns were used as before (40).

[c]Wax (2.17 g) on SiO_2 column (100 g Biosil A, Bio-Rad. Richmond, Calif.). Elution with hexane gave hydrocarbons (0.82 g) and with hexane containing 10-25% $CHCl_3$ gave esters (0.63 g). Polar fraction (0.73 g) obtained by elution with $CHCl_3$.

[d]Carbon numbers of esters are only tentative as hydrolysis products not fully characterized.

[e]Hydrocarbons (0.76 g) chromatographed on an $AgNO_3$-SiO_2 column (80 g, 17, 41). Elution with hexane gave alkanes (0.57 g) and with hexane containing 10% benzene gave alkenes (0.185 g). Alkenes (0.05 g) were oxidized with $KMnO_4$-$NaIO_4$ (42) and products analyzed by GLC (43).

[f]Esters (0.63 g) chromatographed on $AgNO_3$-SiO_2 column. Hexane-benzene (9:1) gave saturated esters (0.11g), hexane-benzene (3:2) gave monounsaturated esters (0.36), hexane-benzene (2:3) gave diunsaturated esters (0.075 g). Ethanolysis of esters and separation of resulting ethyl esters and alcohols on SiO_2 column was as previously described (40). Saturated esters (0.11g) gave ethyl esters (0.055 g) and alcohols (0.078 g), monounsaturated esters (0.36 g) gave ethyl esters (0.13 g) and alcohols (0.26 g), diunsaturated esters (0.084 g) gave ethyl esters (0.029 g) and alcohols (0.064 g).

[g]Remainder of wax (34%) was relatively polar, nonvolatile fraction. This fraction (0.45 g) gave ethyl esters (0.11 g), alcohols (0.05 g) and unidentified gum (0.27 g) on ethanolysis.

hydroxyl group and one at the penultimate position they could arise by reduction of the hydroxy acids though they contain at least 8-12 more carbon atoms (A.P. Tulloch to be published).

The variety of compounds obtained by saponification of beeswax, their peculiar chain length range, the difference in composition of the free and combined acids and the different proportions in which the components are combined to give esters A to G, all suggest complex biosynthetic pathways. Not surprisingly, there have been only a few reports dealing with the biosynthesis of beeswax.

When bees were fed 1-^{14}C-acetate the hydrocarbons and free acids of the wax were strongly labelled but the esters (and the acids and alcohols of which they were composed) were not appreciably labelled (35). It appeared that different wax components were synthe-

TABLE V

Yields Per Cent of Hydrolysis Products of Scale Insect Waxes[a]

Insect	Hydrocarbons	Alcohols	*n*-Acids	Hydroxy acids
Gascardia madagascariensis	0.6	28.0	38.0	33.4
Coccus ceriferus	2.6	47.4	50.0	---
Tachardia lacca	1.8	77.2	21.0	---
Icerya purchasi	26.9	32.3	31.4	9.4
Ceroplastes rusci	11.8	23.6	64.6	0
Pulvinaria flocifera	8.3	39.2	32.2	20.3
Quadraspidiotus perniciosus	9.8	14.7	75.5	0

[a]Faurot-Bouchet and Michel (52,53), with permission.

sized in different tissues. However, when 2-^{14}C-acetate was injected into the body cavity of honey bees, esters and free acids both became labelled in a few hours though the non-saponifiable portion of the wax was more heavily labelled than the acids (36).

Bumble Bee Wax

Wax produced by several species of bumble bee was examined by Sundwik. Wax from *B. muscarum* had mp 35-40 C (7) and this wax and wax from *B. terrestris* (37) gave long chain alcohols on hydrolysis. The alcohols were reported to give a neutral compound on treatment with strong alkali (38) in contrast to the alcohols of a plant louse wax which yielded acids. This could mean that the bumble bee wax alcohol was a secondary alcohol which was dehydrogenated to a ketone, or the neutral material could have been hydrocarbon impurities in the alcohols.

I have investigated wax extracted from honeypots and brood cells of *B. rufocinctus*, which is a native of western North America, and a relatively good wax producer (39). TLC (Fig. 1) shows that hydrocarbons and monoesters are major components, diesters B and esters C and D of beeswax are absent. The TLC pattern was hardly changed by diazomethane treatment of the wax showing that free acids are not present to any extent (methyl esters have an R_f similar to esters B). Fractionation on a silicic acid column gave hydrocarbons (37%), monoesters (29%) and a more polar fraction (34%). The procedures used are shown as footnotes to Table IV.

NMR spectroscopy showed the presence of unsaturated compounds with isolated double bonds (44) in the hydrocarbons but appreciable amounts of branched chain hydrocarbons were absent. The hydrocarbons were separated into alkanes and alkenes ($AgNO_3$-SiO_2) and analyzed by GLC with the results in Table IV. Unlike beeswax hydrocarbons the two fractions had similar chain lengths with the C_{25} hydrocarbon the principal component. Infrared spectroscopy showed that the alkenes were *cis* olefins and oxidative cleavage ($KMnO_4$-$NaIO_4$) gave heptanoic acid and C_{16}-C_{22} fatty acids showing that the double bond is at the 7,8-position. Beeswax olefins contain 10,11-unsaturation (15), but olefins with 7,8-unsaturation have been isolated from rose waxes (45). The composition of the hydrocarbons of bumble bee wax is of interest since Calam (46) has obtained saturated and unsaturated C_{21}-C_{25} hydrocarbons from the heads of males of several species of bumble bee.

The esters are also partly unsaturated and were separated into saturated, monoenoic and dienoic fractions by $AgNO_3$-SiO_2 chromatography. GLC analysis gave the results in Table IV.

Ethanolysis of the saturated esters gave mainly palmitate with a little stearate and a complex misture of saturated primary alcohols. NMR spectroscopy of these alcohols showed them to be branched chain compounds (44) with probably as many as four methyl branches. They may be related to derivatives of the dihydrofarnesols recently isolated from bumble bees (47).

Ethanolysis of the monounsaturated esters gave mainly oleate and saturated primary alcohols which were largely straight chain. The principal alcohols were tentatively identified as tetracosanol and hexacosanol and the minor alcohols as odd-numbered C_{19}-C_{23} alcohols. The components of the diunsaturated esters were not identified.

Ethanolysis of the most polar wax fraction gave a complex misture of esters and alcohols (~30% of weight). The other products were not identified but GLC analysis and NMR spectroscopy showed that 15-hydroxypalmitic acid was absent. There is thus no evidence that yeast

has been involved in hydroxy acid formation in this wax either.

Though wax of only this one species of bumble bee has been investigated in any detail, the available evidence, as mentioned later, at least shows that the physical properties of the waxes of a number of species are similar so that a provisional comparison of bumble bee wax and honey bee wax can be made. My investigation shows that bumble bee wax has a complex composition but one that is considerably different from that of beeswax. The principal differences are as follows:

1. Beeswax contains appreciable proportions of difunctional components, the hydroxy acids and diols, so that about half of the beeswax esters are diesters (or higher esters, or hydroxy esters). Difunctional components are apparently absent from bumble bee wax.

2. Beeswax components are largely straight chain and saturated, the alcohols having mainly 30-32 carbons. Bumble bee wax components are more unsaturated, some are branched chain compounds and the alcohols and hydrocarbons generally contain 4-6 carbons less than the corresponding beeswax components. The physical properties of the waxes are naturally different, particularly the melting point, that of bumble bee wax being about 25 C lower than that of beeswax.

Waxes of Scale Insects

Some scale insects produce enough wax to be commercially important; these are the Chinese wax insect (*Coccus ceriferus*) and the lac insect (*Tachardia lacca*). *C. ceriferus* (in the family Coccidae) is (or was) cultivated in China on branches of the Chinese ash; the insects infest the twigs so closely that they are covered with a thick layer of wax which can be scraped off (2). *T. lacca* (family Lacciferidae) is cultivated on trees in India and is important as the source of lac from which shellac is derived. Crude lac is composed mainly of a resin of cross-linked hydroxy acids, but 5-10% of wax is also present.

Chinese insect wax, was first investigated by Brodie (9) who concluded that it consisted almost entirely of a long chain ester of a long chain alcohol. Lac wax, as a by-product of the shellac industry, contains varying amounts of free alcohols depending on the method used to separate wax from shellac (13). Gascard (11) showed that lac wax and Chinese wax gave long chain acids and long chain alcohols on hydrolysis and these were later found to be C_{26}-C_{30} in the case of Chinese wax and C_{30}-C_{34} in the case of lac wax (13,48,49).

Another commercially interesting scale insect is *Coccus cacti*, the cochineal insect, which lives on a species of Cactus in Mexico and covers itself with a thick layer of hard wax. The wax gives 15-oxotetratriacontan-1-ol and 13-oxo C_{30} and C_{32} acids on hydrolysis (50).

A number of other scale insect waxes have been investigated, particularly in Japan (2), and long-chain monoesters seemed to be the major components of most of them. Wax of *Tachardina theae* (family Lacciferidae) was unusual in yielding 9-dodecenoic and 9-tetradecenoic acids on hydrolysis (51), though these acids may have been derived from glycerides of the body lipids rather than from the waxy shell.

The hydrolysis products of waxes of seven species of scale insect have been separated and analyzed by GLC by Faurot-Bouchet and Michel (52,53) with the results in Tables V to VII. Appreciable amounts of hydroxy acids were obtained from the waxes of *Gascardia madagascariensis* (family Lacciferidae), *Icerya purchasi* (the cottony cushion scale, family Margaroididae or ground pearl) and *Pulvinaria flocifera* (family Coccidae). These three and that of *Coccus ceriferus* also gave approximately equal amounts of acids and alcohols but the waxes of *Ceroplastes rusci* (family Coccidae) and *Quadraspidiotus perniciosus* (the San José scale which attacks deciduous fruit trees, family Diaspididae) gave a large excess of acids and *Tachardia lacca* a large excess of alcohols [as reported earlier by Chibnall (13)].

Hydroxy acids from *G. madagascariensis* were a mixture of C_{30}-C_{34} acids with the hydroxyl group somewhere near the middle of the chain. The other hydroxy acids were not investigated.

Hydrocarbons of the waxes were odd-numbered with 25-35 carbons, the principal components were either C_{27}, C_{29}, C_{31} or C_{33}. In agreement with earlier conclusions of Chibnall et al. (13,49), the acids and alcohols of Chinese insect wax were C_{26}-C_{28} compounds and of lac wax were C_{28}-C_{34}. The original esters of the former wax would then be mainly C_{52} and of the latter C_{56}-C_{62} esters. Acids and alcohols of the other waxes (Table VII) were similar, being mainly C_{26}-C_{30} compounds. Waxes, which gave hydroxy acids on hydrolysis, were probably more complex, perhaps more like beeswax.

There have been conflicting reports about the wax of *Ceroplastes pseudoceriferus*; Hashimoto and Mukai (54) found mainly C_{26} acid and alcohols after hydrolysis, but Tamaki (55) found most of the alcohols to be branched or cyclic and that di- and triunsaturated C_{18} acids were present in addition to saturated C_{26} and C_{28} acids; resin acids were also present.

TABLE VI

Constituents of Coccid Waxes in Per Cent of Each Group[a]

No. of C atoms	*Gascardia madagascariensis*			*Coccus ceriferus*			*Tachardia lacca*		
	Hydro-carbons	Alcohols	Nonhydroxy acids	Hydro-carbons	Alcohols	Nonhydroxy acids	Hydro-carbons	Alcohols	Nonhydroxy acids
10									0.1
11									0.2
12									0.1
13									0.3
14						6.9	--	--	1.0
16	--	--	0.4	--	--	2.4	--	--	3.4
18	--	--	0.1	--	--	3.4	--	--	0.4
20	--	--	0.1	--	--	Traces	--	--	0.3
22	--	--	Traces	--	--	1.2	--	--	0.2
24	--	4.6	0.5	--	7.0	14.4	--	--	0.2
25	3.4	--	--	3.9	--	--	--	--	--
26	1.4	72.0	26.0	0.6	63.0	49.0	--	0.6	0.3
27	72.0	--	--	5.2	--	--	42.0	--	1.2
28	2.2	11.6	4.4	1.0	28.0	15.5	4.1	66.6	18.2
29	20.0	--	--	7.6	--	--	35.1	--	1.7
30	--	5.9	5.8	2.7	2.0	5.6	2.8	21.0	25.1
31	1.0	--	--	42.1	--	--	13.4	--	1.1
32	--	5.9	19.8	1.0	--	1.5	Traces	9.0	27.2
33	--	--	0.1	29.2	--	--	2.6	--	0.2
34	--	--	39.5	0.5	--	--	--	2.8	17.6
35	--	--	--	6.2	--	--	--	--	--
36	--	--	3.2	--	--	--	--	--	0.5

[a]Faurot-Bouchet and Michel (52), with permission.

TABLE VII

Constituents of Coccid Waxes in Per Cent of Each Group[a]

No. of C atoms	*Icerya purchasi*			*Ceroplastes rusci*			*Pulvinaria flocifera*			*Quadraspidiotus perniciosus*		
	Hydro-carbons	Alcohols	Acids	Hydro-carbons	Alcohols	Acids	Hydro-carbons	Alcohols	Acids	Hydro-carbons	Alcohols	Acids
14	--	--	--	--	--	--	--	--	2.5	--	--	--
16	--	--	1.1	--	--	1.9	--	--	11.7	--	--	14.7
18	--	--	4.7	--	--	4.2	--	--	1.9	--	--	4.4
20	--	--	6.1	--	--	5.7	--	--	1.8	--	--	4.7
22	--	0.9	9.4	--	--	--	--	--	1.0	--	0.4	2.9
24	--	7.3	7.4	--	7.0	1.4	--	--	1.7	--	2.7	8.0
25	0.8	--	--	1.0	--	--	3.3	--	--	--	--	--
26	--	49.2	36.4	1.0	81.2	1.2	--	4.1	2.9	--	77.8	12.5
27	7.8	--	--	58.0	--	--	10.6	--	--	1.1	--	--
28	--	33.8	22.7	2.7	11.8	0.9		50.3	48.2	--	19.1	26.4
29	50.4	--	--	26.4	--	--	33.6	--	--	5.9	--	--
30	--	8.8	10.7	1.7	--	15.2	--	35.9	19.8	--	--	12.5
31	32.3	--	--	9.2	--	--	17.2	--	—	72.1	--	--
32	--	--	1.1	--	--	56.3	--	9.7	8.0	2.7	--	12.5
33	8.4	--	--	--	--	--	3.9	--	--	18.2	--	--
34	--	--	0.4	--	--	13.2	--	--	0.5	--	--	1.4
35	0.3	--	--	--	--	--	--	--	--	--	--	--

[a]Faurot-Bouchet and Michel (53), with permission.

TABLE VIII

Melting Points of Some Waxes Secreted by Insects

Wax	Melting point, C
Bumble bee (Ref. 7 and this work)	35-45
Honey Bee (2)	63-65
Chinese Insect (2)	82-84
Lac Wax (2)	72-82
Icerya purchasi (2)	78
Coccus cacti (50)	99-101

But later Hashimoto et al. (56) stated that, while the saturated esters of this wax were true wax esters, containing long straight-chain acids and alcohols, the unsaturated esters were oleates and linoleates of branched (diterpenoid etc.) alcohols. A report that wax of the Comstock mealy bug *Pseudococcus comstocki* (family Pseudococcidae or mealy bugs) gives 10-18% of tetradecanedioic acid (57) on hydrolysis, seems to be the first mention of dicarboxylic acids in waxes secreted by insects.

There seem to have been no investigations of the biosynthesis of waxes of scale insects.

Function of Waxes Secreted by Bees and Scale Insects

Waxes produced by these two groups of insects have entirely different functions but, in both groups, production of large amounts of wax is related to the specialized way of life adopted by the insects. Wax is secreted in wax glands which consist of one or more specialized cells at or near the surface of the abdomen.

Wax of Bees. Honey bees use wax to build the familiar honey comb. Wax is chewed by worker bees until soft and molded piece by piece to form the network of hexagonal cells. Larvae are reared in cells of the comb, different sized cells being used for workers, males and queens. Cells are also used to store honey and pollen.

Since the structural basis of the cell consists only of wax, the wax must have suitable physical properties. Species of *Apis* occur in many tropical countries so that the melting point of the wax must be reasonably high; in most cases it is 62-65 C. Presumably some degree of plasticity and kneadability are also desirable. The unsaturated hydrocarbons of beeswax may act as plasticizers.

The nest of the bumble bee is usually on or under the ground and is much less elaborate than that of the honey bee. It consists of a small group of rounded cells in which the larvae are raised and a few honeypots to store honey. The cells are constructed of wax (58,59), or from a mixture of pollen and wax (60). The larvae also spin cocoons which are later coated with wax and converted to honeypots. Sladen (58), presumably referring to *B. lapidarius* and *B. terrestris*, remarked that the wax was much softer than that of the honey bee, I have found that waxes of *B. rufocinctus* and *B. flavifrons* have mp 35-45 C and Sundwik (7) gave mp 35-40 C for wax of *B. muscarum*.

Bumble bees are commonly found only in temperate climates, the nest temperature rarely exceeding 35 C (61); Hobbs (personal communication) has suggested that this probably accounts for the much lower melting point of bumble bee wax compared to honey bee wax. Also the relatively simple nest does not require a hard strong wax.

Wax of Scale Insects. All scale insects, as members of the order of bugs, have the mouth parts modified for piercing and sucking up fluids. The adult females are degenerate, frequently having lost their legs, and are attached to the host plant by the mouth parts. It is probably because they are stationary that many species protect themselves with a waxy covering. The wax may also protect the eggs and young insects; lac of the lac insect has a similar function. In general scale insects require a hard, high melting wax (particularly as many occur in hot climates) to protect them from insect predators and from the weather.

There is considerable variation in the way in which the wax is attached to the insect and some do not have a true scale. The San José scale (*Q. perniciosus*) has a hard scale of wax and cast skins which shelters the insect and its eggs. The female of *C. ceriferus* and of some species of *Ceroplastes* is covered with thick plates of wax. Other species, such as *C. cacti* and *Pulvinaria spp.*, excrete a cottony mass of wax in which the eggs are laid. Others still have powdery lumps of wax on the surface, examples of these are *I. purchasi* and the Comstock mealie bug (and mealie bugs in general, as their name implies).

One interesting problem which apparently has not been solved is that of how the insect can exude a very high melting wax. This problem applies to honey bees as well as to scale insects although, as Table VIII shows, the latter have the highest melting waxes. Wax of *C. cacti* has a melting point as high as 100 C.

Wax presumably exudes through pores, but this has been disputed in the case of the honey bee (35). Some insects exude cuticle wax containing a volatile solvent (1), but there is no evidence that this method is used by bees or scale insects. It would probably require too much solvent. Beeswax is exuded as a liquid

and hardens to a waxy scale (2). This may be true of all the high melting insect waxes though the manner in which it occurs is not understood. Polymerization and cross linking of unsaturated components cannot be the explanation as such components are found to only a small extent.

CONCLUSION

Though only a minute fraction of the total number of insects has been investigated, it is clear that there is considerable variation in complexity of composition of waxes secreted by insects. All reports indicate that Chinese insect wax has a simple composition (consisting mainly of C_{52} monoester), but some of the other waxes, particularly those of bees and bumble bees, contain a very large number of components. Generally, when they are investigated carefully by the most modern methods, waxes are found to be more complex than was originally thought. In addition to straight chain saturated components, several series of unsaturated and branched chain components may be present, thus it was not until very recently that the exact nature of the hydrocarbons of beeswax was established (15-17). Before any biosynthetic investigations can be carried out, it would seem essential that the exact nature of the major groups of components be established.

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Plant Waxes[1]

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ABSTRACT

The surface of plants is covered with a complex mixture of lipids, often in crystalline form, called plant waxes. The chemistry, biosynthesis, catabolism and function of plant waxes are reviewed. The most common components are hydrocarbons, wax esters, free fatty alcohols and acids. Ketones, secondary alcohols, diols, aldehydes, terpenes and flavones are also found. The major function of the wax appears to be protection of the organism from water loss and other hazards of the environment. The alkanes are formed from fatty acids either by elongation followed by decarboxylation or by head-to-head condensation between two biochemically dissimilar fatty acids followed by specific decarboxylation of one of them. Fatty acyl-CoA is reduced to the aldehyde which in turn is reduced to the alcohol. The alcohol is then esterified with acyl moieties from acyl-CoA or phospholipids. Plant waxes undergo very little catabolism in plants but animals can degrade them to a limited extent and microorganisms readily degrade them.

INTRODUCTION

The main emphasis of this paper will be on the biochemistry of plant wax components. I shall introduce it with a brief summary of the types of compounds most often encountered in plant waxes and conclude with some possible functions of waxes in plants.

The plant cuticle (Fig. 1) consists of a meshwork of polymerized hydroxy fatty acids, called cutin, which is embedded in wax. As described almost a century ago (1), in many plants, such as cabbage, wax is also found in the form of a bloom on the surface. Electron microscopy has recently been used to study the ultra structure of wax deposition (2-5) and its modification by environmental factors and chemical treatment (6-8). The shape of the wax crystals on the surface appears to be characteristic of the plant species (Fig. 2). The specific pattern of distribution of wax constituents has been used in chemotaxonomy (9-16).

Waxes occur in plants primarily on the surface of leaves, fruits and stems. However, smaller quantities of waxy materials have been reported in almost all plant parts: flowers (17-23), roots (24,25), bark (26,27), cork (28-30), wood (31-33), seed oils (34-38), seed coats (39), and even in cell organelles (40).

Plant waxes such as carnauba wax have been used for the manufacture of polishes, candles, etc., for centuries, but investigations into the chemistry of these compounds began only early in this century. The early literature is concerned mainly with such parameters as iodine number and saponification number. Even when the efforts were directed towards separation and identification of individual components in the wax, the techniques available for this purpose posed insurmountable difficulties to the early investigators. Therefore some of the early identifications should be taken with caution.

In spite of such technical inadequacies, pioneers such as Chibnall et al. (41-53) successfully identified components of many plant and animal waxes. With the advent of chromatographic techniques, especially gas liquid chromatography, (GLC) the identification of wax components has become almost a routine matter. Furthermore, technical developments in the area of mass spectrometry, in conjunction with GLC have greatly facilitated structure determination of the components.

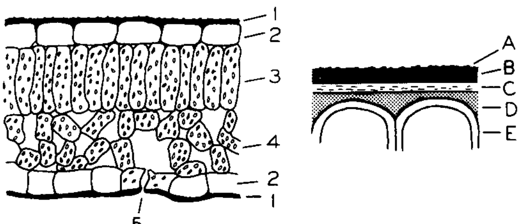

FIG. 1. Left: Cross section of a leaf. 1. Cuticle with surface wax. 2. Palisade cells. 3. Spongy tissue with many air filled spaces (2 and 3 together make up mesophyll tissue). 4. Stomata, the pores through which the exchange of CO_2 and water vapor occurs. Right: Schematic representation of the structure of leaf cuticle. A. Surface wax. B. Cutin embedded in wax. C. A mixed layer containing some cutin, wax and carbohydrate polymers with possibly traces of protein. D. Pectin. E. Cellulose wall of the epidermal cells. (Courtesy of K. C. Clark, Conn. Agr. Expt. Station, New Haven, Conn.).

[1]One of six papers to be published from the Symposium on Natural Waxes, presented at the AOCS Meeting, San Francisco, April 1969.

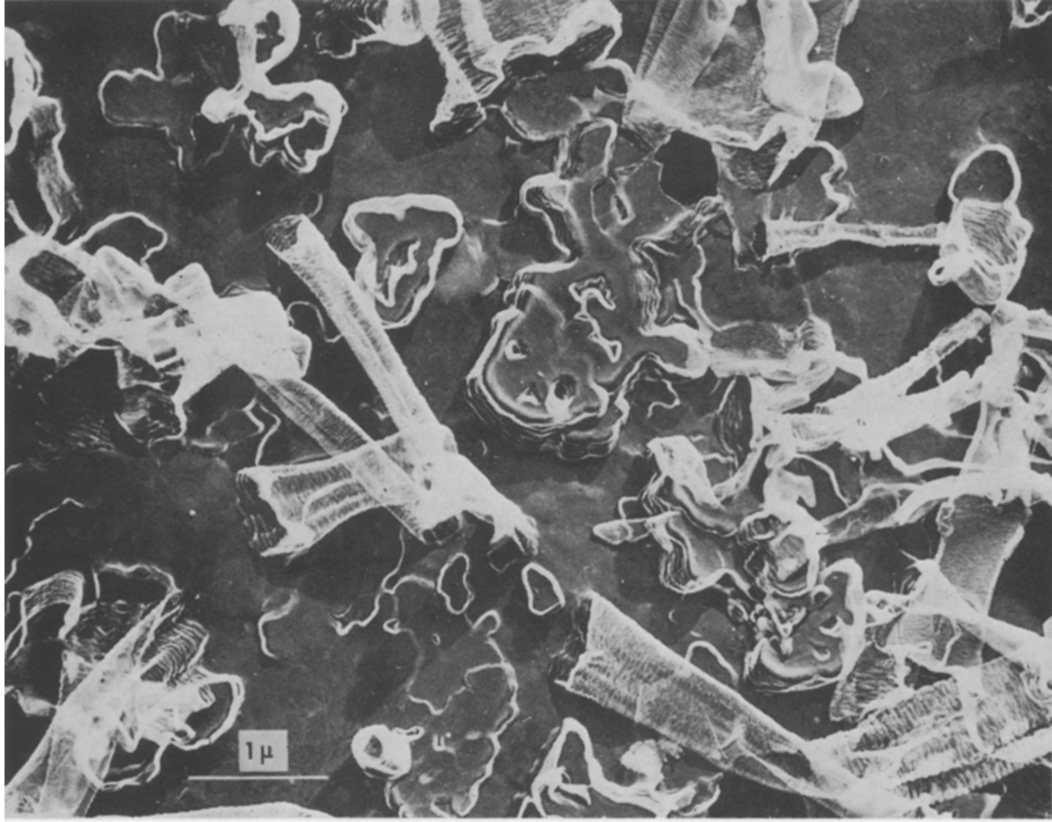

FIG. 2a. Electron micrograph of cabbage "January King" adaxial leaf magnification (X 33,000) (reduced approximately 45%).

CHEMISTRY

Since the chemistry of plant waxes has been reviewed several times in many contexts in recent years (54-57) I shall discuss the matter only briefly. The most common plant wax components are hydrocarbons, wax esters, free fatty alcohols and fatty acids. Table I summarizes the composition of wax obtained from a leaf, a fruit, and a stem.

Usually the plant wax hydrocarbon fraction represents a mixture of *n*-alkanes with 25 to 35 carbon atoms with an overwhelming predominance of odd chains. Very often 90% or more of the paraffin fraction is C_{29} or C_{31}. In this respect, plant waxes are very much simpler than their counterparts in the animal and microbial systems (other papers in this symposium). There are many exceptions to this generalization with respect to the chain lengths, structure and proportion of components. Alkanes much shorter than C_{25} have been reported in some instances, although the more volatile hydrocarbons are likely to be lost from the plant surface as well as during the usual isolation procedures. In most algae, *n*-C_{17} alkane is the major component (58-64). On the other hand much longer hydrocarbons may also be found occasionally–alkanes as long as *n*-C_{62} were reported to be present in a cane grass wax (65). The relative insolubility of such hydrocarbons in the usual organic solvents reduces the chances of detecting them. *Iso* (2-methyl) and *anteiso* (3-methyl) branched alkanes are also found in substantial proportions (up to half) in some leaf waxes such as tobacco (38,66,67) and in many other plants (17,68-72). The *iso* alkanes usually have an odd number of carbon atoms but *anteiso* alkanes usually contain an even number. Exceptions are the *anteiso*-C_{21}, *iso*-C_{20} and *iso*-C_{22} of lilac blossom, and the *iso*-C_{32} of rose and *iso* C_{34} of lavender blossom (17). Alkanes with cyclic structures have been reported (36,73,74). Internal and multiple branches are rare in plant waxes (37,69,75), but recently 4,-7,- and 8-methyl-heptadecanes were identified in some species of blue green algae (64). Unsaturated hydrocarbons occur in some plants such as in

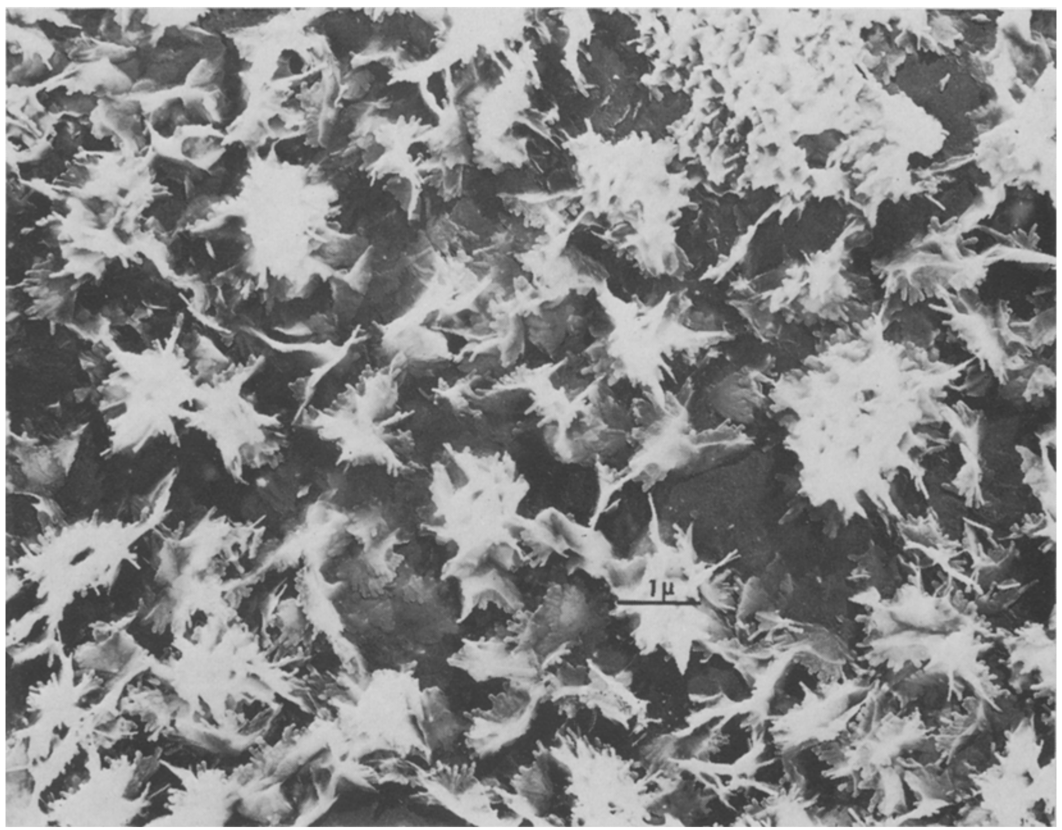

FIG. 2b. Electron micrograph of *Eucalyptus Cloeziana* adaxial leaf magnification (X 20,000) (reduced approximately 45%).

the *Rosaceae*, but only as relatively minor components (17,59,61,68,69,76). Table II summarizes data on olefins found in some plant waxes. Straight chain terminal, *cis*-3, *cis*-5, *cis*-7, *cis*-9, *cis*-10 and *trans* monoenes, branched alkenes, and di- and trienes have been reported.

Table III gives structural formulas of keto and hydroxy compounds most often encountered in plant waxes. Ketones of chain length comparable to the hydrocarbons occur in many plant waxes, the carbonyl group being often in the middle of the chain. For example, nonacosan-15-one (42,52) and hentriacontan-16-one (48,80) are found in waxes where nonacosane and hentricontane, respectively, are the major paraffins. In certain plant tissues, such as rose petals where more complex mixtures of hydrocarbons are found, mixtures of ketones also occur (65,81). In some species such as Eucalyptus, β-diketones are the major components of the wax. The β-diketones, unlike the monoketones, do not correspond in chain length to the major paraffins of the same tissue (82-84). The most common β-diketone appears to be tritriacontan-16,18-dione. Hydroxy derivatives of β-diketones also appear in some plant waxes (85). Secondary alcohols corresponding to the ketones (43,52,86-91), and hydroxy ketones are occasionally encountered (48,92). Occurrence of intermediate length alkan-2-ols (C_{11}, C_{13}, C_{15} and C_{17}) in Eucalyptus wax (84) is reminiscent of the methyl ketones observed in soils (93).

The wax esters are usually made up of *n*-alkanoic acids, and *n*-alkan-1-ols mostly with even numbers of carbon atoms, usually in the range of C_{12} - C_{32}. Unlike animal and microbial waxes, branches and double bonds in this fraction are rare. Other derivatives of fatty alcohols such as glycosides occur in some algae (94). The free fatty acids and fatty alcohols usually correspond in structure to those in the wax esters (Table I). Also occasionally found are many other derivatives of alkanols and alkanoic acids such as α, ω-diols (95,96)

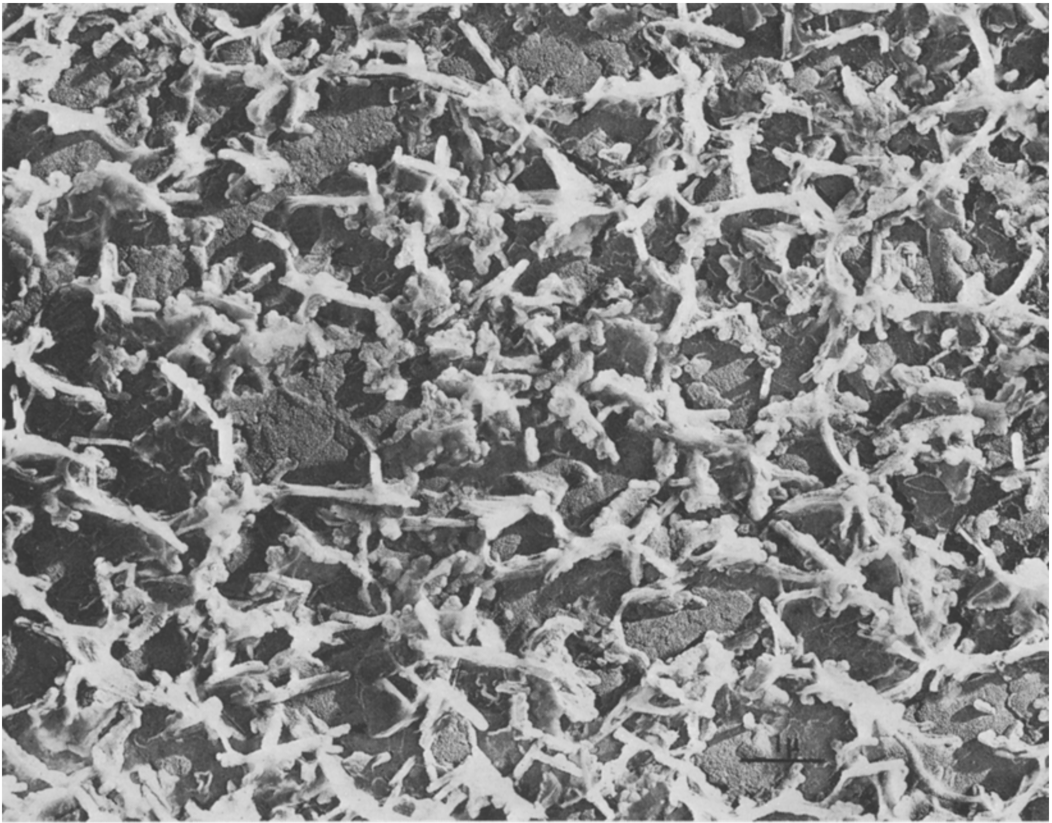

FIG. 2c. Electron micrograph of *Zea mays* adaxial leaf magnification (X 20,000) (reduced approximately 45%).

α-hydroxy, and ω-hydroxy acids (30,65,95,97,98). In recent years the occurrence of aldehydes in many plant waxes has been established (99-101) although they must be unstable when exposed constantly to the atmosphere. Even polymeric aldehydes of the trioxane type have been reported (103).

A variety of alicyclic compounds occur in plant waxes, especially those from fruits and stems. The most common among them are triterpenes (104), such as ursolic acid and oleanolic acid, which occur as major components in the wax of some fruits including apple (105) and grape (101,102). Other components such as mono-, di- and sesquiterpenes, polyisopredoids, and flavones have also been reported occasionally (106-111). In rare cases, such as banana leaf wax, aromatic hydrocarbons of the anthracene and phenanthrene type have been found (112). Olive oil appears to contain a series of polycyclic aromatic hydrocarbons of unknown origin (113).

Analysis of the total nonsaponifiable fraction from many plant lipids often reveals many other components such as sterols and quinones (20).

BIOSYNTHESIS OF PARAFFINS

The first investigation of paraffin biosynthesis was the demonstration of ^{14}C-acetate incorporation onto *n*-heptane in *Pinus gifferii* (114). The paraffins of apple fruit wax (n-C_{29}), on the other hand, did not become radioactive under conditions which allowed incorporation of ^{14}C-acetate into all other wax components (115). However, labeled acetate was readily incorporated into cabbage and broccoli leaf waxes, mostly into the C_{29} compounds (116). Since then labeled precursors were shown to be incorporated into the wax paraffins of pea, spinach, *Senecio odoris* and tobacco plants (117-121).

The Classical Head-to-Head Condensation Mechanism

The demonstration that actively expanding leaves readily incorporate exogenous labeled precursors into hydrocarbons (116) made pos-

TABLE I

Per Cent of Major Constituents in Each Fraction

Fraction	Per cent in total waxes	C_{12}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}	C_{23}	C_{24}	C_{25}	C_{26}	C_{27}	C_{28}	C_{29}	C_{30}	C_{31}	C_{32}	C_{33}	C_{34}	C_{35}
Cabbage leaf (92)																				
Paraffins	36.0	---	---	---	---	---	---	---	---	---	---	1	1	93	1	3	---	---	---	---
Esters { alcohols	12.6	14	14	---	---	---	14	---	8	---	52	---	14	---	---	---	---	---	---	---
Esters { acids		14	28	---	28	---	8	---	8	---	---	---	---	---	---	---	---	---	---	---
Primary alcohols	8.7	6	---	---	24	6	12	---	6	---	36	---	6	---	---	---	---	---	---	---
Free acids	9.2	24	---	24	---	41.5	5.5	---	5.5	---	---	---	---	---	---	---	---	---	---	---
Secondary alcohols	11.1	---	---	---	---	---	---	---	---	---	---	---	---	100	---	---	---	---	---	---
Ketones	13.8	---	---	---	---	---	---	---	---	---	---	---	---	100	---	---	---	---	---	---
Ketols	0.9	---	---	---	---	---	---	---	---	---	---	---	---	100	---	---	---	---	---	---
Grapes berry (102)[a]																				
Paraffins	1	---	T	T	T	T	T	5.7	3.5	17.2	3.8	19.5	2.6	22.1	2.4	14.8	1.1	1.9	---	0.4
Alcohols { free	40	---	---	---	---	0.1	1.3	1.2	14.2	5.7	42.6	5.3	21.3	4.4	3.3	---	T	---	T	---
Alcohols { esterified	9	---	---	---	---	0.2	1.7	1.7	11.6	6.2	44.4	6.6	20.7	2.5	1.2	---	T	---	---	---
Aldehydes	12	---	---	0.2	0.8	1.7	0.4	0.6	12.4	2.8	41.7	2.5	21.8	1.1	7.5	0.5	2.8	---	---	---
Acids { free	7	---	0.4	4.6	9.5	12.2	7.8	1.4	12.8	2.6	18.0	1.2	10.6	1.0	3.3	2.3	2.0	---	---	---
Acids { esterified	9	---	0.4	5.3	18.1	31.7	18.0	1.5	9.8	1.4	3.9	0.7	2.3	0.7	1.2	0.3	1.2	---	---	---
Sugar cane (99)																				
Paraffins	8.5	---		5.4		0.4	0.4	0.8	1.1	7.0	4.9	55.7	2.9	12.8	2.2	4.4	---	1.6	---	---
Alcohols { f	26	---	---	---	---	---	---	---	0.6	0.4	15.0	4.6	72.1	1.7	3.5	0.9	1.2	---	---	---
Alcohols { s		---	---	---	---	---	---	---	0.6	0.2	13.1	2.7	73.0	1.5	6.3	1.0	1.6	---	---	---
Acids	10	---	---	2.9	1.8	1.2	1.9	0.6	2.5	1.9	8.2	8.0	50.5	2.4	8.1	1.0	4.4	1.4	2.5	---
Aldehydes	50	---		0.6		---	---	---	0.25	0.2	7.35	1.7	66.2	2.1	12.25	1.6	4.2	---	3.45	---

[a]Remaining 22% was oleanolic acid. The wax from fresh grape berries was extracted with light petroleum in which oleanolic acid is only slightly soluble; thus the triterpene acid which constitutes more than half of the cuticular wax is underestimated here; f, isolated free alcohols; s, isolated after saponification and thus might include some alcohols derived from the aldehydes as well as those derived from a little wax ester that might have been present in the wax.

TABLE II

Olefins in Plant Waxes

Olefin type	Per cent	Range	Major	Source
Alk-1-ene	5.9	C_{19}-C_{31}	C_{27}	*Senedesmus*
		C_{20}-C_{33}		Sugar cane, Roses
Alk-2-ene (*trans*)		C_{20}-C_{33}		Sugar cane
Alk-3-ene (*cis*)	5.86	C_{17}-C_{33}	C_{27},C_{29},C_{31}	Rose petal
Alk-5-ene, Alk-7-ene, Alk-9-ene (*cis*)	0.5-40.7	C_{17}-C_{33}	C_{27},C_{29},C_{31}	Roses
Alk-10-ene	20	C_{15}-C_{33}	C_{31},C_{33}	Sugar cane
Alk-monoene	61.9	C_{17}-C_{33}	C_{27}	*Anacystis*
Alk-diene	92.8	C_{21}-C_{27}	C_{21}	*Anacystis*
		C_{21}-C_{33}	C_{29},C_{31}	*Botryococcus*
Alk-triene	5.6	C_{25}-C_{29}	C_{25}	*Botryococcus*

sible study of the mechanism of paraffin biosynthesis. When Clenshaw and Smeady-Maclean discovered n-C_{31} alkane in spinach, they suggested a head-to-head condensation between two molecules of n-C_{16} acid as a plausible biosynthetic route for this alkane (122) (Fig. 3a). The occurrence of n-nonacosan-15-one and n-nonacosane in *Brassica oleracea* prompted Channon and Chibnall (42) also to suggest a similar head-to-head condensation mechanism with two molecules of n-C_{15} acid (Fig. 3b). Although the absence of n-C_{15} acid in plants led them to discard this hypothesis (47), the many gas chromatographic demonstrations of the occurrence of odd chain acids in leaves and the relatively recent discovery of active α-oxidation systems in young leaves (123,124) made the Channon-Chibnall hypothesis for the C_{29} synthesis more plausible (116). As expected from such a hypothesis (Fig. 3b) the methyl-carbon of acetate (116) and C_2 of palmitate (125) gave rise to the carbonyl carbon of the ketone. According to this pathway the carboxyl carbon of n-C_{16} acid should not be incorporated into the C_{29} compounds. Thus 1-^{14}C-palmitic acid in comparison to U-^{14}C-palmitic acid should label the C_{29} compounds very poorly at best. However, incorporation of ^{14}C into the C_{29} compounds from carboxyl-labeled n-C_{16} acid was identical to that from uniformly

TABLE III

Structures of Carbonyl Compounds and Derivatives in Plant Waxes

$C_{14}H_{29}COC_{14}H_{29}$
$C_{14}H_{29}CH(OH)C_{14}H_{29}$
$C_9H_{19}CH(OH)C_4H_8COC_{14}H_{29}$
$C_{15}H_{31}COCH_2COC_{15}H_{31}$
$C_{15}H_{31}COCH_2COC_4H_8CH(OH)C_8H_{17}$

labeled C_{16} acid. This shows that the carboxyl-carbon of C_{16} acid was not lost during its incorporation into C_{29} compounds (117). Experiments with ^{3}H and ^{14}C labeled C_{16} acid showed that the exogenous C_{16} acid was not degraded to acetate prior to its incorporation into the C_{29} compounds. Such results are clearly inconsistent with the condensation hypothesis shown in Figure 3.

The hypothetical intermediate of the condensation route for n-C_{31} alkane biosynthesis, hentriacontan-16-one, has been found in some plant waxes (48,80). If two molecules of C_{16} acid condense as shown in Figure 3a, one of them should lose its carboxyl carbon. Thus incorporation of label into the paraffin from 1-^{14}C-palmitic acid should be 50% of that from U-^{14}C-palmitic acid or internally labeled n-C_{16} acids. Young pea and spinach leaves readily incorporated fatty acids into n-C_{31} alkane (120), but in both tissues carboxyl-labeled C_{16} acid was just as efficiently incorporated into the n-C_{31} alkane as was uniformly labeled C_{16} acid. Thus the C_{16} acid did not lose its carboxyl-carbon during its incorporation into the paraffin. Such results are not consistent with the condensation hypothesis shown in Figure 3, unless the two condensing fatty acids are biochemically nonidentical (a possibility discussed later).

One of the most attractive aspects of the condensation mechanism is that the hypothetical intermediates, the symmetrical ketone and the corresponding secondary alcohol are found in some plant waxes. However, all attempts to show interconversion of the ketone, secondary alcohol and alkane in *Brassica oleracea* both in vivo and in vitro failed. For example, an emulsion of labeled nonacosan-15-one applied to the surface of a young broccoli leaf for even as long as four days did not undergo detectable conversion

$$\begin{matrix} C_{14}H_{29}CH_2\text{-}\overset{\bullet}{C}(=O)\text{-}X \\ C_{14}H_{29}CH_2\text{-}C(=O)\text{-}X \end{matrix} \xrightarrow{{}^{\bullet}CO_2} C_{14}H_{29}CH_2\text{-}\overset{\bullet}{C}(=O)\text{-}CH_2C_{14}H_{29} \longrightarrow C_{14}H_{29}CH_2\overset{\bullet}{C}H_2CH_2C_{14}H_{29}$$

PALMITIC ACID PALMITONE n-HENTRIACONTANE

$$C_{14}\text{-}C\text{-}\overset{\bullet}{C}OO \xrightarrow{\overset{\bullet}{C}O_2} C_{14}\text{-}COO \xrightarrow[+C_{15}]{CO_2} C_{14}\text{-}C(=O)\text{-}C_{14} \longrightarrow C_{14}\text{-}CH(OH)\text{-}C_{14} \longrightarrow C_{14}\text{-}CH_2\text{-}C_{14}$$

FIG. 3. (a) Head-to-head condensation mechanism with two molecules of palmitic acid [based on the suggestions of Clenshaw and Smedley-Maclean (122)]; (b) head-to-head condensation mechanism with two molecules of n-C_{15} acid producing the three n-C_{29} compounds of *Brassica oleracea* [an extension of the suggestion by Channon and Chibnall (42)].

into any other wax component (117). The time course of incorporation of labeled acetate into the three C_{29} compounds of broccoli wax did not show any precursor-product relationship. At all times the alkane had the highest specific activity closely followed by the ketone. The secondary alcohol, on the other hand, had a much lower specific activity (117). Confirming such observations the ketone fraction of tobacco leaf was also less radioactive than the alkanes (126). Although the presence of large metabolically inert pools on the surface may complicate matters and enzyme-bound intermediates are possible, the free ketone and secondary alcohol do not act like intermediates in alkane synthesis.

It is crucial for the condensation hypothesis that the carbonyl group of the ketone be derived from the carboxyl group of one of the condensing fatty acids. But the intact carbon chain of n-C_{16} acid was incorporated into nonacosan-15-one (117) with the C_2 of palmitate becoming the carbonyl-carbon of the ketone (125). Furthermore the intact carbon chain of n-C_{18} acid was incorporated into the ketone fraction of broccoli wax three times as well as the C_{16} acid (117,127). Beckmann rearrangement of the oxime prepared from the ketone derived from labeled n-C_{18} acid gave a substituted amide, which on hydrolysis gave exclusively labeled C_{15} acid, showing that the ketone was in fact nonacosan-15-one (127). Thus the carbonyl group of the ketone is not derived from the carboxyl group of one of the condensing fatty acids, a conclusion contrary to the mechanisms shown in Figure 3. Instead it appears that the carbonyl group is introduced into a prebuilt chain of carbon atoms (Fig. 4). Palmitone in the leaves of *Anona senegalensis* was suggested to be formed from n-C_{32} acid by dehydrogenation and hydration followed by oxidation of the resulting secondary alcohol followed by decarboxylation (80). Dehydrogenation at a specific position in a saturated alkane in Nocardia (128) and desaturation at the 9 position of n-C_{18} acid in leaves (129) are known to occur. The direct oxidation of a specific carbon atom in a performed chain is also possible. The source of the oxygen of the ketone may distinguish between these two possibilities.

Elongation-Decarboxylation Mechanism

With such experimental evidence against the condensation mechanism alternate mechanisms were considered. One obvious alternative is elongation of a common fatty acid such as n-C_{16} to C_{30} or C_{32} acid followed by decarboxylation to give the C_{29} or C_{31} paraffin (117). Chibnall and Piper (47) had suggested that biological paraffins originated from corresponding acids by decarboxylation. Fatty acids are generally known to undergo elongation by addition of C_2 units and therefore the above mechanism appeared reasonable. The incorporation of the intact carbon chain of n-C_{16} acid into n-C_{29} and n-C_{31} paraffins in leaves is consistent with this mechanism, as is the observation that C_{18} acid is incorporated into n-C_{29} paraffin in *B. oleracea* and n-C_{31} paraffin in pea and spinach about three times as rapidly as n-C_{16} acid. Moreover, the intact carbon chain of stearic acid was incorporated into these paraffins (118,120).

Attempts to detect possible intermediates of the hypothetical elongation system showed that all tissues (spinach, pea, broccoli, cabbage, *Senecio odoris*) that incorporated labeled fatty acids into the paraffin also produced a range of labeled long chain fatty acids: n-C_{20} to n-C_{28} (117,119,120).

Similar results were obtained for branched

$$C_{14}H_{29}-CH_2-CH_2-CH_2-C(=O)OH$$

↓ ①

$$C_{14}H_{29}-C(=O)-CH_2-CH_2-CH_2-C_{11}H_{23}$$

↓ ②

$$C_{14}H_{29}-COOH + H_2N-CH_2-CH_2-CH_2-C_{11}H_{23}$$

$$C_{14}H_{29}-NH_2 + HOOC-CH_2-CH_2-CH_2-C_{11}H_{23}$$

FIG. 4. Incorporation of the intact carbon chain of stearic acid into *n*-nonacosan-15-one of *Brassica oleracea* (127). 1. Reactions within the leaf leading to the formation of the ketone. 2. Treatment of the ketone with hydroxylamine hydrochloride, Beckmann rearrangement and hydrolysis of the substituted amide. The fate of C_1, C_4 and H on internal carbons of stearic acid are marked.

paraffin synthesis as well. Branched starter chains originate from branched amino acids as shown in Figure 5. Valine yields isobutyrate which would give *iso*-C_{16} and *iso*-C_{18} acids which when elongated and decarboxylated would produce *iso*-branched alkanes with an odd number of carbon atoms. The elongation intermediates should be very long *iso*-branched acids with an even number of carbon atoms. Isoleucine, on the other hand, produces an *anteiso* C_5 starter from which *anteiso* C_{17} and C_{19} acids would result. On elongation these acids should produce very long *anteiso* branched acids with an odd number of carbon atoms and subsequently *anteiso* paraffins with an even number of carbon atoms. When excised tobacco leaves were provided with these labeled amino acids the predicted paraffins (119,121,125) and appropriately branched very long fatty acids were labeled (Fig. 6) (119,125).

Although the exact biochemical relationship between the very long fatty acids and the paraffins still remains uncertain, several lines of evidence suggest that they are related (125): (a) All plant tissues that incorporate labeled fatty acids into paraffins also labeled the very long chain acids, including the appropriately branched acids where branched paraffins were synthesized. (b) Longer acids such as C_{18} were more efficiently converted into both paraffins and very long acids than were the shorter acids. (c) Synthesis of the common fatty acids (C_{16} and C_{18}) in leaves is tightly coupled to photosynthetic reactions and therefore is stimulated by light and inhibited by 3-(4-chlorophenyl)-1,1-dimethyl urea (CMU). On the other hand the synthesis of both paraffin and very long acids is unaffected by light and CMU. (d) Trichloroacetate at low concentrations (10^{-5} to 10^{-4} M) strongly inhibits paraffin synthesis as well as the synthesis of very long acids, while the synthesis of common fatty acids (C_{16} and C_{18}) is not affected by this inhibitor. (e) The epidermal layer of cells which incorporated labeled acetate into paraffins also contained labeled very long acids, while the mesophyll tissue of the leaf failed to synthesize labeled paraffin and did not contain any labeled very long acids. (f) Anaerobic conditions strongly inhibited the synthesis of both very long acids and paraffins, and light partially reversed the inhibition in both cases.

In spite of the above evidence suggesting that the very long acids are biochemically related to the paraffin, a time course of incorporation of label into the very long acids and paraffin failed to show a typical precursor-product relationship (118). Although the very long acids were labeled rapidly, the radioactivity in them did not decrease even several hours after the incorporation of label into paraffins ceased. This suggested that the labeled very long acids encountered in such studies were those that were diverted from the paraffin synthesizing site and trapped into a pool from which they were no longer available for paraffin synthesis. In support of such a suggestion, the very long acids were in fact found in the phospholipid fraction of the broccoli leaf tissue lipids (118).

On the basis of the experimental evidence

VALINE → C–C(–C)–C(=O)S–CoA (*iso* 'starter piece') —C_2–units→ *iso* C_{16} acid —C_2–units, CO_2; Elongation, Decarboxylation→ *iso* C_{29}, *iso* C_{31}, *iso* C_{33} Paraffins; *iso* $C_{20}-C_{28}$ Acids

ISO-LEUCINE → C–C–C(–C)–C(=O)S–CoA (*anteiso* 'starter piece') —C_2–units→ *anteiso* C_{17} acid —C_2–units, CO_2; Elongation, Decarboxylation→ *anteiso* C_{30}, *anteiso* C_{32} Paraffins; *anteiso* $C_{19}-C_{29}$ Acids

FIG. 5. Plausible origin of branched alkanes and branched fatty acids (119,121).

FIG. 6. Left: GLC of alkanes isolated from tobacco leaves that metabolized labeled U-^{14}C-isoleucine, 1-^{14}C-isobutyrate and U-^{14}C-valine. The radioactivity tracings (top three) show that the major labeled alkanes were branched C_{30} and C_{32} from isoleucine, and branched C_{29}, C_{31} and C_{33} from both isobutyrate and valine (119). Right: GLC of the saturated fatty acid fraction isolated from tobacco leaves that metabolized U-^{14}C-isoleucine. The radioactivity tracings (top two) show that the isoleucine labeled branched C_{15} - C_{27} odd carbon numbered acids and isobutyrate labeled branched C_{14} - C_{26} even carbon numbered acids (119).

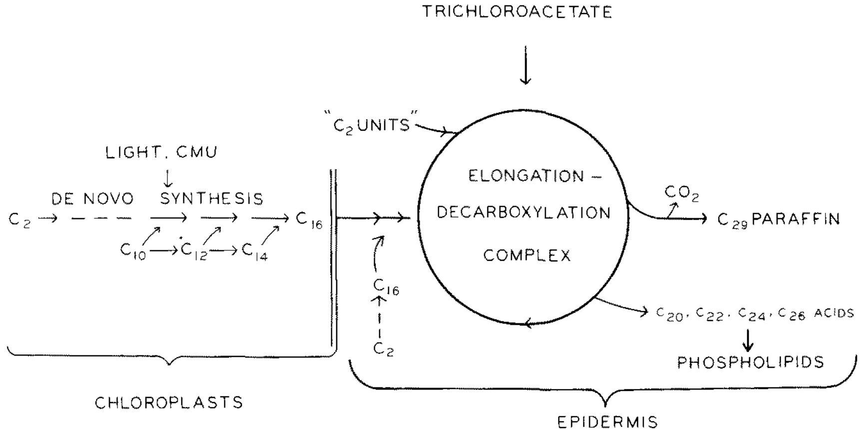

FIG. 7. Elongation-decarboxylation hypothesis illustrated by the *n*-C_{29} alkane synthesis in *Brassica oleracea* (118).

'Acceptor' $C_{17}H_{35}$–C(=O)OH + 'Donor' HO–C(=O)–CH_2–$C_{10}H_{21}$ ⟶ $C_{17}H_{35}$–C(=O)–CH(COOH)–$C_{10}H_{21}$ ⟶ (Reductive Decarboxylation, $-CO_2$) $C_{17}H_{35}$–CH_2CH_2–$C_{10}H_{21}$
C_{29} Paraffin

FIG. 8. Acceptor-donor condensation with specific decarboxylation (125). Note the retention of the carboxyl-carbon of the acceptor C_{18} acid and loss of the carboxyl-carbon of the donor C_{12} acid. Theoretical fate of the various labeled atoms, used in the tracer work discussed in the text, are indicated by the symbols on the atoms (119).

discussed thus far a plausible mechanism for paraffin synthesis in leaves may be schematically represented as shown in Figure 7. The de novo fatty acid synthesis takes place in the chloroplasts which provides the substrate, n-C_{16} acid, for paraffin synthesis. Fatty acids for paraffin synthesis may also be synthesized in the epidermal layer of cells themselves. The C_{16} acid becomes the substrate for an elongation-decarboxylation complex which elongates the acid to the appropriate chain length (C_{30} in broccoli) and then decarboxylates it releasing the paraffin. After each successive addition of C_2 units, some elongated acids may dissociate from the complex and these acids are rapidly esterified into phospholipids. Depending on the specificity of the complex, decarboxylation takes place mainly at the C_{30} or C_{32} levels producing the C_{29} or C_{31} paraffin, the major plant paraffins. The *iso*-branched even acids and *anteiso*-branched odd acids give *iso*-branched odd paraffins and *anteiso*-branched even paraffins respectively. Such a hypothesis readily explains why *B. oleracea* labels primarily n-C_{29} paraffin and pea and spinach leaves give n-C_{31} paraffin irrespective of the size of the labeled precursor. At some stage of synthesis (at the C_{30} acid level or paraffin level), C_{15} is oxygenated to give the nonacosan-15-one and nonacosan-15-ol.

Head-to-Head Condensation Between Acceptor and Donor Acids

Attempts to obtain direct evidence for a decarboxylation have thus far failed. For instance exogenous labeled n-C_{30} acid did not label n-C_{29} paraffin in broccoli leaf slices or homogenates. However such exogenous substrates might not have had access to the metabolic sites. Attempts to get a cell-free system for paraffin synthesis from leaves have not yet been successful. The only cell-free system that labels paraffins even poorly incorporates only labeled acetyl-CoA and malonyl-CoA, but not long chain acyl-CoA (Kolattukudy, unpublished results). Because of such lack of direct evidence for an elongation-decarboxylation mechanism, alternate possibilities were considered.

A modification of the head-to-head condensation is the most obvious alternative. For example, exogenous fatty acids such as C_{16} or C_{18} could condense with other acids of appropriate chain length, followed by decarboxylation and reduction to give the paraffins. However since the intact carbon chains of exogenous C_{16} and C_{18} acids were incorporated into the C_{29} and C_{31} paraffin in leaves, the hypothetical decarboxylation must be specific in such a way that the other condensing acid (donor) must lose its carboxyl carbon and donate its alkyl moiety for the paraffin formation (125). The exogenous C_{16} and C_{18} acids (acceptor) on the other hand accepts only the alkyl moiety while retaining its own carboxyl carbon, which is reduced to methylene level in the process. Incorporation of n-C_{18} acid into n-C_{29} alkane of broccoli might thus be represented as shown in Figure 8. If C_{12} acid, labeled in the carboxyl-carbon with ^{14}C and with ^{3}H on the other carbons, participates in such a reaction the alkane formed should have no ^{14}C but all the ^{3}H of the C_{12} molecule in it. However, since some C_{12} acid can be incorporated into the alkane via C_{18} acid (117), some incorporation of ^{14}C into the alkane should be expected. Even then the ^{14}C:^{3}H ratio of the alkane should be much less than that of the C_{12} acid. However experimental results showed that there was no decrease in the isotopic ratio when the doubly labeled C_{12} acid was incorporated into the alkane by broccoli leaf (119). Attempts to increase the chances of C_{12} acid to condense with C_{18} acid by providing exogenous unlabeled C_{18} acid also gave no decrease in the isotopic ratio. Similar results were obtained with the C_{12} acid as well as the methyl ester of the acid (Kolattukudy, unpublished results). Thus the condensation as shown in Figure 8 cannot account for the results.

However the exogenous C_{12} acid may not be in isotopic equilibrium with the donor pool, and all the C_{12} incorporation observed might have resulted from incorporation of C_{12} acid into the longer acceptor acid. Alternatively, the natural acceptor and donor might be n-C_{16} and n-C_{14} respectively in *B. oleracea*. The doubly

Fatty Acid Synthetase

$C_2 \rightarrow \rightarrow C_{10} \rightarrow C_{12} \rightarrow C_{14} \rightarrow$ Rd $CH_2C(=O)S$-ACP

Ra-C(=O)S-CoA $\longrightarrow$ RdCH_2-CH_2-Ra

C_6, C_8 'Exogenous'

FIG. 9. A plausible biochemical explanation for the acceptor-donor hypothesis. Donor is endogenous acyl-ACP and exogenous acids are postulated to enter the reaction only as the acceptor, namely acyl-CoA or another derivative of it. Ra and Rd denote acceptor and donor respectively.

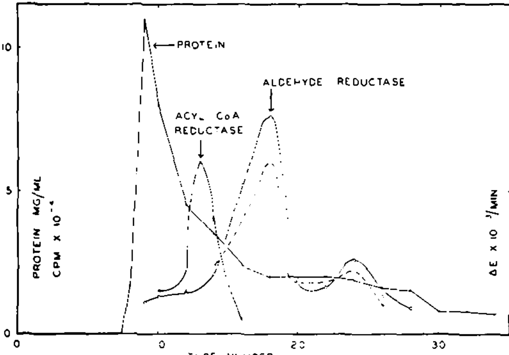

FIG. 10. Sephadex G-100 gel filtration of a protein fraction from broccoli leaves. This protein fraction was obtained from an acetone powder extract by 25-40% saturation with ammonium sulfate. Stearyl-CoA dependent NADH oxidation and palmitaldehyde-dependent NADPH oxidation are shown by X———X and O———O respectively. Broken line represents the aldehyde reductase activity as measured by a tracer-assay with 1-^{14}C-palmitaldehyde (Kolattukudy, manuscript in preparation).

labeled n-C_{12} acid might therefore have been elongated to n-C_{14} and n-C_{16} prior to incorporation into the alkane and thus the isotopic ratio would not be altered. However, 1-^{14}C-U-^{3}H-tetradecanoic acid was incorporated into n-C_{29} alkane of broccoli without any decrease in ^{14}C: ^{3}H ratio. Here again labeled free acid and methyl ester gave similar results (Kolattukudy, unpublished results). Thus either the exogenous acids have no access to the donor pool or the elongation-decarboxylation route is operative in alkane synthesis.

In spinach and pea leaves, which synthesize primarily n-C_{31} alkane (in contrast to C_{29} of broccoli) the acceptor and donor acids are likely to be n-C_{16} acid. Yet exogenous n-C_{16} acid was incorporated into the alkane without any loss of the carboxyl carbon (120). Again the exogenous acid must be incapable of entering the alkane through the donor pool which might be derived strictly from endogenous sources. A possible biochemical explanation would be that the donor is acyl-acyl carrier protein (ACP) and the acceptor is acyl-CoA or a derivative of it (Fig. 9). In leaves, exogenous long chain fatty acids are readily converted into the CoA derivatives (because esterification into other lipids can be readily demonstrated) but not into ACP derivatives (129-131). Because of this lack of trans acylase activity, stearic acid desaturation, for example, could not be demonstrated in leaves until synthetic stearyl-ACP was used as substrate (129,130). In alkane synthesis too, the observed lack of decarboxylation of exogenous acids during their incorporation into alkanes may result because the exogenous acids cannot form acyl-ACP which is the decarboxylating partner (donor) in the condensation process. The chain length of the alkane synthesized by a given tissue does not depend on the length of the exogenous precursor acid. For example all of the labeled acids tested (C_2-C_{18}) yielded primarily n-C_{29} alkane in *B. oleracea* (117). Therefore the specificity with respect to the chain length of the condensing acids must lie in the total number of carbon atoms of the two acids. For example exogenous n-C_{18} acid and n-C_{16} acid should condense with endogenous C_{12} and C_{14} donor acids respectively to give n-C_{29} alkane from either one.

If the alkanes are synthesized by the condensation described above, the relationship of very long acids to the alkanes discussed earlier must be reevaluated. The obvious explanation is that the very long acids are precursors of the other wax components that are synthesized in the epidermal layer of cells, rather than of the alkanes which are also synthesized there (119). Since the epidermis is largely devoid of chloroplasts, wax synthesis, unlike the synthesis of C_{16} and C_{18} fatty acids, is not tightly coupled to photosynthetic reactions and therefore is unaffected by light and CMU. Trichloroacetate on the other hand may not be able to enter the chloroplasts where synthesis of the usual fatty acids takes place but can inhibit the syntheses in the epidermal cells. The inhibition of synthesis of alkanes and very long acids by anaerobic conditions may be a reflection of a reduction in the metabolic energy in the epidermal cells, which derive their energy from respiration rather than photosynthesis. Although 1% to 3% oxygen, which is supposed to be sufficient for respiration (132), failed to sustain C_{29} synthesis, 20% oxygen was sufficient for maximal C_{29} synthesis (Kolattukudy, unpublished results). Somewhat similar effects of oxygen have been reported in other cases; for example, ethylene synthesis in

I $R{-}C(=O){-}S{-}CoA + R'OH \longrightarrow RC(=O)OR' + CoA$

II $R{-}C(=O){-}PL + R'OH \longrightarrow RC(=O)OR' + PL$

III $R{-}C(=O){-}OH + R'OH \longrightarrow RC(=O)OR' + H_2O$

FIG. 11. Three plausible mechanisms for wax ester synthesis. PL-phospholipids (140).

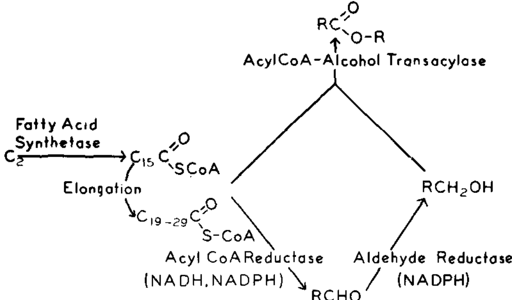

FIG. 12. Pathway for wax ester synthesis in *Brassica oleracea* based on in vivo and enzyme studies. In *Euglena gracilis*, the wax ester was synthesized by the same mechanism except for the elongation step, and the cofactor was NADH (Kolattukudy, manuscript in preparation).

bananas requires oxygen concentrations higher than 7.5% (133). Even though it is difficult to draw conclusions about the reaction sequences from such observations, such high oxygen concentrations should not be required for the condensation reactions per se, but an α-oxidation coupled mechanism (134) for the final decarboxylation of an elongated acid could be consistent with the observed oxygen requirement.

In certain bacteria head-to-head condensation of fatty acids is an established reaction (135) and such a reaction is involved in ascaroside biosynthesis (136). More recently a modification of it has been shown to be responsible for alkane synthesis in *Sarcina lutea* (Albro and Dittmer, this symposium). Algae, on the other hand, might have developed a direct decarboxylation mechanism for the synthesis of hydrocarbons and thus the dominance of n-C_{17} alkane observed in algae. Higher plants evolved an elongation system for the synthesis of very long acids and with such a system a direct decarboxylation would be the simplest route to the alkanes. In any case the elucidation of the actual mechanism of hydrocarbon synthesis in plants still must await further work with cell-free preparations and purified enzymes.

Specificity

Many plants such as broccoli have only n-alkanes, but some plants such as tobacco synthesize n and branched alkanes, whereas others such as roses can synthesize alkenes in addition to n and branched alkanes. This species specificity could reside either in fatty acid synthesis or in the paraffin synthesis itself. When a plant such as broccoli, which normally does not synthesize any branched paraffins, was provided with labeled branched precursors such as isobutyrate or isoleucine, appropriately branched fatty acids were produced, but the formation of no branched paraffin could be detected (137). Thus it appears that broccoli leaves synthesize only straight chain paraffins because the system that synthesizes paraffins is highly specific for straight chains although the fatty acid synthetase is not so specific. Furthermore under conditions that allowed rapid incorporation of n-C_{18} acid into the hydrocarbons of broccoli, oleic acid was strictly excluded from participating in the hydrocarbon synthesis of this tissue (117). Thus structural features of the biosynthetic hydrocarbon of a given organism are apparently determined by the specificity of the paraffin synthesizing system itself.

SYNTHESIS OF FATTY ALCOHOLS, ALDEHYDES AND ACIDS

A special feature of wax acids from plants is that they are much longer (C_{20}-C_{32}) than the usual fatty acids (C_{16}-C_{18}). Many young leaf tissues incorporated labeled exogenous fatty acids of intermediate chain length, most probably by elongation, into very long acids of the size usually encountered in plant waxes (117,119,120). A particulate system from pea cotyledons that elongates usual fatty acids of endogenous source with malonyl-CoA into C_{20}-C_{28} acids has been described (131). Fatty acids C_2-C_{18} were all readily incorporated into fatty alcohols in young broccoli leaves, the longer acids being more readily incorporated than shorter ones (116,117). Recently I have demonstrated the enzymatic conversion of fatty acyl-CoA into fatty alcohol catalyzed by an acyl-CoA reductase and an aldehyde reductase (Kolattukudy, manuscript in preparation). Crude extracts of acetone powder prepared from young broccoli leaves did not reduce acyl-CoA because of the very active thioester hydrolase and the rapid endogenous NADH oxidation which was inhibited by acyl-CoA. Fractionation by Sephadex G-100 gel

filtration (Fig. 10) made it possible to demonstrate fatty acyl-CoA-dependent NADH oxidation. This acyl-CoA reductase activity was confirmed by the isolation of a labeled product with an R_f identical to that of palmitaldehyde from the reaction mixture containing labeled palmityl-CoA and NADH. The enzymatic product on treatment with sodium borohydride at room temperature gave a product with an R_f identical to hexadecanol. The acyl-CoA reductase preferred NADH to NADPH.

Fatty aldehyde reductase activity could be more easily shown in the acetone powder extracts prepared from young broccoli and pea leaves with either a spectrophotometric assay or in a tracer assay starting with labeled palmitaldehyde. This reductase has been partially purified (10-12 fold) by ammonium sulphate fractionation and Sephadex G-100 gel filtration. The pH optimum was about 5.6 and the enzyme was quite specific for NADPH although NADH did function poorly. The half maximal velocities were obtained at 5×10^{-5} M for NADPH and 7×10^{-5} M for palmitaldehyde under saturating conditions of the other substrate. Like other aldehyde reductases (138,139), the fatty aldehyde reductase was a sulfhydryl enzyme, since thiol reagents such as iodoacetate, N-ethyl maleimide and *p*-chloromercuribenzoate inhibited the enzyme.

A partially purified enzyme preparation from etiolated *Euglena gracilis* Z catalyzed the reduction of C_{14} and C_{16} fatty acids to the corresponding alcohols with ATP, CoA and NADH (but not NADPH) as cofactors (Kolattukudy, manuscript in preparation). The pH optimum was near 6.5 and the enzyme system had thiol groups essential for activity. The apparent Km was 1.6×10^{-5} M for myristic acid and 2.4×10^{-4} M for NADH. An aldehyde intermediate could be trapped with phenyl hydrazine and the enzyme preparation also catalyzed reduction of exogenous 1-^{14}C-palmitaldehyde.

SYNTHESIS OF WAX ESTER

Acetone powder preparations of young broccoli leaves readily incorporated labeled fatty alcohols into wax esters using endogenous acyl moieties (140). Further examination of the nature of the acyl moieties revealed three mechanisms (Fig. 11) analogous to cholesterol esterification in animals. At high concentrations of alcohol and acid and at low pH there was a substantial synthesis of wax ester by a direct esterification presumably catalyzed by an esterase type enzyme. Broccoli acetone powder also catalyzed an apparent acyl transfer from phospholipids to the hydroxyl group of fatty alcohol, a mechanism analogous to the synthesis of cholesterol ester in animal serum where acyl moieties are transferred from lecithin of lipoprotein to the hydroxyl group of cholesterol (141). A third mechanism for wax ester synthesis became apparent when a protein fraction first precipitated with ammonium sulphate was subjected to Sephadex G-100 gel filtration. This partially purified enzyme catalyzed an acyl transfer from acyl-CoA to fatty alcohol (140). Also in *Euglena* extracts esterification of fatty alcohol with fatty acids required ATP and CoA as cofactors (Kolattukudy, manuscript in preparation). A similar mechanism is known to be involved in cholesterol esterification in adrenal and liver homogenates (142-144). Thus the pathway for fatty alcohol and wax ester synthesis may be summarized as shown in Figure 12.

CATABOLISM OF WAXES

Plant waxes especially the very long ones appear to undergo little turnover or interconversion. Analysis of waxes from Brussel sprout and runner bean leaves at various stages throughout the life of these plants led Chibnall and Piper to suggest that all of the components of the wax are end-products of metabolism (47). More recently time course studies of incorporation of labeled precursors and changes in specific activity of various wax components in *B. oleracea* (116,117) suggested a lack of interconversion. No interconversions were apparent in similar experiments with tobacco (126). Such studies however might have failed to detect enzyme bound intermediates and interconversions at the actual site of synthesis because the measurements were made on essentially metabolically inert pools on the surface of the leaves. Utilization of wax could not be shown even when plants were starved (47), although disappearance due to weathering may take place (6,145). In lower forms such as *Euglena*, on the other hand, wax apparently serves as a food reservour and thus under adverse conditions it can be utilized (146); similar situations may be encountered in algae as well.

Attempts to detect the conversion of wax components into any other product by providing excised leaves, leaf slices, homogenates or subcellular particles with labeled biosynthetic *n*-C_{29} alkane or nonacosan-15-one have failed (117). The difficulty involved in getting such large insoluble molecules to the site of metabolic activity was at least partially responsible for such failures. Therefore a much smaller alkane, *n*-hexadecane, labeled with ^{14}C was

administered to a variety of plant tissues, including bacteria-free tissue slices. A small but significant catabolism was observed. The terminal methyl carbon was oxidized producing the corresponding fatty acid (147). Thus plants do possess the ability to oxidize at least certain types of compounds found in waxes. Some of the more reactive components such as aldehydes and medium length fatty acids are undoubtedly catabolized by plants.

The waxes synthesized by plants do not accumulate to a large extent in nature because they are degraded to a limited extent by animals and readily by bacteria. Rats can absorb and catabolize at least part of the cabbage wax paraffin (148) and wax esters (149) ingested. *Micrococcus cerificans* isolated from soil readily degraded cabbage paraffin (n-C_{29}) (150) and alkanes up to C_{36}, and grew on such alkanes as the sole source of carbon (Kolattukudy and Hankin, unpublished). A *Pseudomonad* species isolated from soil in an apple orchard grew on the major component of apple wax, ursolic acid, as the sole source of carbon (151). Undoubtedly many more microorganisms in the soil utilize the various plant waxes and thus only a limited accumulation of such compounds occurs in the soil (152).

Little is known about the breakdown of unusually long carbon chains, a salient feature of wax degradation. When labeled n-C_{29} alkane isolated from broccoli leaves was metabolized by rats (148) or *M. cerificans* (150) the major radioactive product was n-C_{17} acid in phospholipids. Either an oxidative attack on the C_{17} of the alkane or a β-oxidation type chain-shortening of the C_{29} acid produced by the oxidative attack on the terminal methyl carbon of the C_{29} alkane could explain the results. Randomly ^{3}H-labeled C_{28} acid, when fed to rats, was absorbed and catabolized (Kolattukudy, unpublished results). Time course experiments showed that the C_{28} acid was readily converted into n-C_{16} and n-C_{18} alkanoic acids; 10% of the administered radioactivity was incorporated into liver lipids, 90% of the ^{3}H being in C_{16} + C_{18} acids in 3 hr after the feeding. From the relative amounts of ^{3}H found in water and the C_{16} + C_{18} fatty acids it appears that the conversion of C_{28} acid into C_{16} + C_{18} was not by a complete β-oxidation and resynthesis but by a more direct conversion to the C_{16} or C_{18} level. *Micrococcus cerificans* which readily grows on very long chain alkanes also converted the tritiated C_{28} acid to C_{16} + C_{18} acid (Kolattukudy and Hankin, unpublished). Time-course of labeling of C_{16} + C_{18} acid and water showed that the formation of labeled C_{16} + C_{18} acids was not by degradation into acetate and resynthesis, but a more direct route is involved. An oxidative attack on an internal methylene group and subsequent split of the chain or a β-oxidation type chain shortening to C_{16} or C_{18} level is involved. In any case oxidation of C_{28} acid by cell-free preparations of this bacterium required ATP and CoA indicating that it is the CoA derivative that undergoes this catabolism. Small amounts of stearic acid are known to undergo a chain shortening to C_{16} acid in rats (153) and the major catabolic route for acids much longer than C_{18} may be a β-oxidation type chain shortening to the C_{16} or C_{18} level.

FUNCTION

It is common knowledge that wax waterproofs and protects surfaces and this is true also of plant surface waxes. The cuticular wax waterproofs the plant so efficiently that only a very small portion (less than 10%) of the water loss from a plant takes place by cuticular transpiration (157). Lack of a clear correlation between surface wax and xeromorphic adaptation has been taken to mean that the wax has little survival value (2). However removal of wax causes a several-fold increase in water loss (145,155-157). Although techniques used to remove wax may cause some other damage to the surface, it is quite clear that the cuticular wax is responsible to a large extent in maintaining the cuticular transpiration at a minimum. Measurements of reduction in water loss through artificial membranes treated with various isolated wax components indicate that the aliphatic components, such as hydrocarbons, alcohols, aldehydes, and wax esters, are far more effective in reducing transpiration than the alicyclic components such as triterpenes (157). Surface wax also increases resistance to abrasive damage, which can facilitate the entry of pathogens and toxic chemicals into the plant (8). It obviously plays a role in controlling the entry of foliar sprays and other chemicals from the environment and therefore the wax layer is often a factor in the usefulness of various chemical sprays in agriculture.

Defense against pathogenic microorganisms is another possible function often assigned to plant waxes. The nonwetting nature of the surface may make it difficult for the organisms to establish themselves on the surface, but the role of the wax as a physical barrier to penetration by the organism is not considered important because most organisms can push their way through the wax (158). It is possible that the wax also protects chemically by being toxic to pathogens. There are some observations in the

literature to support such a view (158). For instance an ether-soluble acid from apple wax is toxic to apple mildew. However, the significance of such observations with respect to actual defense itself is questionable because equal quantities of fungitoxic materials could be isolated from both susceptible and resistant varieties. Similarly fractions that were toxic to withertip fungus could be found in young citrus lime leaves susceptible to withertip disease as well as in mature resistant leaves (159). Thus it is far from clear how significant a role such toxic materials play in defense of plants.

In some lower forms, such as *Euglena*, wax appears to serve as a food reservoir (146); However in higher plants the waxes are deposited primarily on the outside and these waxes do not appear to be utilized as an energy source even in adverse conditions. In some cases waxes may play specialized roles. For example the alga *Botryococcus* apparently excretes large amounts of highly branched unsaturated hydrocarbons which presumably function as a flotation sack, protection device and food reservoir (77). Certain wax components such as long chain alcohols show hormone-like activities in plants (160) as well as in animals (161), but little is known about the significance or the mechanism by which such effects are brought about.

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SHORT COMMUNICATIONS

Binding of ^{14}C-Phosphatidylcholine to Sodium Chloride Crystals

ABSTRACT

^{14}C-Phosphatidylcholine in benzene was found to bind to sodium chloride crystals. The magnitude of this binding was greatest when the crystal size was the smallest, namely when the crystals were prepared from frozen and lyophilized aqueous solution.

In the course of studying the binding of ^{14}C-labeled lecithin to erythrocyte membranes (1) it was found that binding of the lipid took place to sodium chloride crystals. This observation was explored to assess the magnitude of this binding and factors affecting it. It was found that significant binding of the lipid occurred from benzene solution only when the sodium chloride crystals were prepared from frozen and lyophilized aqueous solution.

Sodium chloride crystals in unaltered form were allowed to incubate at 37 C for 10 min with 1 μmole of labeled phosphatidylcholine in benzene. The benzene was removed by a stream of nitrogen. The salt crystals were washed with 5 ml of benzene and centrifuged two to three times. The samples were dried by vacuum sublimation or in a stream of nitrogen and extracted twice with 5 ml of chloroform-methanol (1:4 v/v). No significant counts were left in the sodium chloride crystals after chloroform-methanol extraction. The extracts were combined and counted in a well-type scintillation counter (Nuclear Chicago). No significant binding was observed under these circumstances (Table I).

The previous experiment was repeated with the modification that the sodium chloride crystals were ground as finely as possible using an agate mortar and pestle. With this procedure the binding increased slightly.

In the following experiment the sodium chloride crystals were placed in tubes, water was added to 1 ml and samples were then rapidly frozen and lyophilized. A benzene solution of 1 μmole ^{14}C-lecithin was added, the reagent removed, and the crystals washed with benzene. Chloroform-methanol extraction of these crystals resulted in the finding of comparable binding to that observed when the lipid was added in aqueous dispersion (Table I).

It appeared then that significant binding did not necessarily require the salt to be dissolved in water, since the binding also occurred under these conditions from the benzene solution. The magnitude of the binding appeared to depend on the surface area and in the case of lyophilized samples the sodium chloride was present in a microcrystalline form. Microscopic examination of the crystals indicated that those produced by lyophilization of a quick-frozen aqueous solution were much smaller than those prepared by grinding with a mortar and pestle.

It is interesting that this binding would take place in benzene solution since it is currently thought phosphatidylcholine has a zero net

TABLE I

Binding of ^{14}C-Lecithin to Sodium Chloride Crystals[a]

NaCl, mg	^{14}C-Lecithin Per cent Uptake, Sodium chloride crystals		
	Untreated	Ground[b]	Lyophilized[c]
0	NS[d]	NS	NS
10	NS	NS	5.9
20	NS	NS	8.8
30	NS	NS	12.8
40	NS	NS	15.9
50	NS	2.2	20.9
100	NS	2.9	36.0
500	NS	9.0	36.0

[a] ^{14}C-Phosphatidylcholine produced by the feeding of $^{14}CO_2$ to chlorella algae was obtained from Applied Science Laboratories (State College, Pa.) and was further purified to more than 95% radiochemical purity by elution from silicic acid impregnated glass fiber (ChromAR-Mallinkrodt Chemical Works). Cold carrier bovine or egg lecithin (Applied Science Laboratories) was added and the specific activity measured using the phosphorus determination (2). The purified labeled phospholipid was stored at 4 C for no more than seven days.

[b] By hand using an agate mortar and pestle.

[c] From aqueous solution.

[d] NS, not significant.

charge in the usual pH range and also that this phospholipid is present in benzene as an internal salt with polar ends removed from contact with the solvent (3). Significant binding was also observed when 1,4-dioxane was substituted for benzene throughout.

We were unable to demonstrate binding of the salt to the phospholipid in aqueous solution. Indeed it has been known for some time that in a biphasic system of chloroform, methanol and water the presence of salt in the aqueous phase increases the amount of phospholipid in the lower chloroform-methanol phase (4). This is the opposite that one would expect if binding of the lipid to sodium chloride in the aqueous phase were important. In aqueous solution other factors may be operative. We also found no evidence of binding in a biphasic system consisting of benzene and ethylene glycol. When labeled phosphatidylcholine was partitioned in this system it was found that 10% was recovered in the upper benzene layer and 90% in the lower ethylene glycol layer. Sodium chloride was added and dissolved in the lower phase, but the partition of phosphatidylcholine was not altered. This ruled out tight binding of the phospholipid to the salt in ethylene glycol solution.

We also found ^{14}C-lecithin binding of a comparable degree to potassium chloride crystals, indicating that sodium chloride was not unique in this respect. It is probable that charge-charge interaction at the crystal surface was responsible for the binding of the phosphatidylcholine to the sodium chloride crystals. It may be of interest to point out the possibility that the lipid might actually penetrate the crystal lattice of sodium chloride.

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[Received August 1, 1969]

Unusual Olefinic Fatty Acids in Seed Oils from Two Genera in the Ranunculaceae

ABSTRACT

Oil from one of the two species of *Anemone* investigated contained 20% of γ-linolenic acid but the other had none. Linoleic acid was the major component in both oils. Four of the five species of *Ranunculus* produced oil containing 2% to 4% of a component identified in one sample as *cis*-7,*cis*-10-hexadecadienoic acid. Major components were linoleic and linolenic acids.

Gas liquid chromatography (GLC) of methyl esters prepared from seed oils of selected species within two genera of the Ranunculaceae indicated that the oils contained unusual fatty acids. A component constituting 19% of the esters from *Anemone cylindrica* seed oil had equivalent chain lengths (ECL) (1,2) identical to those of methyl all-*cis*-6,9,12-octadecatrienoate (γ-linolenate). Esters from four species of the *Ranunculus* genus had an unusually high content of 16:2. Further examination of one oil sample from each genus reported here provided definite identification of the unusual fatty acids.

Oil was extracted from the seeds and analyzed as previously described (3,4). Methyl esters were prepared from the oils (5) and analyzed by GLC (1). The esters from *A. cylindrica* and *R. sericeus* oils were also separated according to chain length by preparative GLC, and then each GLC fraction was further separated according to degree of unsaturation by preparative silver nitrate thin layer chromatography (6). Double bond positions in the un-

charge in the usual pH range and also that this phospholipid is present in benzene as an internal salt with polar ends removed from contact with the solvent (3). Significant binding was also observed when 1,4-dioxane was substituted for benzene throughout.

We were unable to demonstrate binding of the salt to the phospholipid in aqueous solution. Indeed it has been known for some time that in a biphasic system of chloroform, methanol and water the presence of salt in the aqueous phase increases the amount of phospholipid in the lower chloroform-methanol phase (4). This is the opposite that one would expect if binding of the lipid to sodium chloride in the aqueous phase were important. In aqueous solution other factors may be operative. We also found no evidence of binding in a biphasic system consisting of benzene and ethylene glycol. When labeled phosphatidylcholine was partitioned in this system it was found that 10% was recovered in the upper benzene layer and 90% in the lower ethylene glycol layer. Sodium chloride was added and dissolved in the lower phase, but the partition of phosphatidylcholine was not altered. This ruled out tight binding of the phospholipid to the salt in ethylene glycol solution.

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Oil was extracted from the seeds and analyzed as previously described (3,4). Methyl esters were prepared from the oils (5) and analyzed by GLC (1). The esters from *A. cylindrica* and *R. sericeus* oils were also separated according to chain length by preparative GLC, and then each GLC fraction was further separated according to degree of unsaturation by preparative silver nitrate thin layer chromatography (6). Double bond positions in the un-

TABLE I

Fatty Acid Composition of Seed Oils From Two Genera in the Ranunculaceae

Source	Methyl ester composition, area % by GLC[a]								
	14:0	16:0	16:1	16:2	18:0	18:1	18:2	18:3	20:0
Anemone cylindrica Gray	0.1	9.0	0.2	---	2.8	8.5[b]	59	20[c]	0.4
A. decapetala Ard.	0.1	13	0.2	---	2.3	9.2	75	---	---
Ranunculus arvensis L.	0.1	7.3	2.6	2.3	1.3	18	28	40	---
R. constantinopolitanus D'Urv.	---	8.3	1.1	3.0	2.5	17	41	27	---
R. falcatus L.	Trace	7.2	0.2	---	1.9	16	9.8	65	0.1
R. sardous Crantz.	0.1	16	0.1	2.9	1.9	10	25	43	---
R. sericeus Poir.	---	9.9	2.4[d]	3.8[e]	1.2	17[f]	39	26	---

[a]Relative abundance of positional isomers calculated from oxonolysis products (7).
[b]Includes 8.0% $18{:}1^{9}$ and 0.5% $18{:}1^{11}$.
[c]Includes 19% $18{:}3^{6,9,12}$ and 0.7% $18{:}3^{9,12,15}$.
[d]Includes 1.9% $16{:}1^{7}$ and 0.5% $16{:}1^{9}$.
[e]*cis*-7, *cis*-10-Hexadecadienoate.
[f]Includes 16% $18{:}1^{9}$ and 1.0% $18{:}1^{11}$.

saturated fractions were located by reductive ozonolysis (7). Infrared analyses were performed on either liquid films or carbon disulfide solutions of the samples.

Fatty acid compositions of the oils, based on GLC analyses of their methyl esters, are given in Table I. Since infrared spectra of the oils, the esters and the ester fractions had no bands in the 10 to 11 μ region, unsaturation in the oils presumably has the *cis* configuration.

The two *Anemone* samples investigated are markedly different in fatty acid composition. The presence of γ-linolenic acid in *A. cylindrica* oil was confirmed by ozonolysis of the isolated trienoates. The monoene, 18:1, included oleate and *cis*-vaccenate but the dienoate, the most abundant component in the esters, proved to be only linoleate. Although γ-linolenic acid has been reported in seed oils from *Oenothera* (8) (Onagraceae), *Humulus* (9) (Moraceae), two genera of the Liliaceae (10), and many genera of the Boraginaceae (11,12), this report is believed to be the first of its presence in the Ranunculaceae. The proportion of γ-linolenic acid in *A. cylindrica* oil is comparable to that found in those Liliaceae and Boraginaceae oils richest in this acid.

Four *Ranunculus* seed oils, other than that of *R. falcatus*, contained relatively large amounts of 16:2. Since the esters of *R. sericeus* were the highest in 16:2 of the four, they were selected for further study. Separation and ozonolysis of these esters established the structures of the unusual acids as $16{:}1^{7}$, $16{:}2^{7,10}$, and $18{:}1^{11}$. The other unsaturated acids were oleic, linoleic and linolenic. The 7-hexadecenoic and 7,10-hexadecadienoic acids have previously been found in plant lipids of *Ginkgo biloba* leaves and *Anacardium occidentale* (cashew) nuts (13).

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Fatty Acids of the Alkane Diol Diesters of Vernix Caseosa

ABSTRACT

The fatty acid monoenes esterified to the alkane diol diesters of vernix caseosa lipid form two patterns of homologues starting from either $C_{16}\Delta 9$ or $C_{16}\Delta 6$ and adding (or subtracting) an integral number of C_2 units at the carboxyl group. Although components of the $\Delta 6$ pattern are the predominant monoenes of sebaceous gland ester lipid classes, for these diol diesters $\Delta 9$ pattern components are preferentially used.

This paper reports the composition and double bond patterns of the fatty acid monoenes that occur in the alkane diol diesters of the lipids of vernix caseosa, the greasy material covering the human newborn. The alkane diol diesters constitute ~3% of the total lipids. The fatty acid monoenes of these diesters show what we call the $C_{16}\Delta 6$ and the $C_{16}\Delta 9$ patterns, two unusual patterns of homologues present in human skin surface lipids (1-4). We define these patterns in terms of a postulated three step biosynthetic process. (1-3). These steps for the $C_{16}\Delta 6$ pattern are: step 1, build up of saturated fatty chains predominantely, but not exclusively, to C_{16} (i.e., smaller amounts of C_{14}, C_{15}, C_{17}, C_{18}, branched, and others are also formed); step 2, desaturation of all chains at $\Delta 6$; and step 3, extension of the resultant monoenes to various lengths by addition of C_2 units at the carboxyl group. The same three steps apply to the $C_{16}\Delta 9$ pattern of acids, except that the desaturation of step 2 occurs at $\Delta 9$. These two patterns of skin fatty acid monoenes are in contrast to still a third, the $C_{18}\Delta 9$ pattern (i.e., oleic acid and its C_2 extension products), which

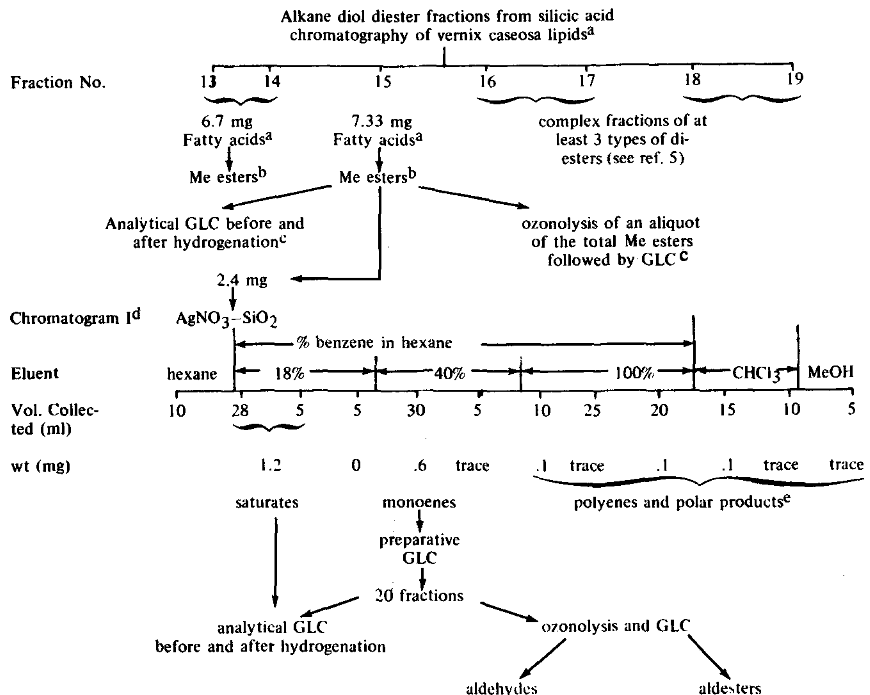

FIG. 1. Analysis of fatty acid methyl esters from diol diester diester fractions of vernix caseosa.
[a] Obtained as in Figure 1 (5).
[b] By BF_3 methanol.
[c] GLC and ozonolysis as in (2).
[d] Column 0.6 cm i.d. x 13.4 cm bed height packed with 2.8 g CABN as in (2).
[e] No attempt was made to analyze these products.

TABLE I

Fatty Acid Methyl Esters From Alkane Diol Diesters of Vernix Caseosa[a]

No. of C atom	Saturates				Monoenes			
	Straight		Branched		Straight		Branched	
	Even	Odd	Iso[b]	Anteiso[b]	Even	Odd	Iso[b]	Anteiso[b]
12	.02		.02		ND			
13		.4		.3		ND		ND
14	2.1		3.4		.03		ND	
15		3.8		6.5		.1		.1
16	28.3		9.7		5.4		.03	
17		.8		1.8		.9		.1
18	1.6		1.4		23.9		.4	
19		.1		.2		.5		.03
20	.3		1.4		1.5		.4	
21		.02		.6		.04		.02
22	.07		.9		.2		.01	
23		.02		.4		.001		ND
24	.2		.9		.1		ND	
25		.02		.5		.03		
26	.04		.03		.06			
27		.09		.002		ND		
28	.02		ND		.05			
29		.001		ND				
30	.007				.03			
31		ND						
32	.001				.02			
33								
34	.001				.001			
35								
36	ND				ND			
	32.7	5.2	17.8	10.3	31.3	1.6	.8	.3
	37.9		28.1		32.9		1.1	
Total	66				34			
	100							

[a]Expressed as per cent of total saturates plus monoenes; ND, not detected (less 0.001%).

[b]The assignment of the terms iso and anteiso are based solely on carbon number data when the sample was run on the nonpolar liquid phase OV-101. The carbon numbers for (iso) 0.64 and for anteiso 0.71 with average deviations of ±0.03.

is the major pattern of monoenes of most animal and plant tissues.

Figure 1 shows the manner in which the fatty acids were obtained and analyzed. Fraction 15, which gave only one spot by TLC (5), accounted for more than 50% of the total diols (hence diesters), whereas Fraction 13 plus 14 (accounting for ~35% of the diols) as well as 16 through 19 (accounting for ~12% of the diols) had other components besides alkane diol diesters (5). We have therefore examined the fatty acids from Fraction 15 in detail, and assume that its composition is a representative fraction of this ester class.

The usual four types of chains (straight even, straight odd, iso and anteiso) found in other skin lipid samples are also present in the fatty acids of the alkane diol diesters (Table I). These data are in approximate agreement with those of Karkkainen et al., who reported on the fatty acid composition of the total diesters of vernix caseosa (6).

Table II gives the distribution of double bond positional isomers for the even and the odd chain monoene fatty acids and presents a possible mode of formation for each even chain monoene. For the odd chain monoenes, an analogous scheme of formation could be written, since the isomers of C_{14}, C_{16} and C_{18} are distributed in a manner parallel to those of C_{15}, C_{17} and C_{19}, respectively. Other schemes involving decarboxylation are also possible for the odd chain monoenes.

These data clearly show that in the alkane diol diesters of vernix caseosa both the $C_{16}\Delta 6$ and the $C_{16}\Delta 9$ patterns occur, the latter pattern predominating by a factor of at least threefold. Isomer distribution of the various even chain lengths indicates that elongation of $C_{16}\Delta 9$ apparently occurs to a far greater extent than elongation of $C_{16}\Delta 6$. For example, at a chain length of C_{16}, the $\Delta 9$ and the $\Delta 6$ isomers

TABLE II

Position Isomers of Fatty Acid Monoenes of Alkane Diol Diesters of Vernix Caseosa

Even chain length monoenes				Odd chain length monoenes		
Fatty acid structure	wt %[a]	Isomer distribution[b]	Possible mode of formation	Fatty acid structure	wt %[a]	Isomer distribution[b]
14:Δ6	.03	11	14:0 −2H →14:Δ6	15:Δ6	.1	27
14:Δ7		8	16:Δ9 − C_2→14:Δ7	15:Δ7		2
14:Δ8		2		15:Δ8		10
14:Δ9		72	14:0 −2H →14:Δ9	15:Δ9		46
14:Δ11		7		15:Δ10		5
				15:Δ11		10
16:Δ6	5.4	17	16:0 −2H →16:Δ6			
16:Δ7		1	18:Δ9 − C_2→16:Δ7	17:Δ6	.9	2
16:Δ8		6	14:Δ6 + C_2→16:Δ8	17:Δ7		1
16:Δ9		57	16:0 −2H →16:Δ9	17:Δ8		6
16:Δ10		3	14:Δ8 + C_2→16:Δ10	17:Δ9		48
16:Δ11		16	14:Δ9 + C_2→16:Δ11	17:Δ10		2
				17:Δ11		36
18:Δ6	23.9	3	18:0 −2H →18:Δ6	17:Δ12		2
18:Δ7		2	20:Δ9 − C_2→18:Δ7	17:Δ13		3
18:Δ8		7	16:Δ6 + C_2→18:Δ8			
18:Δ9		40	18:0 −2H →18:Δ9	19:Δ6	.5	Trace
18:Δ10		Trace	14:Δ6 +2C_2→18:Δ10	19:Δ8		5
18:Δ11		48	16:Δ9 + C_2→18:Δ11	19:Δ9		44
18:Δ12		Trace	14:Δ8 +2C_2→18:Δ12	19:Δ10		6
				19:Δ11		35
20:Δ10	1.5	5	16:Δ6 +2C_2→20:Δ10	19:Δ12		3
20:Δ11		30	18:Δ9 + C_2→20:Δ11	19:Δ13		7
20:Δ12		3	18:Δ10+ C_2→20:Δ12	19:Δ14		Trace
20:Δ13		61	16:Δ9 +2C_2→20:Δ13			
20:Δ14		1	14:Δ8 +3C_2→20:Δ14	21:Δ6	.04	2
				21:Δ7		3
22:Δ9	.2	Trace	22:0 −2H →22:Δ9	21:Δ8		6
22:Δ11		9	20:Δ9 + C_2→22:Δ11	21:Δ9		31
22:Δ13		30	18:Δ9 +2C_2→22:Δ13	21:Δ10		9
22:Δ14		8	14:Δ6 +4C_2→22:Δ14	21:Δ11		25
22:Δ15		53	16:Δ9 +3C_2→22:Δ15	21:Δ12		7
				21:Δ13		19
24:Δ11	.1	3	22:Δ9 + C_2→24:Δ11	21:Δ15		Trace
24:Δ13		4	20:Δ9 +2C_2→24:Δ13			
24:Δ15		40	18:Δ9 +3C_2→24:Δ15			
24:Δ17		53	16:Δ9 +4C_2→24:Δ17			

[a]Expressed as per cent of total saturates plus monoenes.
[b]For each chain length.

constitute 58% and 17%, respectively, whereas of all the C_{20} isomers, the Δ13 and the Δ10 isomers (which represent $2C_2$ extensions each of $C_{16}\Delta 9$ and $C_{16}\Delta 6$, respectively) constitute 61% and 5%, respectively. In an analysis of the total fatty acid monoenes of vernix caseosa, Downing and Greene (4) reported that $C_{16}\Delta 9$ and its C_2 extension products are major positional isomers of chains above C_{19}. These higher chains however, do not constitute the bulk of the total monoenes. The major fraction of fatty acid monoenes are below C_{19} and have the $C_{16}\Delta 6$ pattern.

Although lipids of vernix caseosa could be derived from either the fetal sebaceous gland or from its keratinizing epidermis, it is highly probable that the alkane diol diesters are products of human sebaceous glands. The skin surface lipids of many animals and the preen gland lipids of birds, both lipids primarily of sebaceous gland origin, have alkane diol diesters as major components (7). This would imply that the synthesis of $C_{16}\Delta 9$ and its elongation products is a sebaceous gland process. This assumption is further supported by the fact that surface lipids of rat (also primarily of sebaceous gland origin) show almost exclusively this pattern (2). Thus, human sebaceous glands appear to synthesize two highly characteristic double bond patterns of fatty acids, the $C_{16}\Delta 6$ and the $C_{16}\Delta 9$ patterns, and, strikingly, despite the presence of large amounts of components

from the $C_{16}\Delta6$ patterns in human sebaceous glands, components of the $C_{16}\Delta9$ pattern are preferentially used to esterify the alkane diols.

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[Received July 3, 1969]

In Vivo Transfer of ^{14}C-4-Cholesterol From Mouse Host To Encapsulated *Trichinella Spiralis* Larvae

ABSTRACT

Mice infected for four months with the parasite *Trichinella spiralis* were fed 4-^{14}C-cholesterol. The encapsulated larvae were then collected from the mice, and the lipids of the larvae were extracted and fractionated by thin layer chromatography (TLC). One of the TLC bands known to contain cholesterol was subfractionated; the resulting bands were assayed for radioactivity. It was found that the pure cholesterol fraction contained radioactive cholesterol, presumably from the host.

Trichinosis in humans results from the ingestion of insufficiently cooked pork which is infected with the cysts of *Trichinella spiralis*. Larvae are excysted and released in the duodenum. Maturing sexually within seven days, each female worm liverates a thousand or more larvae into the blood and lymph vessels. In the striated muscles the larvae mature within three weeks and become encapsulated by the host's connective tissue remaining there in an infective state for several years (1).

It is generally thought that the encysted *T. spiralis* metabolizes at a very low rate, and that host substances are unable to permeate the thick capsule wall. However, Stoner and Hankes in 1955 fed ^{14}C-labeled amino acids to mice which had been infected with this parasite for 56 days and were able to recover significant amounts of radioactivity in the extracted larvae (2). Also, the in vivo experiments of Frayha showed that cholesterol passes freely from the host tissue to the hydatid cysts of the parasite *Echinococcus granulosus* (3). The above findings suggest that *T. spiralis* exists in an active metabolic state in which host substances do permeate the capsule. The question remains as to whether the parasite is actually dependent on an exogenous source of cholesterol as has been shown with certain insect species (4,5).

Recently, the authors have observed the passage of ^{14}C-labeled cholesterol from the host to encapsulated *Trichinella* larvae. Two mice were infected with infective larvae of this worm. The infection was administered by stomach tube and 25 larvae per gram of body weight were the infecting dose. Four months after infection, they were fed for a week on food pellets (Ralston-Purina lab show) soaked with 3 μc, equivalent to 4.66 x 10^6 counts per minute (c.p.m.) of an ethanolic solution of 4-^{14}C-cholesterol (Radiochemical Centre, Amersham, England). The encysted *T. spiralis* larvae were recovered by digestion of the skinned, eviscerated hosts in a 1% pepsin-0.7% hydrochloric acid digestion solution. Tissue was soaked at 37 C (1 liter of digestion solution per gram of tissue) until all the muscle was digested. After repeated washings in sterile saline, until no radioactivity could be detected in the washings, the larvae were lyophilized to a dry weight of 0.100 g and then extracted with chloroform-methanol (2:1 v/v). [The methods for the extraction and fractionation of the lipids and the isolation of the cholesterol were similar to those described previously (6)]. The extract was subsequently dried over Na_2SO_4,

from the $C_{16}\Delta 6$ patterns in human sebaceous glands, components of the $C_{16}\Delta 9$ pattern are preferentially used to esterify the alkane diols.

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concentrated and spotted on thin layer chromatography (TLC) silica gel for the fractionation of the lipids, into three bands designated as Bands 1, 2 and 3 with methylene chloride-acetone (92:8 v/v) as developing solvent.

Band 1 was further fractionated on TLC (chloroform-acetone 90:10) into Bands 1a, 1b, 1c and 1d with corresponding R_f's of 0.00, 0.58, 0.87 and 0.98. The identities of these bands were unknown except for Band 1c which was shown to be cholesterol by infrared spectroscopic examination and R_f values in four solvents when in a previous experiment larvae were extracted from infected mice not fed radioactive material.

The four bands were subsequently scraped from the TLC plate, eluted with chloroform, and assayed for radioactivity in a Packard-Tricarb liquid scintillation counter (Model 3003). Most of the radioactivity of Band 1, 328 c.p.m., was found to reside in 1c, there being none in 1d and only negligible amounts in 1a and 1b. The eluates of Bands 2 and 3 (origin and front, respectively, of the TLC plate) were found to contain no radioactivity.

That the radioactivity did, indeed, reside in the cholesterol was confirmed by mixing 3 mg of the Band 1c residue with 37 mg of pure, unlabeled cholesterol and recrystallizing it twice from 95% ethanol and twice from ether-absolute ethanol (1:1 v/v) to the constant specific activities of 9.9, 8.1, 8.1 and 8.2 c.p.m./mg. The theoretical specific activity of the cholesterol mixture was 8.2 c.p.m./mg. Although the incorporation of radioactive cholesterol into the larvae by transfer from the host was small, the fact that it did occur is fairly certain.

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Sterol Galactosides and Sterol Esters of the Cotton Bud

ABSTRACT

About 60% of the total sterols in the cotton bud appeared in the free state; the esterified sterol glycosides contained about 50% saturated fatty acids, largely palmitic acid; the principal unsaturated fatty acid was linolenic acid. β-Sitosterol was the major sterol in all classes of sterol derivatives. The sugar moiety of the esterified sterol glycosides and the sterol glycoside was galactose. Efforts are continuing to evaluate the minor sterols of cotton buds, some of which appear to be hydroxylated ecdysones, and to study their relationship to the development of the Boll weevil, *Anthonomus grandis* Boheman.

Sadykov and Padkudina (1) isolated a sterol galactoside from cotton flowers. The presence of sterol glycosides and esterified sterol glycosides in a variety of plants has been reported (2-4). A previous study in this laboratory of the polar lipids of the cotton bud (square) (5) revealed the presence of a sterol glycoside. This report gives a detailed characterization of this glycoside, and also of the 3-hydroxysteroids and their esters.

Sterols and sterol derivatives were extracted by the method of Folch et al. (6) from freshly picked Deltapine Smoothleaf cotton buds containing the calyx and corolla. The total extract (4.2 g) was applied to a silicic acid column (100 g, 4 x 15 cm) in pentane. The lipids were eluted from the column with pentane (carotenoids, sterol esters, triglycerides), pentane-

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ether (1:1) (fatty acids, sterols, esterified sterol glycosides), chloroform-methanol (90:10)(phospholipids), and methanol (phospholipids, galactolipids). Final purification was performed by thin layer chromatography (TLC) on standard 20 x 20 cm plates coated with 250 micron layers of silica gel and calcium sulfate (9:1 w/w). The esterified sterol glycoside was purified by irrigation of the silica gel plates with chloroform-methanol (10:10 v/v), the sterol glycoside plates were developed in chloroform-methanol-water (85:15:1 v/v/v), and the components were eluted from the silica gel with chloroform-methanol (1:1 v/v). The sterol derivatives were made visible on the plates by spraying with water and observing the light bands.

Methyl esters were produced by alkaline hydrolysis of the esterified sterol glycoside (5,7). GLC was performed on a 10 ft, 1/8 in. o.d. column packed with 10% DEGS on 60/80 mesh HMDS-treated Chromosorb-W. The injector, column and detector temperatures were 210, 185 and 185 C, respectively. The instrument was calibrated with authentic standard mixtures of fatty acid methyl esters.

The major fatty acids found in the esterified sterol glycosides of the cotton bud were palmitic (40.2 μmole %), oleic (12.3 μmole %), linoleic (17.6 μmole %), and linolenic (29.7 μmole %). About equal quantities of saturated and unsaturated fatty acids were present in esterified sterol glycoside, with palmitic acid ($C_{16:0}$) the only saturated acid and linolenic acid ($C_{18:3}$) the major unsaturated fatty acid. Free sterols comprised 57.5%, esterified sterols 24.7%, esterified sterol glycosides 5.8%, and sterol glycosides 12.0% of the total sterols in the cotton bud. The sterols and sterol derivatives consitituted 0.019% of fresh weight of the cotton buds.

Sterol glycosides and esterified sterol glycosides were hydrolyzed with 1% sulfuric acid in ethanol under reflux for 22 hr (8), ethanol was removed in vacuo, and water was added to the residue. The aqueous suspension was extracted three times with ether, and the ether extracts were combined, concentrated and analyzed by GLC for sterols. GLC of the sterols was performed on a 6 ft, 1/8 in. glass column packed with 3% XE-60 on 100/120 mesh Gas-Chrom Q, and a 6 ft, 1/8 in. glass column packed with 1.5% SE-30 on 60/80 mesh Chromosorb-W (4,9). The injector, column and detector temperatures for both columns were 260, 240 and 240 C, respectively. Individual sterols were identified by comparing relative retention times of the samples with those of authentic sterols.

The distribution of β-sitosterol in free sterols was 59.3%, esterified sterols 49.4%, sterol galactoside 80.5%, and esterified sterol galactoside 78.7%. Campesterol and stigmasterol were present in the free sterols 37.2%, sterol galactoside 11.2%, esterified sterol galactoside 4.7%, and absent in the esterified sterols. Cholesterol was present in the free sterols at 1.6%, esterified sterols 50.6%, sterol galactoside 4.3%, and esterified sterol galactoside 7.5%. Over 50% of the sterols in the cotton bud were β-sitosterol, and most of the campesterol and stigmasterol were present as free sterols. Most of the cholesterol (50%) was esterified.

The aqueous acid phase from the ether extraction of the sterols was neutralized with barium hydroxide, filtered, vacuum dried at 40 C, and the trimethylsilyl derivatives of the sugar were prepared (10). Total sugar was determined by the method of Dubois et al. (11). The sugar was identified as galactose from the relative retention time of its trimethylsilyl derivative, measured by GLC on a 4 ft, 1/8 in. stainless steel column packed with 1% SE-30 on HMDS-treated Chromosorb-P. The injector temperature was 220 C, column and detector temperatures both were 185 C, and inlet pressure was 14 psi. The ratio of sugar-sterol-fatty acid was 1:1:1 for the esterified sterol glycoside, and the ratio of sterol-sugar was 1:1 for the sterol glycosides. These ratios are common in plants, but the substitution of galactose is unusual.

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The *Trans*-6 Fatty Acids of *Picramnia sellowii* Seed Oil[1]

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ABSTRACT

The C_{18} monoenoic acids in *Picramnia sellowii* Planch. seed oil include both *cis*- and *trans*-6-octadecenoic acids, as well as oleic acid. The hexadecenoic acids are also the *cis*- and *trans*-Δ6-isomers, and the eicosenoic acids have Δ6-unsaturation of undetermined geometric configuration. The C_{18} polyenoic acids detected are 9,12- and 6,9-octadecadienoic and 9,12,15- and 6,9,12-octadecatrienoic acids. Partial investigation of another species, *P. pentandra* Sw., revealed its oil to have a similar fatty acid composition.

INTRODUCTION

In screening a variety of plant seed oils, infrared (IR) spectroscopy has provided a means to detect unusual composition. For example, the IR spectrum of *Picramnia sellowii* (Simarubaceae) oil in carbon disulfide solution had a weak band at 10.4 μ. This band is usually associated with isolated *trans* unsaturation. The rest of the spectrum was consistent with those of normal seed oils. Methyl esters prepared from the oil also showed the *trans* absorption band. Although *Picramnia* seed oils are rich in 6-octadecynoic (tariric) acid (1), fatty acids containing *trans* bonds have not been reported in them. Since the oil of another species, *P. pentandra*, exhibited a similar IR spectrum (2) and since *trans* double bonds occur infrequently in seed oils, fatty acid compositions of the *Picramnia* oils were investigated.

EXPERIMENTAL PROCEDURES

Oil was extracted from the ground seed and analyzed as previously described (3,4). Methyl esters of the fatty acids were prepared (5) and analyzed by both gas liquid (GLC) and thin layer (TLC) chromatography (3,6). TLC on plates coated with a 1 mm thick layer of Silica Gel G containing 20% silver nitrate and developed with benzene was used to separate *P. sellowii* esters according to degree of unsaturation. These fractions were also analyzed by GLC. Another portion of these esters was first separated on the basis of chain lengths by preparative GLC (7) and then examined by $AgNO_3$-TLC.

Double and triple bond positions were located by ozonolysis procedures, which utilize GLC to identify and relate the cleavage fragments quantitatively (8,9). IR spectra of carbon disulfide solutions were recorded on a Perkin-Elmer Model 137 or a Model 337 spectrophotometer in 1 mm sodium chloride cells.

RESULTS AND DISCUSSION

Identification of Acids Containing Trans Bonds

TLC of the esters from *P. sellowii* oil on a plate impregnated with silver nitrate is shown in Figure 1. Spot II, slightly above the monoenes of the soybean esters (Sample B), is in the area of the plate to which *trans* monoenes migrate. After the esters had been separated by prepara-

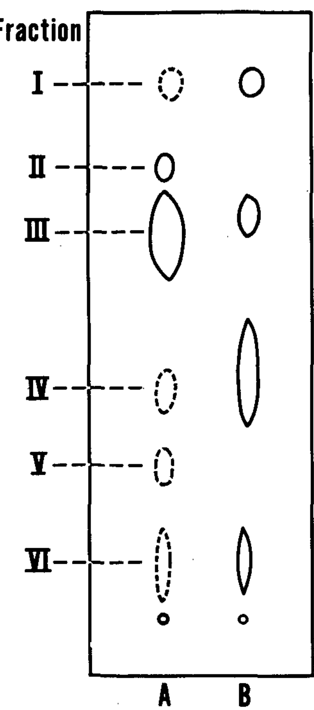

FIG. 1. Thin layer chromatography of *Picramnia* esters (Sample A) and soybean esters (Sample B) on 20% $AgNO_3$ in Silica Gel G with benzene as the development solvent.

[1]Presented in part at AOCS Meeting, New York, October 1968.

[2]No. Utiliz. Res. Dev. Div., ARS, USDA.

TABLE I

Ester Composition of Fractions From Preparative TLC

Fraction[a]	Composition (Per cent by GLC)
I (saturates)	C_{14}-2.1; C_{16}-9.3; C_{18}-83.4; C_{20}-5.1
II (*trans*-monoenes)	C_{16}-2.6; C_{18}-93.5; C_{20}-0.2; tarirate-3.7
III (*cis*-monoenes)	C_{16}-0.5; C_{18}-3.7; tarirate-96.0
IV (diene)	C_{18}-74.3 (ECL 18.9); tarirate-25.6
V (diene)	C_{18}-91.6 (ECL 18.7); C_{18}-5.4 (ECL 19.3); C_{18}-3.0 (ECL 19.7)
VI (diene and triene)	C_{18}-30.3 (ECL 18.7); C_{18}-57.8 (ECL 19.3); C_{18}-11.8 (ECL 19.7)

[a]Identification based on ECL from LAC-2-R-446 column (6).

tive $AgNO_3$-TLC into Fractions I through VI (Fig. 1), IR analyses indicated that only Fraction II contained *trans* unsaturation. The composition of each fraction by GLC analysis is given in Table I.

Preliminary identifications of the components in the fractions were based on their equivalent chain lengths (ECL) (6). The ECL of the diene in Fraction IV is identical to that of methyl linoleate. Fraction V is composed of a diene which has an ECL 0.2 units less than that of linoleate along with small amounts of the two trienes which, together with this unusual diene, make up Fraction VI. The ECLs of the trienes in Fraction VI suggest that one is linolenate (ECL = 19.7) and the other (ECL = 19.3) a triene analogous to the unusual diene found in Fraction V.

$AgNO_3$-TLC of the fractions collected from preparative GLC of the esters showed spots corresponding to both *cis*- and *trans*-hexadecenoates and octadecenoates. The eicosenoate fraction, however, migrated to a single spot in the *trans*-monoene region of the plate.

TABLE II

Composition of *Picramnia sellowii* Esters

Component	Per cent by GLC
14:0	Trace
16:0	0.1
$16{:}1^{6c}$	0.4
$16{:}1^{6t}$	0.2
18:0	1.5
$18{:}1^{6c}$	2.9[a]
$18{:}1^{6t}$	7.4
$18{:}1^{9c}$	0.3[a]
$18{:}2^{6,9}$	0.1
$18{:}2^{9,12}$	0.9
$18{:}3^{6,9,12}$	0.3
$18{:}3^{9,12,15}$	0.1
20:0	0.3
$20{:}1^{6}$	0.2
Tarirate (6-octadecynoate)	85.3

[a]Percentages based on peak areas of ozonolysis fragments (8) (see text).

The IR spectrum of a highly concentrated solution of Fraction III had no bands in the 10-11 μ region. At such a concentration *trans* unsaturation at levels of less than 1% would have been detected. It was assumed, therefore, that Fraction III contained no *trans*-monoenes. Since Fraction III was by far the largest fraction collected and Fraction IV was very small, Fraction III contained essentially all the tarirate in the mixed esters. The proportions of the *cis*-hexadecenoates and *cis*-octadecenoates in the mixed esters could therefore be calculated using the tarirate as an internal standard. The proportions of *trans*-monoenes in the mixed esters were obtainable by difference. The ratios thus established between *cis*- and *trans*-hexadecenoates were corroborated by GLC of the esters on a capillary column (Ackman, private communication).

Establishment of Bond Positions

Ozonolysis-GLC of Fraction II yielded C_6 aldehyde-ester (AE), C_{12} aldehyde (A), and C_{10} A. Since C_6 AE was the only aldehyde-ester found, the *trans*-monoenes are all $\Delta 6$ unsaturated. C_{14} A was tentatively identified (one column only) in the reduced ozonides from Fraction II; therefore, the eicosenoate is probably the $20{:}1^{6t}$ ester although neither the geometry nor the position of the double bond is firmly established. The C_{10} A and C_{12} A were found in the same relative abundance as the parent esters ($16{:}1^{6}$ and $18{:}1^{6}$) in Fraction II (Table I).

Fraction III was more complex than Fraction II and yielded C_9 AE and C_9 A, as well as C_6 AE, C_{10} A, and C_{12} A upon ozonolysis. Oleate is therefore present in the *cis*-monoenes together with the $18{:}1^{6}$ and $16{:}1^{6}$. Under the low temperature ozonolysis conditions, triple bonds were not cleaved (9). Ozonolysis in methanol at room temperature, however, identified the major component in Fraction III as methyl tarirate (9).

The major component in Fraction IV was proved to be methyl linoleate by interrupted

ozonolysis (8).Similarly, the diene in Fraction V was shown to be 6,9-octadecadienoate. The trienes in Fraction VI were examined by the interrupted ozonolysis method and the fragments delineated the parent esters to be 6,9,12-octadecatrienoate (γ-linolenate) and 9,12,15-octadecatrienoate (α-linolenate). The relative peak areas of the fragments are in good agreement with the proportions of the parent esters shown in Table I, after correction for detector response and for the amount of 6,9-octadecadienoate (Fraction V) which carried over into Fraction VI.

The total fatty acid composition of *P. sellowii* oil is given in Table II. The percentages are based on the GLC of the mixed esters and of the ozonolysis products from the unsaturated esters. Although not so rigorously analyzed, *P. pentandra* seed oil appears to contain *trans*-6-hexadecenoic and octadecenoic acids in nearly the same abundance as *P. sellowii* oil.

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R. G. Ackman, Fisheries Research Board, Halifax, N.S., provided the capillary column GLC analysis of *Picramnia* esters.

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trans-6-Hexadecenoic Acid in the Atlantic Leatherback *Dermochelys coriacea coriacea* L. and Other Marine Turtles

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ABSTRACT

Depot fat from the Atlantic leatherback turtle (*Dermochelys coriacea coriacea* L.) was shown to contain ~3% of *trans*-6-hexadecenoic acid. Structural details were elucidated through comparative aspects of isolation techniques, NMR, IR, hydrogenation, oxidative fission, etc., and confirmed by similarity of properties with those of a sample of fatty acid of this structure isolated from the seed oil of *Picramnia sellowii*. One additional leatherback turtle oil sample, and depot fat from two other marine turtles, the loggerhead (*Caretta caretta caretta*) and ridley (*Lepidochelys olivacea kempi*) contained this acid, that from the ridley in a lower proportion. No corresponding C_{18} acid was detected in the leatherback oil.

INTRODUCTION

The lipids of marine reptiles have received scant attention in recent years, although several turtle oils were at one time studied by classical distillation techniques (1). The oil of the Atlantic leatherback turtle *Dermochelys coriacea coriacea* L. was examined in this laboratory by gas liquid chromatography (GLC) on packed columns and found to conform broadly in fatty acid composition to a general marine oil pattern (2). Oil from this species was recently reinvestigated on open tubular (capillary) GLC columns as part of a continuing study on monoethylenic fatty acid isomer compositions of marine lipids (3-8; also, Ackman et al., submitted for publication).

It is known that the elution sequence of the methyl esters of monoethylenic fatty acids, when all are of the same configuration (either *cis* or *trans*), is nominally similar on both polar and nonpolar GLC liquid phases when only the common range of fatty acid positional isomers is concerned. Basically this range, in the C_{18} acids for example, would include the 6-octadecenoic to the 15-octadecenoic acids. It is also apparent from published data that the elution order of the components in a mixture of *cis* and *trans* monoethylenic acids from this range, as well as for the other positional isomers, may be somewhat different on a nonpolar liquid phase as compared to a polar liquid phase (9-11). Comparison of the C_{16} monoethylenic fatty acid complex in a whole-oil GLC analysis of leatherback turtle oil on a polar column (Fig. 1) and a nonpolar column (Fig. 2) showed transposition of the two major 16:1 components. This observation of a nonconforming isomer stimulated a detailed investigation and resulted in the identification of *trans*-6-hexadecenoic acid in the leatherback and two additional marine turtle oils.

EXPERIMENTAL PROCEDURES

Detailed examination of the leatherback turtle oil was primarily based on the sample examined previously (2). The oil (a depot fat) was recovered from its fibrous matrix by extraction with chloroform. An additional sample of leatherback turtle fat, although somewhat oxidized, was treated similarly. These fats came from turtles recovered in Nova Scotian waters (12). Body fat samples from the loggerhead (*Caretta caretta caretta*) and ridley (*Lepidochelys olivacea kempi*) came from specimens captured respectively at Veracruz, Mexico, and in the Gulf of Mexico off Tamaulipas. Seed oil was recovered from *Picramnia sellowii*.

All oil samples were saponified and nonsaponifiable materials were extracted from soap solutions by AOCS methods. The fatty acids were recovered and converted to methyl esters by brief treatment with 5% BF_3-MeOH solution.

Basic analytical GLC was carried out with open tubular columns (150 ft x 0.01 in i.d.) coated with butanediol succinate (BDS) polyester or Apiezon-L (Ap-L) grease. The columns were purchased from the Perkin-Elmer Corp. and operated with a high split ratio (No. 1 or 2) in either Model 226 or Model 900 GLC units of this firm. Injection port temperatures were 250 C, and operating conditions for the respective columns were: BDS, 170 C and 50 psig helium; Ap-L, 190 C and 80 psig helium. Preparative GLC was carried out with an Aerograph A-90 (thermal conductivity) unit fitted with a 10 ft x 1/4 in. column packed with Chromosorb G (DMSC), 80/100 mesh, coated with 10% SE-30 silicone gum.

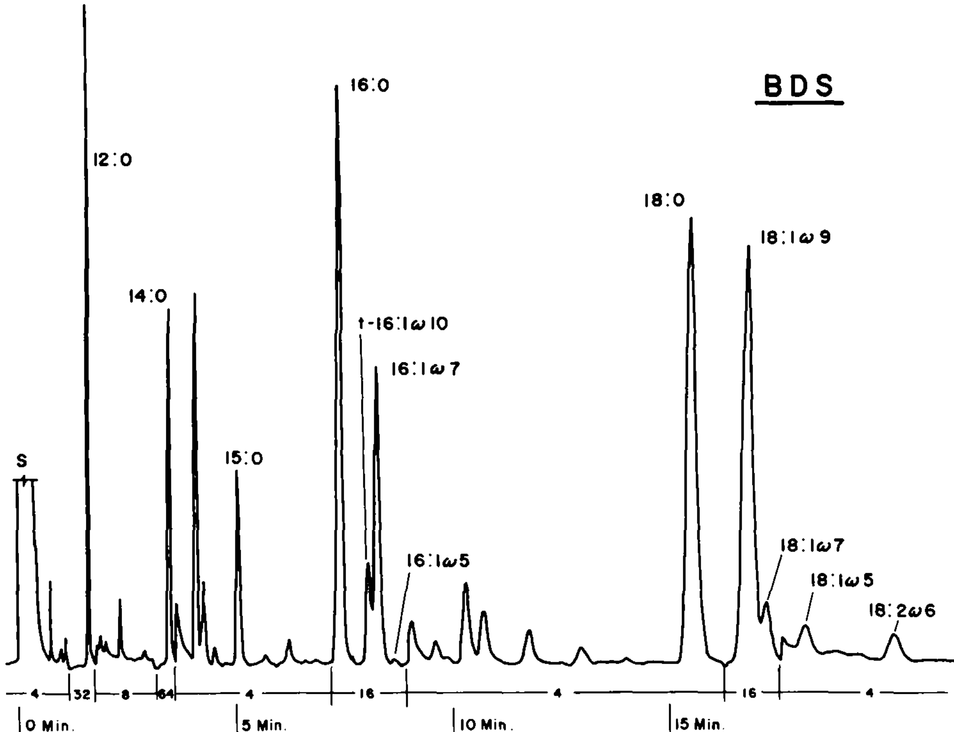

FIG. 1. Partial chromatograph recording from GLC analysis on an open-tubular column, with BDS coating, of methyl esters of fatty acids of leatherback turtle oil. S, solvent. Time and attenuations noted at bottom.

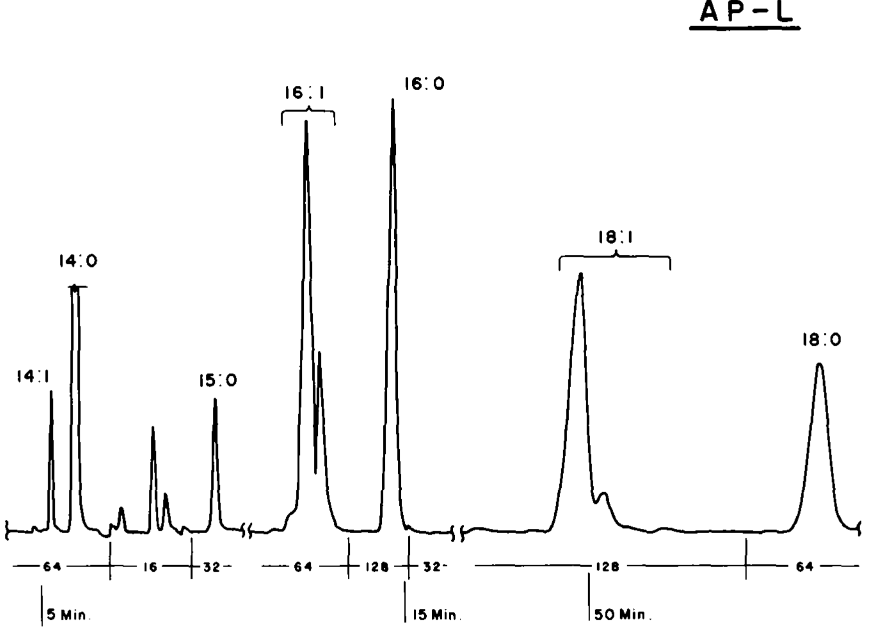

FIG. 2. Partial chromatograph recording from GLC analysis on open-tubular columns, with Ap-L coating, of sample shown in Figure 1.

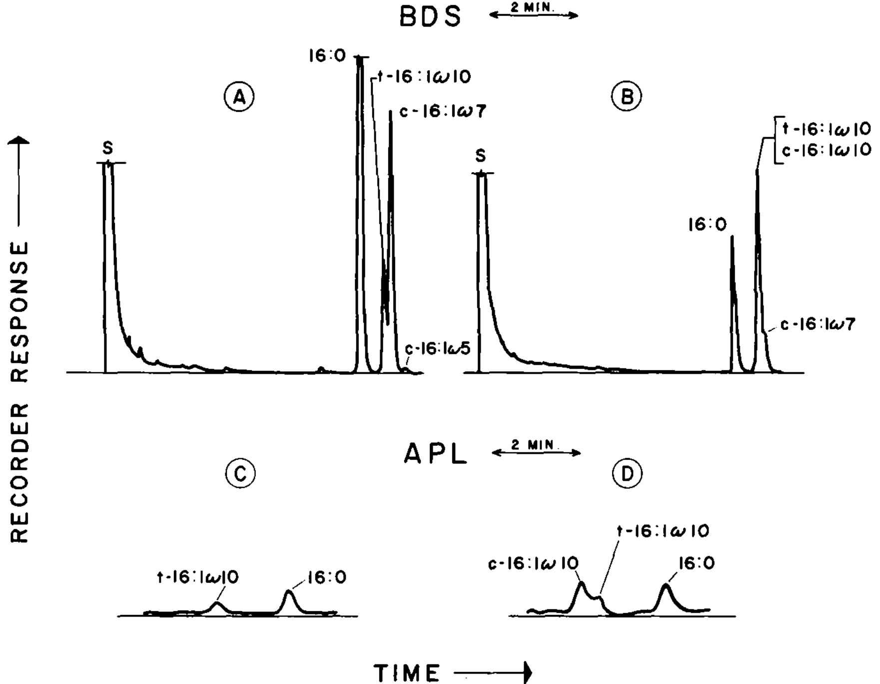

FIG. 3. (A) Chromatograph recording from GLC analysis on open-tubular column, with BDS coating, of methyl esters of C_{16} fatty acids from loggerhead turtle oil as isolated by preparative GLC (note: polyenes not obvious at this attenuation). (B) Chromatograph recording from GLC analysis on open-tubular column, with BDS coating, of methyl esters of C_{16} fatty acids from *P. sellowii* seed oil as isolated by preparative GLC. Note that both *cis*- and *trans*-6-hexadecenoic acid are present in the first monoene peak. (C) Partial chromatograph recording from GLC analysis on open-tubular column, with Ap-L coating, of *trans*-6-hexadecenoic acid after GLC and TLC-$AgNO_3$ isolation (16:0 added for reference). (D) Partial chromatograph recording from GLC analysis on open-tubular column with Ap-L coating of sample shown in B.

Silver nitrate chromatographic procedures were either large scale (~100 mg) by column, using Florisil-$AgNO_3$ as described elsewhere (3), or semipreparative thin layer chromatography (TLC), using plates prepared with Supelcosil 12D Lot 45E (Supelco Inc., Bellefonte, Pa.). Plates were activated for 1 hr at 100 C before use and developed at room temperature in benzene-hexane, 1:1. Visualization was by spraying with 0.2% 2',7'dichlorofluorescein and scanning under UV light.

NMR spectra were measured at 60 m H_z with a Varian A 60 spectrometer. Solutions in $CDCl_3$ (with TMS standard) were made up in 40 μl integral sphere microcells. IR spectra were obtained on samples of pure film (NaCl plate) with a Perkin Elmer 237.

Ozonolysis was carried out in methanol with oxidative work-up (13). Products were identified through direct GLC of C_5 - C_{12} monocarboxylic acids (14) and by in situ esterification with 2,2-dimethoxypropane (13) for study of methyl esters of mono- and dicarboxylic acids.

RESULTS

Preparative Florisil-$AgNO_3$ chromatography failed to give an adequately clean separation of the methyl ester of the unknown (*trans*-6-hexadecenoic acid) from total marine oil fatty acid esters owing to the rapid elution behavior of certain of the range of marine oil *cis* isomers in several chain lengths commonly found in marine oils (3-8, 15). More economy of effort was achieved by isolation of the C_{16} methyl esters by preparative GLC followed by TLC on the Supelcosil plates. The unknown C_{16} material gave a clearly defined spot between 16:0 and a major component presumed to be palmitoleate (*cis*-9-hexadecenoate) on the basis of an R_f similar to that of methyl oleate (*cis*-9-octadecenoate) (16). The R_f value of the unknown was essentially the same as that of methyl elaidate (*trans*-9-octadecenoate). The unknown recovered from the TLC plates was identical in GLC behavior to the unusual C_{16} component illustrated in Figures 1 and 2. This

isolative technique was reproducible and applicable without difficulty to all samples. Column chromatography was however applicable to the C_{16} fraction (order of elution: 16:0, unknown, palmitoleate), but was used only for a few large-scale preparations on pooled preparative GLC effluents.

On hydrogenation the unknown gave a material indistinguishable from 16:0 by various GLC techniques. The IR spectrum showed a strong absorption band at 10.33 μ. On a semi quantitative basis this absorption, and that of the rest of the spectrum, were similar to that of methyl elaidate, and the general absence of specific NMR details suggested a double bond in the central portion (approx Δ^4 to Δ^{11}) positions (17). There was no spectral evidence for methyl branching or methyl substitution at the double bond. Oxidative fission of material recovered from chromatographic steps indicated >96% purity in terms of 10:0 monocarboxylic acid (identified as acid and as methyl ester) and 6:0 dicarboxylic acid (identified as methyl ester). An ozonolysis of total C_{16} fraction from the preparative GLC gave 16:0, 10:0, 7:0 mono- and 6:0 and 9:0 dicarboxylic acids in approximately the proportions indicated by open-tubular GLC analysis of the total C_{16} fraction.

The above results indicated that the unknown methyl ester from leatherback turtle oil should be the methyl ester of *trans*-6-hexadecenoic acid. The melting point of the acid recovered after saponification of ester (poorly defined crystals from petroleum ether) was 33-34 C, and is consistent with this proposed structure on the basis of similarity to melting points listed for various C_{18} monoethylenic acids (18). The GLC behavior of the ester on BDS and Ap-L vis a vis methyl palmitoleate was also compatible with this structure when comparisons were made with published retention data for methyl esters of C_{18} monoethylenic acids (9), and with data obtained in our laboratory for these materials on BDS and Ap-L open-tubular columns supporting the broad applicability of the literature data (10).

Subsequent to our identification of this acid in leatherback turtle oil a sample of *P. sellowii* seed oil became available. Recovery of the methyl ester of *trans*-6-hexadecenoic acid, previously indicated as a component of this oil (19,20), gave complete coincidence of components in several TLC and GLC systems tested (see below).

DISCUSSION

Trans-6-hexadecenoic acid amounted to 2-3% of the total fatty acids in the initial sample of oeatherback turtle oil, about the same in a different sample (somewhat oxidized), and in loggerhead depot fat, but was not as obvious (<1%) in the fat from the ridley. The presence of this acid in three different marine species of diverse origin would seem to indicate the deposition of this acid from a common food source. There is no apparent occurrence of either *trans*-6- or *trans*-8-octadecenoic acid which might be related. A preliminary screening of a number of other marine lipids for *trans*-6-hexadecenoic acid suggests that the occurrence of *trans*-6-hexadecenoic acid is limited to animals such as the marine turtles and the ocean sunfish (*Mola mola*) which are known to feed heavily on jellyfish (21). Further research on this basic source of this acid is planned.

Comparative features of the GLC behavior of some methyl esters of hexadecenoic acids are shown in Figure 3. A precise study of retention data remains to be carried out, but it may be noted that the *cis*- and *trans*-6-hexadecenoates (ratio 2:1) fail to separate from each other on this BDS column and effectively occupy the position usually assigned to *cis*-7-hexadecenoate in analyses of the methyl esters of fatty acids from marine lipids. In the alternative viewpoint of structure and GLC retention times, where significance is assigned to the ω value (carbon chain moiety terminating in the methyl group), this means that, in the 16:1 acids, *cis* ω10 and *cis* ω9 do not really separate from each other, but separate from *cis* ω7. Paralleling this, in the 18:1 acids, *cis* ω12 (petroselinate) and *cis* ω9 (oleate) show no separation on BDS columns with as many as 50,000 plates, although a small but useful separation of *cis* ω11 and *cis* ω9 is observed (3,22), but all of these 18:1 acids separate from *cis* ω7. In screening seed oils for petroselinic and related acids by open tubular GLC (polar columns) it might therefore be useful to examine the minor C_{16} acids as indicators of the probable presence of C_{18} analogues not detectable directly.

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An Investigation of the Lipid Metabolism of Dog Kidney Medulla and Cortex

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ABSTRACT

Unanesthetized, resting dogs were infused through a leg vein for approximately 90 min with 1-^{14}C-palmitate and oleate-9,10-H^3 complexed to albumin. The animals were then anesthetized, exsanguinated, and the kidneys removed. Lipids were extracted from the medullae and cortices with chloroform-methanol (2:1) and the lipid classes were analyzed for radioactivity content and fatty acid composition. Triglyceride subclass species were determined by silver nitrate thin layer chromatography. The triglyceride content of the medulla was much higher than that of the cortex while the phospholipid level was slightly higher in the cortex. The fatty acid composition of triglyceride and phospholipid was not demonstrably different in the medulla or cortex. The amount of monounsaturated triglyceride species (001) was lower and the tetra- (022) and more unsaturated species were higher in the cortex than in the medulla. Most of the radioactivity was found in the phospholipid fraction and a large portion of the remainder was in triglyceride. Cortical phospholipids contained 80% of the radioactivity versus a value around 60% in the medulla, whereas triglyceride, in the medulla, was approximately 26% compared to the cortical content of 15%. The results, with respect to the per cent distribution of radioactivity, were similar for radiocarbon and tritium. The cortex possessed a higher specific activity for oleate and palmitate in both triglyceride and phospholipid. The total kidney retained 0.25% of the infused palmitate and 0.08% of the infused oleate.

INTRODUCTION

The clear demonstration by Chinard et al. (1) that glucose uptake cannot provide the energy required by the oxygen consumed in the kidney made it obvious that lipids had to be considered for this role. Hohenleitner and Spitzer (2) were among the earliest investigators to demonstrate that free fatty acids (FFA) could be taken up from the blood stream by the kidney under in vivo conditions in anesthetized dogs, with catheters placed in a systemic artery and renal vein. Using a similar preparation, Gold and Spitzer (3) infused various radioactive fatty acids complexed to albumin. They measured the radioactive carbon dioxide produced and by gas liquid chromatography measured the arteriovenous plasma free fatty acid composition. They noted a consistent uptake and oxidation of palmitate and a quite variable response for oleate, leading to a statistically nonsignificant uptake and oxidation for this metabolite.

In the meantime, the concept was rapidly becoming accepted that the renal medulla and cortex had quite different metabolic capacities. Kramer et al. (4) reported that in the dog, the

TABLE I

Lipid Composition of Dog Kidney Medulla and Cortex

Dog no.		Wet weight,[a] g	Triglyceride (μM/g wet wt.)	Phospholipid (μM/g wet wt.)
46	Medulla	16.6	12.6	7.3
	Cortex	83.4	8.8	11.3
47	Medulla	8.0	9.6	9.1
	Cortex	56.5	1.2	11.2
48	Medulla	13.6	20.6	11.2
	Cortex	98.2	1.5	11.7
Average	Medulla	12.7	14.3	9.2
	Cortex	79.4	3.8	11.4

[a]Weight of both kidneys.

TABLE II

Triglyceride and Phospholipid Fatty Acid Distribution of Dog Kidney Medulla and Cortex (Mole %)

Dog No.			14:A[a]	14:0	16:A	16:0	16:1	18:A	18:0	18:1	18:2	18:3	20:3	20:4
46	Medulla -	triglyceride	---	1.2	---	28.4	4.7	---	7.4	41.9	16.5	0.7	1.5	3.5
		phospholipid	0.4	0.3	2.4	32.0	1.1	1.2	10.1	14.6	12.4	0.2	0.7	24.6
	Cortex -	triglyceride	---	1.9	---	24.7	3.7	---	11.7	48.0	8.4	0.9	---	0.3
		phospholipid	0.2	0.8	3.8	24.3	1.2	2.8	14.7	18.0	12.1	---	0.4	21.8
47	Medulla -	triglyceride	---	3.0	---	20.9	5.4	---	7.8	42.9	17.3	0.8	---	0.6
		phospholipid	---	---	2.4	21.9	1.0	2.2	16.2	18.0	15.2	---	0.9	22.3
	Cortex -	triglyceride	---	2.0	---	22.0	5.7	---	7.3	40.4	19.5	1.3	---	1.8
		phospholipid	---	---	2.2	23.3	1.2	1.6	10.8	18.1	18.6	0.4	0.9	22.5
48	Medulla -	triglyceride	---	2.2	---	22.5	3.4	---	11.2	45.9	12.8	1.0	---	0.4
		phospholipid	---	---	3.9	22.1	1.0	2.7	13.2	13.7	15.6	1.6	1.3	25.7
	Cortex -	triglyceride	---	1.9	---	25.9	3.2	---	10.2	43.8	12.2	1.2	---	1.0
		phospholipid	---	0.4	2.8	34.2	2.0	1.9	8.5	12.1	13.1	0.4	0.7	23.9
Average	Medulla -	triglyceride	---	2.1	---	23.9	4.5	---	8.8	43.6	15.5	0.6	0.5	1.5
		phospholipid	0.1	0.1	3.6	25.3	1.0	2.0	13.2	15.4	14.4	0.6	1.0	24.2
	Cortex -	triglyceride	---	1.9	---	24.2	4.2	---	9.7	44.1	13.4	1.1	---	1.0
		phospholipid	0.1	0.4	2.9	27.3	1.5	2.1	11.3	16.1	14.6	0.3	0.7	22.7

[a]Carbon number: double bonds; A stands for aldehyde.

TABLE III

Percentage Distribution of Major Lipid Classes Based on Radioactivity in Kidney

Dog No.		Phospholipid		Triglyceride		Diglyceride		FFA		Cholesterol		Cholesterol ester	
		^{14}C[a]	H^3	^{14}C	H^3	^{14}C	H^3	^{14}C	H^3	^{14}C	H^3	^{14}C	H^3
47	Medulla	51.6	74.2	26.1	14.3	2.0	0.1	5.8	8.2	13.5	3.0	0.1	0.1
	Cortex	82.3	79.4	12.4	14.0	1.2	0.1	0.5	4.1	3.0	2.2	0.5	0.1
48	Medulla	50.5	63.9	38.6	29.2	0.1	0.1	5.3	4.0	5.5	2.5	0.1	0.1
	Cortex	73.2	83.9	19.5	13.1	1.8	0.9	0.2	0.1	4.3	1.9	0.8	0.1
Average	Medulla	51.1	69.1	32.4	21.8	1.0	0.1	5.6	6.1	9.5	2.8	0.1	0.1
	Cortex	77.8	81.7	16.0	13.6	1.5	0.5	0.4	2.1	3.7	2.1	0.7	0.1

[a] ^{14}C (1-^{14}C-palmitate infused), H^3 (oleate-9,10-H^3 infused).

medullary blood flow and oxygen consumption was only about 5% of that in the cortex. Lee et al. (5), using rabbit kidney medulla and cortex slices, observed that fatty acids appeared to be used predominantly by the cortex while glucose seemed to be the major fuel for the medulla. The reports of low oxygen consumption along with a high rate of glycolysis in the medulla thus correlated well, as did the high oxygen consumption with a large utilization of fatty acid by the cortex.

Since the above mentioned investigators have found FFA utilization by the dog kidney, it was of interest to ascertain if the dichotomy of metabolism would lead to marked differences in the lipid pattern in the medulla and cortex, especially under essentially normal conditions. Therefore, a study was initiated in which a mixture of 1-^{14}C-palmitate and oleate-9,10-H^3 complexed to albumin was infused into a leg vein of unanesthetized resting dogs for 90 min; the dogs were then anesthetized, exsanguinated, and the renal medulla and cortex removed for analyses.

MATERIALS AND METHODS

To form the FFA-albumin complex 1-^{14}C-palmitic acid (Tracer Lab., Inc., specific activity 41 mc/mmole in hexane) and oleic acid-9,10-H^3 (Nuclear-Chicago, specific activity 750 mc/mmole in benzene) were placed in a flask, the solvent evaporated off with nitrogen, and 1.5 mmoles of sodium hydroxide in 5 ml of physiological saline added. Four grams of Fraction V human serum albumin were dissolved in 20 ml distilled water. The flask containing the fatty acids was swirled in warm water to dissolve the soaps and the albumin solution was then added with mild agitation. The amount of palmitate added to the albumin was 0.31 μg/mg and the corresponding value for oleate was 0.19.

Three male mongrel dogs weighing 20-27 kg were used after fasting overnight (approximately 16 hr) with free access to water. The animals had previously been fed Purina Dog Chow ad lib. The day prior to the experiment the dogs were anesthetized with sodium pentothal (20 mg/kg) and a small catheter placed into the superficial plantar metatarsal vein. A cardboard collar was placed around the dog's neck to prevent gnawing at the catheter site. The following day the dog was placed into a resting position and infused with 20 ml of FFA-human albumin mixture at the rate of 0.2 ml/min. The infusion mixture consisted of 11.4 μc/ml of 1-^{14}C-palmitate and 84.4 μc/ml oleate-9,10-H^3. Ten minutes before the end of the

TABLE IV

Kidney Triglyceride and Phospholipid Fatty Acid Total and Specific Radioactivity

Dog No.		Triglyceride fatty acid S.A.[a]		Phospholipid fatty acid S.A.		Total tissue Triglyceride Radioactivity (mμc)		Total tissue Phospholipid Radioactivity (mμc)	
		^{14}C DPM / μM Palmitate	H^3 DPM / μM Oleate	^{14}C DPM / μM Palmitate	H^3 DPM / μM Oleate	^{14}C	H^3	^{14}C	H^3
47	Medulla	370	463	1461	6861	8.1	20.8	21.2	81.7
	Cortex	3102	4627	3328	9908	63.1	172.8	446.0	1031.6
48	Medulla	150	520	723	3402	12.9	91.2	22.1	64.5
	Cortex	2005	2253	1350	8293	104.4	198.2	482.4	1047.9
Average	Medulla	260	492	1092	5132	10.9	56.0	21.7	73.1
	Cortex	2554	3440	2339	9101	83.8	185.5	464.2	1039.8

[a]S.A., specific activity.

infusion the dog was anesthetized with phenobarbital (30 mg/kg) and exsanguinated by catheters placed in the femoral and carotid artery. The kidneys were exposed by laparotomy, excised, and the cortex and medulla separated by cutting along the visible demarcation line. The capsule and adhering tissue were carefully removed.

Medullae and cortices were weighed, homogenized, and then lipids extracted by the method of Folch, et al. (6). The corresponding kidney segments were pooled so data presented in the Result Section do not specify right or left medulla or cortex. The lipid classes were separated by the thin layer chromatographic (TLC) procedure of Christophe and Matthijs (7), which employed a solvent system of petroleum ether-acetone (85:15). This method separated the major lipid classes in the following sequence of increasing polarity: cholesterol esters, triglycerides, diglycerides, cholesterol, free fatty acids, monoglycerides and phospholipids. Lipids were identified by means of standards purchased from Applied Science, Inc. and visualized by spraying with Rhodamine 6G dye. The lipid bands were scraped from the thin layer plate into counting vials and their radioactive content determined by the method of Bray (8) in a Packard Liquid Scintillation Spectrometer. The triglyceride and phospholipid classes were also analyzed for their fatty acid compositions by scraping the silica gel with these lipids into screw-cap tubes containing 5 ml methanol, 0.2 ml, 2,2-dimethoxy-propane and 0.25 ml concentrated sulfuric acid, sealing with a teflon-lined cap, and heating at 70 C for 2 hr. The samples were then extracted with three 5 ml portions of hexane after the addition of 10 ml water. The triglyceride and phospholipid bands were quantitated by adding, as an internal standard, heptadecanoic acid (Applied Science, Inc., better than 99% pure by gas chromatographic assay) to the methylation mixture. The methylated derivatives were analyzed in a Beckman GC-4 gas chromatograph using a flame ionization detector. The column was 6 ft x 1/8 in stainless steel tubing packed with 10% diethylene glycol succinate polymer on Chromsorb W (80-100 mesh, acid-washed and silanized) with helium the carrier gas (9). The areas of each peak were calculated from measurements of peak height and width at half height. The areas were corrected for attenuation differences and also for the response differentials by the flame ionization detector. The areas were compared to that of the internal standard and its concentration, then the micromoles of each component were calculated.

Standards were used to identify the

TABLE V

Triglyceride Double Bond Species in Dog Kidney Medulla and Cortex (Mole %)

Component	Dog no. 48		Dog no. 45
	Medulla	Cortex	Whole kidney[a]
000	2.2	1.9	2.9
001	21.0	14.7	17.6
011	30.3	31.9	27.5
002	6.2	4.3	4.1
111	9.8	6.2	9.9
012	15.5	13.6	17.7
112	9.0	9.1	10.5
022	1.9	5.7	3.5
Others	4.1	12.7	6.4

[a]Reference 11.

retention times of the fatty acids and fatty aldehydes (Applied Science, Inc., Supelco, Inc.). The chromatographic assays were terminated when arachidonic acid appeared, so no longer chain components were assayed. The individual phospholipids, e.g., lecithin, cerebrosides, etc., were not separated and the data reflects the total phospholipid class, as found in the chloroform phase of the Folch et al. (6) extraction procedure. Micromoles of triglyceride were calculated by dividing three into the total number of micromoles of triglyceride fatty acid. A similar computation was made for phospholipid concentration by using two as a close approximation to the actual number of fatty acid or aldehyde groups present in the phospholipid molecules. This calculation will be in error by 0.5 μmole for each micromole of a component containing only one fatty acid or aldehyde group in the phospholipid band.

Sufficient triglyceride for double bond species determinations were prepared by running several plates using the separation system described above (7). The triglyceride double bond species were assayed using silver nitrate-silica gel TLC as detailed in a previous publication (9).

Efficiency, overlap and quench corrections were applied to the radioactive data to calculate disintegrations per minute. Specific activities of the individual fatty acids were computed utilizing the data from gas chromatography and liquid scintillation spectrometry. In referring to triglyceride species the following designations were used: O-saturated fatty acid, 1-monoene, 2-diene. The subclass fatty acid position was not assigned.

RESULTS

The total phospholipid and triglyceride content of the medulla and cortex is illustrated in Table I. The medulla was only 14% of the kidney wet weight. The phospholipid content was fairly constant and slightly higher in the cortex. The triglyceride level was quite disparate, being, on the average, almost four times higher in the medulla.

Table II contains the data on fatty acid distribution of triglyceride and phospholipid in the medulla and cortex. The variation among the animals was as large as the differences between the medulla and cortex and, as can be noted by the close average values, a large series of animals would be required to demonstrate any significant disparity in the two anatomic sites.

The radioactivity distribution among the lipid classes is shown in Table III. Most of the radioactivity was found in phospholipid and a large portion of the remainder was in triglyceride. More counts were found in the phospholipid of the cortex than the medulla whereas the reverse was true for triglyceride. Radiocarbon and tritium were similarly distributed among the various lipid classes.

The specific radioactivities of the individual fatty acids were calculated for triglyceride and phospholipid and the results are listed in Table IV. The cortex in each animal exhibited a higher specific activity for triglyceride and phospholipid palmitate and oleate. When the ratios of specific activities or counts are compared between palmitate and oleate it can be seen that in one animal (No. 47) the triglyceride values were similar while in another (No. 48) they were not. Conversely, the phospholipid ratios were more alike in dog No. 48 than dog No. 47. The total radioactivity was greater in the cortex, as shown in Table IV.

In dog No. 48 the triglyceride double bond species were determined in the medulla and cortex, as shown in Table V. The 011 species was present in the greatest amount in both

sites, and the medulla 001 percentage was larger than that of the cortex. The cortex contained more polyunsaturated species than the medulla.

DISCUSSION

Since the number of animals used in this study is limited, conclusions must be drawn with a degree of caution. However, it is clear that this set of animals showed a consistently elevated medulla triglyceride level compared to the cortex. One might expect to find, in consonance with FFA uptake being proportional to blood flow, a greater triglyceride content in the cortex. The fact that more triglyceride was present in dog medulla could be interpreted to mean that less fatty acid was oxidized due to the lower oxygen consumption, with more going into fatty acid-containing structures. The cortex value may simply reflect larger oxidation of fatty acids. Evans (10) showed with rat heart perfusion that hypoxia diverted FFA into triglyceride formation. A similar situation may prevail here.

The fatty acid composition data would lead to the conclusion that the enzymes involved in triglyceride and phospholipid metabolism have the same order of specificity in the medulla and cortex. The radioactive fatty acids are incorporated predominantly into phospholipid constituents.

The specific activity of the radioactive fatty acids found in the cortex was clearly greater than that in the medulla. Also, the total radioactivity was much larger in the cortex than the medulla. This data leads to the inference that cortical incorporation was more active. Since the size of the precursor pool was not measured it is not possible to tell if unequal dilution of the fatty acids in this pool produced these results. The rate of incorporation as calculated from the specific activity of an immediate precursor could not be computed from this data.

The 001 triglyceride species in dog No. 48 was lower in the cortex while the more unsaturated components, 022 and others, were higher. It is interesting to compare this data to the results reported from this laboratory (11) for the triglyceride double bond species in whole kidney. As most of the triglyceride was found in the medulla it would be anticipated that the total kidney triglyceride subclasses would be similar to those of the medulla. This can be observed to be the case.

The total kidney, after 90 min of infusion in the unanesthetized resting state, retained 0.25% of the infused palmitate and 0.08% of the infused oleate. The cortex contained 89% of the radioactivity, which is interesting since the cortex represented 86% of the wet weight of the kidney. The lower content of the oleate may be due to either a lower uptake of this fatty acid compared to palmitate or a relatively greater oxidation, or both possibilities. The previously reported lack of consistent uptake and oxidation for oleate in the dog kidney under in vivo conditions (3) implies less rather than greater oxidation. The mechanism leading to this disparity remains to be discovered.

When seeking to compare this data to those in the literature there were few reports available for the dog. However, an extensive study of rabbit cortex and medulla composition was published by Morgan et al. (12). They found much less phospholipid in the medulla and twice as much triglyceride and cholesterol than in the cortex. The medulla was 20% of the wet weight of the rabbit kidney. They also noted less linoleic acid in medulla than cortex with a corresponding increase in longer chain fatty acids, in both neutral and phospholipids.

Karlsson et al. (13) studied the sphingolipid distribution in bovine kidney. They noted the sulfatides were present in much greater concentration in the medulla than the cortex while the sphingomyelin decreased in level from the cortex to papillae. The authors suggested that the localization of sulfatides to the outer zone of the medulla implicated these compounds as possible carriers or receptors in sodium ion transport.

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Diester Waxes in Surface Lipids of Animal Skin[1]

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ABSTRACT

The literature is surveyed on two types of diester lipid that occur on the skin surfaces of animals: Type 1, a hydroxy fatty acid of which the hydroxyl group and the carboxyl group are esterified respectively with another fatty acid and a fatty alcohol, and Type 2, an alkane α,β-diol of which each OH group is esterified with a fatty acid. New data presented here show that the cow, rabbit and cat produce Type 1, whereas the dog, mouse, guinea pig and baboon produce Type 2 diesters. Each occurs as a major component of the surface lipid. The homologue distribution is given for Type 1 diesters of cow, rabbit and cat as well as the Type 2 diesters of dog and mouse. Distribution of long chain fatty acids of Type 1 diesters parallels that of the fatty alcohols suggesting a biogenetic relation between the two types of compounds. GLC of total diesters for the cow suggests that the components are assembled randomly during biosynthesis. Molecular weight of these diesters are in the range of those of natural triglycerides composed mainly of C_{16} and C_{18} fatty acids.

INTRODUCTION

Analysis of the lipid classes from the skin surfaces of many animal species shows a diversity of compounds, (Fig. 1-4 of Ref. 1 reproduced here). Except for the surface lipids of man, where triglycerides and their breakdown products predominate, these lipids consist primarily of a variety of mono- and diester waxes. The monoester waxes are chiefly of two types: (a) fatty acid esters of fatty alcohols, and (b) fatty acid esters of sterols. Diester waxes are also primarily of two types: Type 1, a hydroxy fatty acid of which the hydroxyl group and the carboxyl group are esterified, respectively, with another fatty acid and a fatty alcohol, and Type 2 an alkane α,β-diol of which each OH group is esterified with a fatty acid. In this paper we shall consider only the diester waxes, presenting first a survey of their literature, then new data on diesters of both types, obtained from various species.

In 1952 Tiedt and Truter (2) remarked that, despite the enormous amount of work that had been done on the analysis of wool wax up to that time, no single type of ester had yet been identified from this source. From the molecular weight of total wool wax and its high content of hydroxy acids they reasoned that wool wax must be largely composed of diesters. Then, by a systematic and laborious program of fractional crystallization, they finally succeeded in isolating what turned out to be a mixture of diesters of Type 1.

Both alkane diols and hydroxy fatty acids were long known to exist in the unsaponifiable

TABLE I

Type and Amount of Diester in the Surface Lipids of Different Animals

Species	R_f[a]	Per cent of each diester type in its total surface lipids	
		Type 1	Type 2
Cow	.58	35	
Rabbit	.60	66	
Cat	.58	66	
Dog	.50		4
	.48		46
Mouse	.54		61
	.43		6
Guinea pig	.52		18
	.43		23
Rat[b]	.57	12	
	.55		14
Baboon	.51		21
Chicken[c]	.52		90
Man (vernix caseosa)[d]	.48		3

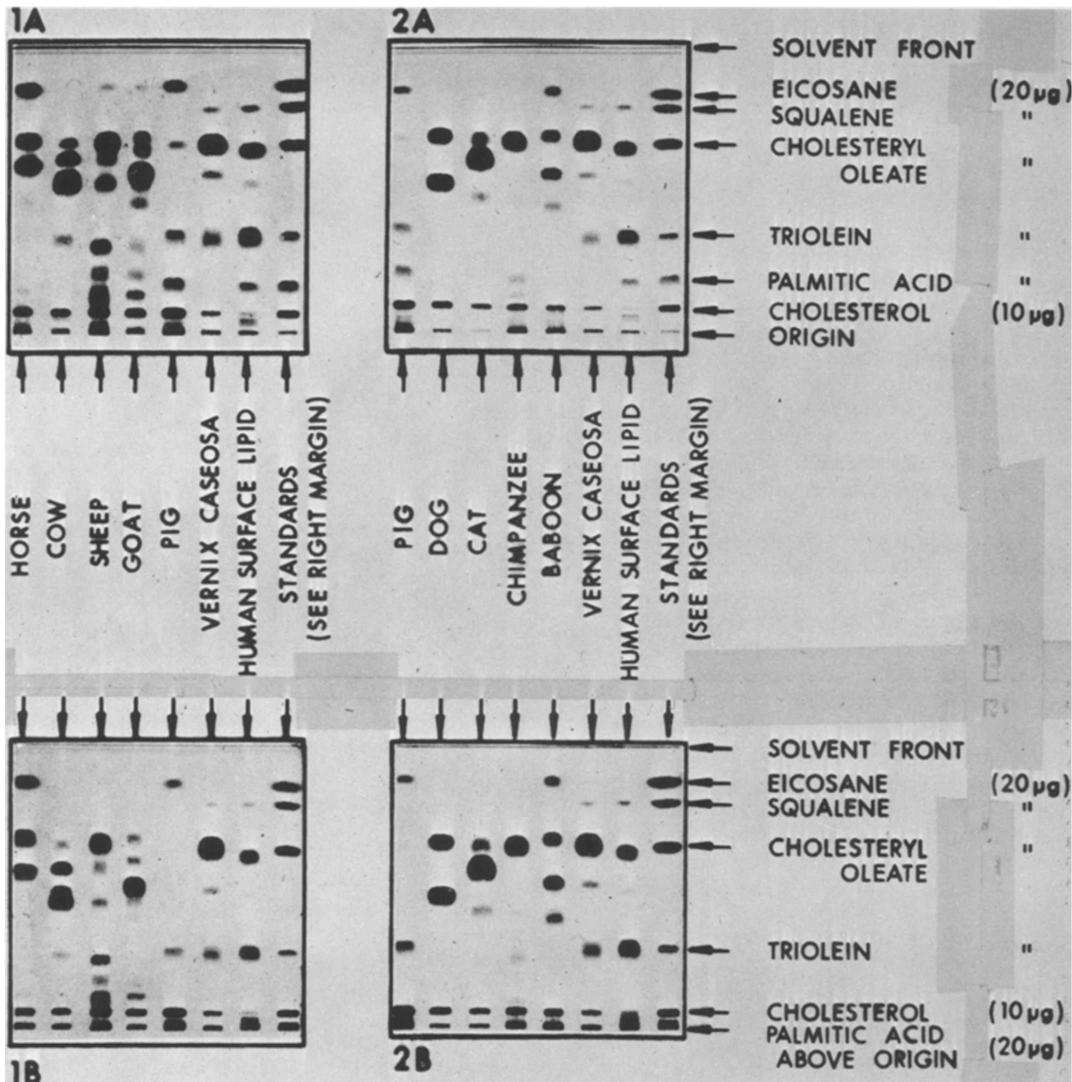
[a]R_f is the relative migration of each diester in the systems used for Figures 1-4 (three successive ascending developments of the plate were used). They are listed here merely for the purposes of identification and are quoted since they do not have the same meaning as for development with a single solvent. The R_f of cholesterol oleate and of triolein in this system of multiple linear development are 0.64 and 0.27, respectively.

[b]Data are estimated from Ref. (8).

[c]The positions of the hydroxyl groups of the diols of this diester are at 2,3 as reported in (9) rather than at 1,2 as we find for the rest of the diols of this ester type.

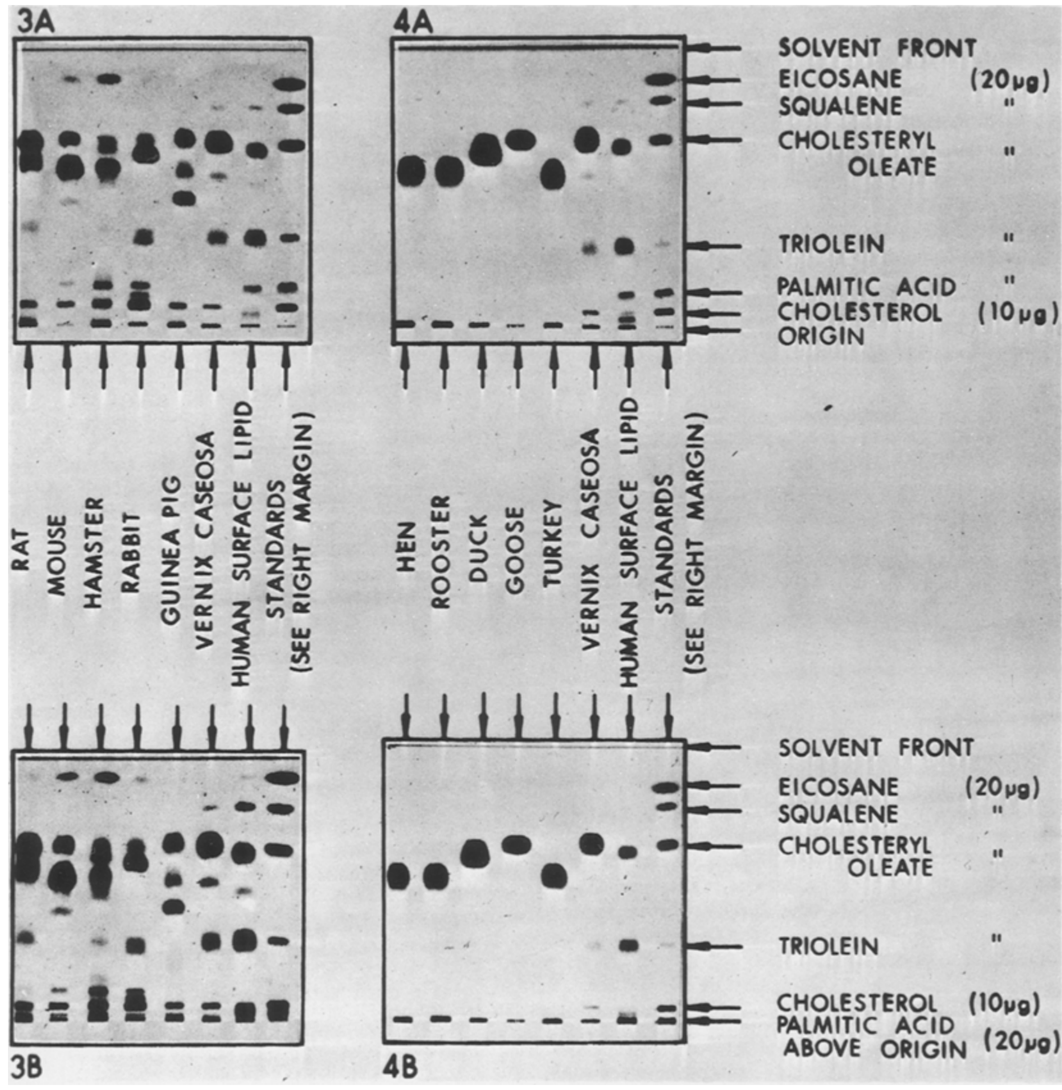
[d]From Ref. (13).

[1]Presented at the 60th AOCS Annual Meeting, San Francisco, April 1969, as part of a Symposium on Natural Waxes.



FIGS. 1-4. Photographs of thin layer chromatograms of the surface lipids and preen gland lipids of the species of animals and birds indicated. Standards are in the farthest lane to the right on each plate. Conditions for the preparation of the plates and their multilinear development were as in (1). For the "A" figures the plates were developed first with hexane-ether-acetic acid (80:20:1) to half the plate, then with hexane-ether (95:5) to the top, and finally with pure hexane again to the top. Plates of the "B" figures were developed in an identical manner except acetic acid was omitted from the first solvent. In the "A" figures, free acids migrated to an R_f of about 0.25, whereas they remained at the origin in the "B" figures, all the other constituents migrating about to the same position in both the A and B systems. The components migrating between cholesteryl oleate and triolein were the diesters. Figures 1-4 are reproduced from reference (1) with permission.

and saponifiable portions, respectively, of various skin surface lipid samples (for a review of the earlier literature see Ref. 3), however, it was not known in what form these substances were present in the unhydrolyzed lipids. With the development of chromatographic techniques enabling the analysis of unhydrolyzed lipids, components migrating in the region between mono- and triesters suggested an existence of diester lipids. Thus Nikkari (4) and Nicolaides (5) independently presented evidence for the possible existence of both types of diesters in rat skin surface lipid. Additionally, Nikkari et al. (6) gave evidence



for the occurrence of both types in vernix caseosa (the greasy material covering the human new-born), and also Type 2 in the preen gland lipids of birds (7). A rigorous demonstration that diesters of Types 1 and 2 do, in fact, occur in rat skin surface lipids was made by Nikkari and Haahti (8). It was also shown by Haahti and Fales (9) that the hydroxyl groups of the diols of diester Type 2 from the preen glands of the domestic chicken were in the 2,3-positions (a mixture of threo and erythro isomers), in contrast to their occurrence in the 1,2-positions, for instance, in the diols of wool wax (10,11). Hansen et al. (12) confirmed the work of Haahti and Fales and reported that turkey secreted a Type 2 diester wax with 2,3-diols having only the erythro configuration. The diols of the Type 2 diesters of vernix caseosa were shown to be in the 1,2-positions and the chains primarily iso and anteiso (13).

EXPERIMENTAL PROCEDURES

Lipids from the skin surfaces of cow, rabbit, cat, dog, mouse, guinea pig and baboon were obtained and analyzed for their diester content as shown in Figure 5. The substances analyzed migrated in what we are calling the diester region on thin layer chromatography (TLC), and had R_f's between those of cholesteryl oleate and triolein, (Fig. 1-4, Table I).

RESULTS AND DISCUSSION

Proof of the structures of these diesters is based on the following: (a) The unhydrolyzed

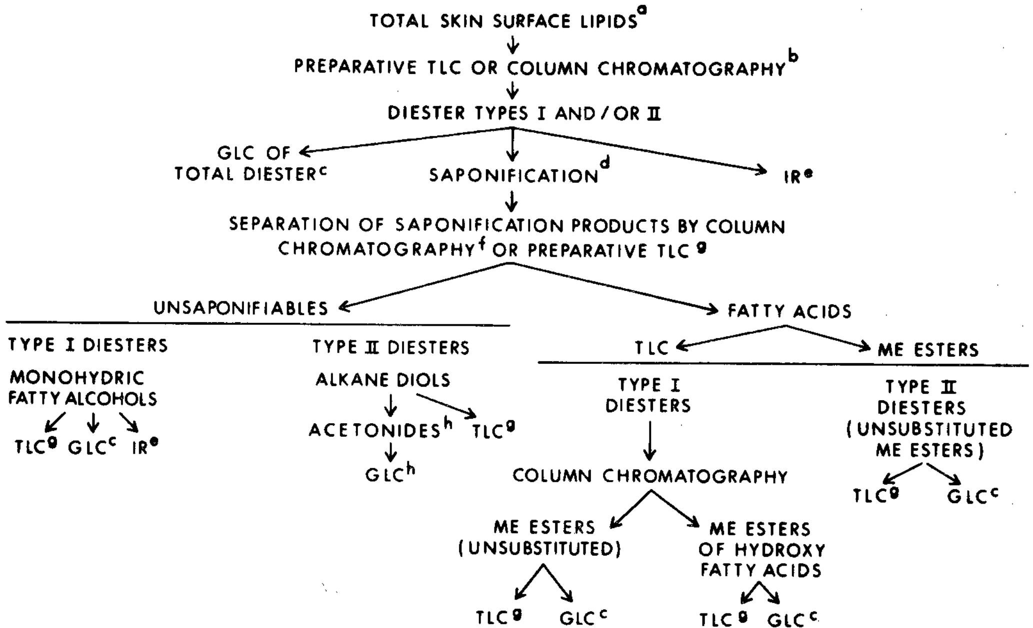


FIG. 5. Isolation and analysis of diester waxes from the skin surface lipids of various animals.

[a]Obtained by hexane extraction of the skin surfaces of various animals as previously described (1).

[b]For the diester of cat, cow, dog, mouse, guinea pig and baboon we used preparative TLC procedures as in footnote d Fig. 1 of Ref. 13. From 250 mg of rabbit skin surface lipids, applied to a column packed with Unisil 100-200 mesh (Clarkson Chemical Co., Williamsport, Pa.) with bed dimensions 20 x 1.6 cm, we eluted successively Fraction 1 (a trace), with 128 ml 15% benzene in hexane, then with 40% benzene in hexane we eluted Fraction 2 (29.9 mg), Fraction 3 (138.1 mg), Fraction 4 (10.8 mg), Fraction 5 (1.8 mg), and Fraction 6 (5.48 mg) with 180, 140, 90, 110 and 470 ml respectively, and finally, 63 mg more with several polar solvents. Fraction 3 showed one spot by TLC in the diester region, whereas Fraction 2 showed a spot in the sterol ester and another in the diester regions. Fractions 4 and 5 also showed primarily diesters, thus Fraction 3 represents a middle portion of the total diesters and was considered a representative sample.

[c]GLC of total diesters was carried out in a Beckman GC-4 gas chromatograph on a 12 in x 1/8 in o.d. stainless steel column packed with 1.5% OV-101 on Chromosorb G, 100-200 mesh, acid washed, DMCS treated (Johns Manville, Manville, N.J.), and temperature programmed from 300 to 400 C in 16 min with He flow of 35 ml/min. Peaks were identified as described in the text. GLC of fatty alcohols was done on a 6 ft x 1/4 in o.d. stainless steel column packed with 3% OV-101 on Gas-Chrom Q (Applied Sciences, Inc., State College, Pa.) programmed from 200-300 C 1n 16 min with He flow of 75 ml/min. For the unsubstituted me esters and those of the hydroxy fatty acids the same conditions as for alcohols were used except the temperature program was 180 to 280 C in 16 min. Carbon numbers were determined as in (14) and standard alcohols, me esters of unsubstituted, α hydroxy fatty acids and triglycerides were purchased from Applied Sciences, Inc.

[d]As in Ref. 13 except 3.5 hr of reflux rather than 2 hr was used.

[e]KBr pellet technique.

[f]Column separations were carried out on alkaline silicic acid as in footnote f, Figure 1 of Ref. 13 except that a maximum load of 15 mg of saponification products per gram adsorbent packed in a 1 cm (i.d.) column was applied. Columns were packed as a slurry of alkaline Unisil in chloroform containing 3% methanol and the sample applied to the column with the same solvent, then the unsaponifiables eluted until zero residue was obtained (~45 ml for a 5 g column). The acids were eluted with formic acid-methanol-chloroform in volume proportion of 10:30:60 and the elution contomied until no fatty acids were further removed (~45-50 ml for a 5 g column). The eluate of acids was washed with water (twice) in a separatory funnel to remove acid and salts and the solvent was removed under a nitrogen stream. All separations of fatty acids from unsaponifiable matter were quantitative as judged by TLC.

[g]TLC procedures were as those described in Ref. 13. To observe hydroxy fatty acids, the plates were developed with 1% glacial acetic acid in ether or chloroform-acetone-acetic acid 80:20:1. Standards to establish that the position of the OH group was α, were a series of hydroxy fatty acids with the OH group substituted on the 2nd to the 16th C atoms (gifts of Robert J. Meyer, Morton Salt Co., Woodstock, Ill.). α Hydroxy fatty acids showed a lower R_f than any of the isomers.

[h]Prepared and gas chromatographed as in Ref. 13.

TABLE II

Fatty Moieties of Diester Type 1 From Cow, Rabbit and Cat Skin Surface Lipids

	Unsubstituted fatty acids			α Hydroxy fatty acids			Fatty alcohols		
Carbon No.	Cow %	Rabbit %	Cat %	Cow %	Rabbit %	Cat %	Cow %	Rabbit %	Cat %
8	Trace								
9	Trace	Trace							
10	Trace	Trace	0.3						
11	Trace	Trace	0.3						
12	3.5	4.2	3.8						
13	Trace	10.3	4.0						
14	89.4	33.0	38.5	9.9	3.0	13.1			
15	0.7	8.7	8.5	Trace	20.2	23.1			
16	5.1	34.9	16.9	90.1	76.5	60.8			
17	Trace	5.3	1.2		0.3	1.3			
18	0.7	1.4	1.5			0.4			Trace
19	Traces = ~0.3% (C19–C30)[a]	Traces = ~1% (C19–C32)[a]	0.5 (C19–C30)[a]						0.3
20			1.9				Trace	1.5	10.9
21			1.0					0.8	10.2
22			2.7				6.4	2.4	20.8
23			1.7				0.2	1.5	10.2
24			4.8				62.5	13.5	22.9
25			2.3				0.3	33.0	10.2
26			3.0				27.8	23.4	7.8
27			0.8				Trace	9.2	1.4
28			1.1				2.8	5.2	1.4
29			0.6					2.6	Trace
30			1.0				Trace	2.5	Trace
31								0.7	
32								0.6	
Totals	99.7[b]	98.8[b]	96.6[b]	100.0	100.0	98.7[b]	100.0	96.9[b]	96.1[b]

[a]The pattern of these minor components match the relative distribution of the fatty alcohols of the species in question (see text).

[b]The difference between these totals and 100% is due to small amounts of branched chain substances distributed over the entire range of the homologues listed.

material behaved as diesters in chromatography (GLC, TLC and liquid chromatography). (b) Hydrolysis of both types of diester gave only the predicted products (Fig. 5) in almost quantitative yield. (c) Infrared spectra of the unhydrolyzed material of both types showed presence of ester carbonyl and absence of free OH, and spectra of the hydrolysis products (alkane 1,2-diols, α-hydroxy fatty acids and unsubstituted fatty acids) were identical to known standards. (d) The OH groups of the diols were at the 1,2- rather than the 2,3-positions as established by GLC retention data of the acetonides (footnote h, Fig. 5). (e) The position of the OH group of the hydroxy fatty acids was found to be at α as judged by TLC of standards (footnote g, Fig. 5).

Table I gives relative amounts of diesters of both types found in the surface lipids of the animals investigated in this study as well as relevant data from the literature. Note that for many animals diesters constitute major components and that some animals produce only Type 1, others only Type 2 and the rat, both types. Diesters of Type 2 are also produced by man, (in vernix caseosa) but in considerably smaller proportions. From unpublished chromatographic studies of unhydrolyzed adult skin surface lipid, we have seen material that migrates in the diester region. If these substances are diesters they are present in amounts even less than those of vernix caseosa, i.e., ~1%. It is noteworthy that another primate, the baboon, produces 21% diester Type 2 in its surface lipid.

Table II lists our data on the fatty components of diesters of Type 1 from three widely differing species, namely, the cow, the rabbit and the cat. The unsubstituted fatty acids are nearly all saturated, straight chain acids with both odd and even numbered carbon chains ranging from C_8 to C_{32}. The occurrence of relatively large amounts of C_{14} and shorter chain lengths is noteworthy, in that the usual

TABLE III

Fatty Acids and Diols of Diesters (Type 2) From Dog Skin Surface Lipids[a]

	Fatty acids					Alkane 1,2-diols			
	Straight		Branched			Straight		Branched	
Carbon No.	Even %	Odd %	Carbon No.	%	Carbon No.	Even %	Odd %	Carbon No.	%
12	Trace								
			12.60	Trace					
13		Trace							
			13.65	2.4					
14	3.0								
			14.65	10.0					
15		1.9							
			15.76	7.6					
16	21.8				16	0.2			
			16.70	7.0				16.65	0.2
17		0.9			17		0.6		
			17.65	2.4				17.60	18.4
18	2.5				18	5.8			
			18.70	6.6				18.63	18.6
19		0.4			19		1.3		
			19.67	16.2				19.65	8.5
20	0.7				20	9.7			
			20.67	13.9				20.65	16.1
21		0.1			21		0.9		
			21.65	1.1				21.68	13.4
22	0.1				22	0.8			
			22.68	0.4				22.68	4.5
23		0.1			23		0.1		
			23.61	0.2				23.62	0.9
24	0.4								
			24.62	0.2					
25		0.1							
			25.60	Trace					
26	Trace								
	28.5	3.5		68.0		16.5	2.9		80.6
Totals	32.0					19.4			

[a]These are the major dog diesters, R_f = .48 of Table I.

products of fatty acid synthetase is C_{16}.

For all three species the chain lengths of the acids from C_{19} to C_{32} parallel those of the fatty alcohols. Even though these acids occurred in trace amounts for the rabbit and cow, this parallel was strikingly shown. For example, in the rabbit, C_{24}, C_{25} and C_{26} were the largest traces of acids, C_{25} being the maximum, and this distribution is exactly what Table II lists for alcohols of rabbit. These correlations suggest that the fatty acids above C_{19} and the fatty alcohols, which are also nearly all above C_{19}, are probably derived from common precursors. If these precursors are built up as they are for the fatty alcohols of human skin surface lipid (15), they are very likely chain extensions of C_2 units above C_{14} or C_{16}.

In contrast to the saturated odd and even numbered carbon chains of the fatty moieties of diester Type 1, the diester chains of Type 2 show a variety of structural features, including branching and unsaturation. For example, the fatty acids and diols of the major group of dog surface lipid diesters (R_f = 0.48, Table I) show more than two thirds branched chain saturated substances with carbon numbers in the iso or anteiso region or both (Table III). The minor group of dog diol diesters (R_f = 0.50, Table I), although not tabulated here, shows the same general distribution of chain length except that the chains are somewhat longer. Longer chain lengths in our TLC systems are faster migrating.

The mouse also shows two diesters of Type 2 (R_f = 0.54 and 0.43, Table I), and their fatty moieties are tabulated in Tables IV and V, respectively. Note that the alkane diols for both diesters show very little unsaturation whereas more than half the fatty acids of the diesters of

TABLE IV

Fatty Acids and Diols of the Faster Migrating[a] Diesters (Type 2) From Mouse Skin Surface Lipid

	Fatty acids						Alkane 1,2-diols				
	Straight				Branched			Straight[b]		Branched	
	Even		Odd								
Carbon No.	Sat. %	Unsat. %	Sat %	Unsat. %	Carbon No.	%	Carbon No.	Even %	Odd %	Carbon No.	%
13											
					13.6	0.1					
14	0.9										
					14.65	0.4					
15			0.6	0.1			15		0.4		
					15.70	1.7				15.62	0.7
16	7.4	16.5					16	11.6			
					16.71	0.4				16.65	2.4
17			1.0	0.2			17		3.9		
					17.70	0.5				17.63	1.9
18	1.9	5.9					18	15.6			
					18.71	0.6				18.65	0.4
19			1.1	0.1			19		4.9		
					19.67	4.0				19.61	1.6
20	12.0	7.3					20	27.2			
					20.68	1.9				20.63	4.6
21			2.8	0.8			21		3.8		
					21.67	2.7				21.62	4.7
22	6.4	8.3					22	13.4			
					22.70	0.4				22.62	0.9
23			1.1	Trace			23		0.4		
					23.66	0.4				23.62	0.5
24	2.9	1.0					24	0.8			
					24.71	0.2				24.6	Trace
25			0.5				25		0.1		
					25.65	0.2				25.6	Trace
26	1.0	0.3					26	0.2			
27			0.3				27		Trace		
28	0.7	0.4									
29			0.4								
30	1.3	Trace									
31			0.3								
32	1.4										
33			0.2								
34	1.3										
	37.2	39.7	8.3	1.2		13.5		68.8	13.5		17.7

[a]Faster migrating here refers to the diester of R_f of 0.54 of Table I.
[b]Showed ~5% unsaturation mainly at C_{16} and C_{18}.

R_f = 0.54 and almost three fourths of the diesters of R_f = 0.43 are unsaturated. It also appears that nearly all of the alkane diols of the slower migrating diesters show branching whereas only 17.7% of the diols of the faster migrating diesters show branching. The two diesters may be parts of the same biosynthetic pool but may have separated in our TLC systems because branching and unsaturation in the chains result in slower migration.

GLC of the total diesters gave some insight into what was the combination of component chain lengths in the intact diester molecule. Particularly instructive was cow diester Type 1, since it consisted of relatively few molecular homologues of each component. GLC of these diesters showed six base line separated peaks. Standards used were single fatty acid triglycerides. Their retention times were close to those of wax monoesters of the same carbon content. It was therefore assumed that the diesters of a given carbon content would also have similar retention data. Such was indeed found to be the case, for the major peak was at C_{54}, exactly what one would expect for a diester composed of a C_{14} fatty acid, a C_{16} α hydroxy fatty acid, and a C_{24} fatty alcohol, each of which makes up 89.4%, 90.1% and 62.5% respectively of that

TABLE V

Fatty Acids and Diols From the Slower Migrating[a] Diester (Type 2) From Mouse Skin Surface Lipid

	Fatty acids		Alkane 1,2-diols			
	Straight		Straight		Branched	
	Even					
Carbon No.	Sat %	Unsat %	Even %	Odd %	Carbon No.	%
12	0.1					
13						
14	0.2					
15						
16	1.6	0.8	0.7			
17				Trace		
					17.60	Trace
18	3.5	2.7	1.2			
					18.68	Trace
19				1.1		
					19.60	15.3
20	12.2	62.3	1.0			
					20.69	1.0
21						
					21.61	47.6
22	4.1	6.0	Trace			
					22.65	Trace
23						
					23.60	23.0
24	0.8	2.0				
25						
					25.58	8.1
26	Trace					
27						
	22.5	73.8			27.59	1.0
	96.3[b]		2.9	1.1		96.0

[a]"Slower migrating" here refers to the diester of R_f 0.43 of Table I.

[b]The difference between these totals and 100% is due to small amounts of branched and odd chain length substances distributed over the entire range of homologues listed.

type of substance (Table II). Furthermore, the amounts of diester one would expect to find from a random distribution of chains are close to what was found, i.e., diesters ranging in total carbon contents from C_{50} to C_{60}, (Table VI). This Table also shows the internal consistency of the data. For the other diesters of Type 2 the GLC peaks were not so clearly resolved because of a wider distribution of homologues of each type. Maxima for other diesters were at C_{54} to C_{56} for cat and rabbit, and C_{58} to C_{60} for mouse diester of R_f = 0.54. These data are similar to those reported for rat diesters Types 1 and 2 (8).

COMMENT

The spread of molecular weights of these diesters is approximately the same as that of the average group of triglycerides found in nature, which are generally made up of C_{16} and C_{18} fatty acids. One wonders then what advantage there is to the animal, if any, in the investiture of all the extra biochemical apparatus required for the synthesis of these unusual types of fatty chains into diesters of these kinds, rather than utilization of the already existing and widely used enzymatic machinery for synthesis of triglycerides as actually occurs on the skin of man. Part of the answer might be the selection of waxy compounds that have the proper melting requirements, i.e., fluid enough to be secreted but solidifying when on the skin surface. [This and other possible functions of skin surface waxes has already been discussed (1,16)]. Another part of the answer might lie in the instability of triglycerides. Because of the widespread occurrence of lipases, especially of bacterial origin, triglycerides would not remain on the skin for long but would be broken down

TABLE VI

Distribution of Molecular Species of Diester (Type 1) of Cow Surface Lipids

Total carbon no. of diester	Amount[a] %	Random distribution[b] %	Possible diesters of a given carbon content[c]
50	1	1	12-14-24; 12-16-22; 14-14-22
52	13	13	12-16-24; 12-14-26; 14-14-24 14-16-22; 16-14-22.
54	55	55	12-14-28; 12-16-26; 14-14-26 14-16-24; 16-14-24; 16-16-22 18-14-22.
56	27	26	12-16-28; 14-14-28; 14-16-26; 16-14-26; 16-16-24; 18-14-24; 18-16-22.
58	3	4	14-16-28; 16-14-28; 16-16-26; 18-14-26; 18-16-24.
60	1	1	16-16-28; 18-16-26; 18-14-28.

[a]Obtained from GLC of total diester as such.

[b]Calculated from the data of Table II assuming random distribution.

[c]Sequence of numbers of each group represents the carbon contents respectively of unsubstituted fatty acid-hydroxy fatty acid--fatty alcohol. The sum of these numbers equal the total carbon number (column 1).

into lower glycerides and free fatty acids as they are in man. Animals do not have appreciable amounts of free fatty acids in their surface lipids which means that these diesters are not broken down. It is also known that free fatty acids such as oleic acid are not tolerated well by the skins of some animals (17). The skin of man is unique in this regard for he can tolerate rather large amounts of free fatty acids, e.g., ~30% occurs in average adult skin surface lipid. But even here, a substantial body of evidence exists that free fatty acid of human skin lipid is one of the factors in the pathogenesis of acne (18). As far as we are aware, acne is not seen in animals.

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Occurrence, Function and Biosynthesis of Wax Esters in Marine Organisms[1]

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ABSTRACT

Wax esters occur as a major lipid-type in at least 30 species of marine animals, distributed among 17 orders and 3 phyla. They are of limited usefulness as a chemotaxonomic character, since only in two suborders, the calanoid copepods, Calanoidei, and the toothed whales, Odontoceti, do the wax esters occur in all members so far examined. In bony fishes their occurrence in muscle correlates better with mesopelagic habitat and vertical migration patterns than with taxonomy. Homologs with 21 to 44 total carbon atoms have been reported, but the usual range for the wax esters in copepods and fish is C_{30}-C_{42}. In fishes the muscle wax esters contain predominantly one and two double bonds per molecule, while in roe lipids up to 65% of the homologs contain three or more double bonds. The component alcohols are saturated and monounsaturated, with 16:0 and 18:1 as the usual major constituents. The fatty acids are more diverse, but 18:1 is most often the main component, and 16:1 and 20:1 are frequent major constituents; polyunsaturated acids make up 1-12% in fish muscle and whale oils and up to 45% in fish roe wax esters. Possible functions of the wax esters are for buoyancy, as energy reserves and for thermal insulation. In vitro, various tissues of marine bony fishes synthesize wax esters from long chain alcohols and fatty acids, without activation. A competing pathway for the long chain alcohols in vivo is their catabolic oxidation to the corresponding fatty acids. The key to the accumulation of wax esters is to be sought in the metabolism of the long chain alcohols, their biosynthesis and esterification vs. their catabolism.

INTRODUCTION

Esters of fatty acids with long chain alcohols, the wax esters, occur in many marine animals, but only one, the sperm whale, is the source of a commercial wax. The greatest amounts of wax esters in the oceans are probably those produced by calanoid copepods, small crustaceans which are the principal food of herrings, sardines, anchovies and other fishes harvested in great quantities; thus the copepods are indirectly of considerable importance to man from both nutritional and economic standpoints. This paper is essentially a review of the literature published to July 1969, but includes some new data.

The history of our knowledge of wax esters in the marine environment began with the recognition of the true chemical nature of sperm whale oil. In 1815, M. E. Chevreul found that spermaceti wax did not contain glycerol (5). Two years later he isolated the long chain alcohols liberated by saponification, partially characterized them, and named the mixture cetyl alcohol; this trivial name is used today for the principal alcohol present, 1-hexadecanol. In 1925 Kimura (22) reported that the lipids of the castor oil fish, *Ruvettus pretiosus*, were largely wax esters; his article was never abstracted by Western journals and so was unknown to Cox and Reid, who repeated the work in 1932 (8). The first identification of wax esters in invertebrates was the isolation of cetyl palmitate from stony corals by Bergmann et al. in 1941 (26). Bolstered by the growing evidence for wax esters as normal constituents of many species of toothed whales and marine bony fishes, Lovern in 1964 (29) reinterpreted his 1935 (28) analyses of *Calanus* finmarchicus lipids as implying that these crustaceans were rich in wax esters. However, the first isolation of wax esters from a copepod, the deep-sea species *Gaussia princeps*, was made only in 1967 (Lee et al., unpublished information).

OCCURRENCE

Wax esters have not been identified in marine plants, perhaps because they have not been looked for specifically, but probably because plants living submerged in sea water do not need the same surface structures as terrestrial plants and so do not have the cuticle waxes of the latter. A more promising tissue to examine for wax esters would be spores or other reproductive bodies, analogous to the

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TABLE I

Systematic Classification (74) of Marine Animals Containing Wax Esters

Phylum	Class	Order	Family	Genus	Species
Protozoa	Flagellata	Euglenoidea			
	Sporozoa				
Coelenterata	Actinozoa	Actinaria (sea anemones)			
		Madreporaria (stony corals)			
		Zoantharia (zoanthid corals)			
		Gorgonaria (gorgonians)			
Arthropoda	Crustacea	Copedoda	Calenidae	*Calanus*	*helgolandicus*
Mollusca	Cephalopoda (octopuses and squid)				
Chordata;	subphylum Vertebrata				
	Elasmobranchii (sharks and rays)				
	Teleostei (bony fish)				
	Aves (birds)				
	Mammalia	Cetacea (whales and dolfins)			

seeds of the jojoba, *Simmondsia chinensis*, in which wax esters are the principal energy reserve (75). Nor have marine bacteria been described which produce wax esters, although several genera of soil bacteria are known to metabolize exogenous *n*-alkanes to wax esters (82).

The following paragraphs will therefore discuss only marine animals and will be organized according to the systematic classification accepted by zoologists (74). Table I summarizes those phyla, classes, and, in a few cases, orders in which at least one species has been reported to contain wax esters or long chain alcohols. Only a fraction of the recognized phyla are represented, and only four classes (sea anemones and corals, copepods, bony fishes, and whales) include more than one or two species. All marine animals containing wax esters as a principal lipid type are listed in Table II, which also extends the classification of the bony fishes and marine mammals to orders and families. A briefer list was given by Malins in 1967 (31).

There remain to be considered the numerous identifications in marine animals of small amounts of wax esters or, after saponification, of long chain alcohols. Some of these reports are of doubtful value because of the inadequate techniques employed during isolation and characterization. In others the amounts of wax esters found are mere traces, thus raising the question of whether they are significant endogenous metabolic products in the species under consideration or perhaps as only transient intermediates of digestion. Where pertinent, these reservations will be mentioned in the discussion below. Criteria for inclusion of a species in the compilation now to be presented are: (a) identification of wax esters, including identification by thin layer chromatography (TLC); (b) identification of long chain alcohols; or (c) the presence of high percentages (20-50%) of uncharacterized nonsaponifiables in the lipids, if related species have been shown to contain wax esters. All the available evidence implies that the long chain alcohols do not occur free in living animal tissues, for example, in none of the species of Table II were more than traces of free alcohols detected in the total crude lipid, and in those animal surface lipids (sebums) which contain major amounts of wax esters, free alcohols are not present at significant levels (76). Therefore, the identification of free alcohols in the lipids implies the original presence of wax esters in the tissue.

No data are available for marine euglenoid protozoans, but fresh water *Euglena* species do make wax esters, especially when grown heterotrophically (47). A marine sporozoan was reported to contain long chain alcohols in appreciable amounts (46), although there is some question whether the lipid analyzed was produced by the microorganism. Three additional sea anemones were reported by Toyama and Takagi (83) to contain small amounts of alcohols, namely *Anthopleura japonica*, *A. stella*, and *A. pacificus*. Other coelenterate classes have been investigated in lesser detail, but small amounts of wax esters or long chain alcohols were identified in a stony coral, *Madrepora cervicornis* (26), and in some gorgonians, including *Plexaura* flexuosa (6,21). In copepods (phyllum Arthropoda) several investigators have found presumptive evidence for the occurrence of wax esters; Collin et al. (7),

TABLE II

Occurrence of Wax Esters in Marine Animals

Order / Family / Genus & species	Tissue	Wax esters (Per cent of total lipid)	Reference
Invertebrates, Coelenterates			
Actinaria (sea anemones)			
Actiniidae			
Bolocera tuediae	Whole animal	35	2
Condylactis gigantea	Whole animal	40	2, 3
Actinostolidae			
Actinostola collosa	Whole animal	<25	2
Zoantharia (zoanthid corals)			
Palythoa mammilosa	Whole animal	<30	3
Zoanthus proteus	Whole animal	<30	3
Invertebrates, Crustaceans			
Copepoda			
Calanidae			
Calanus helgolandicus	Whole animal	30-40	26
Metridiidae			
Gaussia princeps	Whole animal	73	26
Vertebrates, Bony Fishes			
Salmoniformes			
Myctophidae (lantern fishes)			
Sympolophorus evermanni	Whole animal	ca. 10	46
Stenobrachius leucopsarus	Muscle	90	46
Triphoturus mexicanus	Muscle	74	46
Lampanyctus ritteri	Muscle	87	46
Gadiformes			
Gadidae (codfishes)			
Merluccius capensis	Roe (eggs)	<25	37
Moridae (deep-sea cods)			
Lotella phycis	Liver	30	23
Laemonema morosum	Muscle	50	24
	Liver	<60	25, 67
Beryciformes			
Trachischthydae			
Hoplostethus islandicus	Muscle	90	20
Zeiformes			
Oreosomatidea (oreos)[a]			
Allocyttus verrucosus		76	38
Perciformes			
Sciaenidae (croakers)			
Cynoscion nebulosus	Roe (eggs)	40	18
Mugilidae (mullets)			
Mugil japonicus	Roe (eggs)	70	19, 37, 60, 65
Mugil cephalus	Roe (eggs)	67	18
Gempylidae (escolars)			
Ruvettus pretiosus	Muscle	92	8, 22, 39, 44, 65
Lepidocybium flavobrunneum	Muscle	89	35, 39, 44, 80
	Spleen	74	80
Osphrenomidae (gouramis)[b]			
Trichogaster cosby	Roe (eggs)	70	52
Crossopterygii			
Latimeridae (coelacanths)			
Latimeria chalumnae	Muscle	93	45
	Adipose tissue	97	45
Vertebrates, Birds			
Procellariiformes			
Procellariidae (petrels)			
Puffinus tenuirostris	Stomach oil	90	88
Vertebrates, Mammals			
Odontoceti			
Ziphiidae (beaked whales)			
Ziphius cavirostris	Blubber	55	14
Berardius bairdii	Blubber	80	49, 50, 59, 61, 66
Hyperoodon ampullatus	Blubber	70	36
Physeteridae (sperm whales)			
Physeter catodon	Blubber	66	13, 36, 41, 53, 56, 58, 64
	Head (spermaceti organ)	73	10, 13, 40, 41, 55, 62, 71
Delphinidae (dolphins, porpoises)			
Globicephala melaena	Head	<30	63
Tursiops truncatus	Jaw	<35	9

[a]Cf. Fitch and Lavenberg (78).
[b]Fresh-water fishes.

working with zooplankton collections which were largely *Calanus finmarchicus*, found 28-32% of a nonsaponifiable fraction, and identified a saturated C_{16} alcohol and what were probably monounsaturated C_{18} and C_{20} alcohols. Similar results were reported by Lovern (28); Saiki et al. (50) found in *C. cristatus* 43.1% nonsaponifiables, but did not identify any alcohols; tables in Yamada's review article (72) gave values of 28-51% for the nonsaponifiable fractions of some additional marine calanoid copepods, implying that the wax esters were probably significant components in all. For the large phyllum Mollusca only one investigation has suggested the presence of wax esters, namely Hatano's (11) finding of long chain alcohols in the liver oil of *Octopus* dofleini.

Wax esters are not major components of the lipids of elasmobranch fishes (i.e., sharks and rays), but Toyama reported that the liver oil of *Chlamydoselachus anguineus* (suborder Hexanchoidea) was rish in octadecenol and hexadecanol (56). From the liver of the spiny dogfish, *Squalus acanthias* (suborder Squaloidea), Malins (30) isolated trace amounts of wax esters which were biosynthesized by the shark, as shown in experiments using radioactive precursors. The egg lipids of marine or euryhaline fishes have been investigated by Japanese workers. Only trace amounts of wax esters were reported for two salmons (order Salmoniformes), suborder Salmonoidei) *Onchorhyncus keta* (73) and *O. kisutch* (36); the fresh water carp (order Cypriniformes), *Cyprinus carpio* (17); a flying fish (order Atheriniformes), *Prognichthys agoo* (36); a pollack or whiting (order Gadiformes, suborder Gadoidei), *Theragra chalcogrammus* (16,36,41,68); and the following four members of the superorder Acanthopterygii: the oreo (order Zeiformes), *Allocyttus verrucosus* (36), the three-spine stickleback (order Gasterosteiformes), *Gasterosteus aceleatus* (67), a blenny (order Perciformes, *Stichaeus grigorjewi* (12), and a flatfish (order Pleuronectiformes), a *Limanda* species (35). From a fifth member of this superorder, a puffer fish of the family Tetraodontidae (order Tetraodontiformes), saturated C_{16} and C_{20} and monounsaturated C_{18} and C_{20} alcohols were identified (69). Much of the data given above for fish eggs were derived from a paper by Mori & Saito (36) in which trace amounts of wax esters were detected by TLC. Their figure showing a developed TLC plate, however, indicates that their system would not distinguish between wax esters and sterol esters; small amounts of the latter are probably universal constituents of animal tissues.

The presence of wax esters in the muscle of a teleost fish appears to preclude their presence in the roe of that species: wax esters are the major lipid type in the muscle of *Allocyttus verrucosus* (37) but are present only as a trace, if at all, in its eggs (36); the reverse situation prevails with the mullet, where the wax esters occur only in the roe and not in the muscle (18).

Besides the species listed in Table II, another oceanic bird, the Australasian petrel, *Aestrelata lessoni*, has been reported to contain "cetyl esters" (85). Recent work (Hansen, unpublished data) did not confirm the presence of wax esters in the stomach oil of the fulmar, *Fulmaris glacialis*, which were deduced by Rosenheim & Webster (86) on the basis of the 40% of nonsaponifiables found. Perhaps the main lipid of *F. glacialis* will prove to be diacyl glyceryl ethers, as were found for Leach's petrel, *Oceandroma leucorhoa* (87). All of these species are members of the order Procellariiformes, which are notorious for spewing out oil and regurgitated stomach contents, as a defense mechanism (88). Finally, two additional marine mammals have been reported to contain small amounts of long chain alcohols. One is the only baleen whale (suborder Mysticeti) represented, namely the sei whale, *Baleaenoptera borealis*, in which the alcohols occur in the heart lipids (15). In the porpoise, *Phocoena phocoena* (suborder Odontoceti), alcohols have been found in the jaw oil and blubber (27).

The presence of wax esters is not a generally useful chemotaxonomic character of marine animals, judging by the data of the preceding paragraphs and Table II. In part this is because of the varied functions served by this lipid type, to be discussed in the next section. Apparently the use of wax esters as energy reserves (in copepods and fishes) or as thermal insulation (in the toothed whales and dolphins) are characteristics that developed at an early stage in the evolution of certain families and suborders. On the other hand, their use for buoyancy in the muscle of mesopelagic fishes appears to be related to the specific life patterns or ecologic niches of individual species and probably developed independently in at least six orders of teleost fishes. Therefore, only in certain groups are the wax esters helpful taxonomically. For example, in the free-living pelagic copepods of the suborder Calanoidei the sparse available evidence is compatible with this lipid type being a universal constituent of at least the marine families; no information has been published on the lipids of attached or parasitic species of related suborders. Also for the toothed whales, suborder Odontoceti, the

TABLE III

Wax Esters of Marine Animals (Wt. %)

Species Tissue Reference Homolog	*Gaussia princeps* whole animal 80	*Lampanyctus ritteri* muscle 46	*Mugil cephalus* roe[a] 18	*Latimeria chalumnae* muscle 45	*Physeter catodon* head oil 92
26					2.3
28					6.1
30:0				1.6	
30:1	6.1	0.6	5.0	0.4	10.8
30:2	0.9				
31			6.2		1.4
32:0		0.6		2.0	
32:1	22.9	9.8	23.0	8.7	18.3
32:2	6.3			1.6	
33:1			10.2		3.1
34:0		1.8		Trace	
34:1	40.8	61.1	17.9	27.6	24.9
34:2	8.6	9.2		11.5	
35			5.4		2.9
36:1	4.0	7.8	12.4	8.8	19.5
36:2	7.3	8.2		30.4	
37			4.4		1.0
38:1		0.3	11.7	1.2	8.0
38:2	2.7	0.4		4.9	
39			1.1		
40:2			1.9	1.2	

[a]After hydrogenation.

wax esters seem to be ubiquitous components, although in certain species they are quantitatively important only in some tissues, e.g., in the jaw oil of the porpoise (27). The teleost fishes are less consistent: of the seven genera of lantern fishes examined (45), three had 80-90% and four had 10% or less of wax esters; for the escolars, family Gempylidae, of five genera examined (38,43; also, Nevenzal, unpublished data) only two contained wax esters; of the members of the family Moridae, the deep-sea cods, for which data are available, two genera contain wax esters (23-25,66) and one does not (Nevenzel, unpublished data).

ANALYSES

Gas liquid chromatographic (GLC) analyses of the native (unhydrolyzed) wax esters from seven representative species are given in Table III. Including those seven, comprehensive analyses have been published for the derived alcohols and particularly for the fatty acids of some 20 marine species; values for six of these, from a copepod to the sperm whale and including one example of roe wax esters, are collected in Table IV.

The following generalizations emerge from the data. The chain lengths of the wax esters are in the range C_{30}-C_{42} in invertebrates and fishes, and as short as C_{21} in the marine mammals [hexadecyl isovalerate was reported from the head oil of the pilot whale (62)], although the usual range for the sperm whale is probably C_{26}-C_{38} (1,10,89). The component alcohols are overwhelmingly saturated and monounsaturated; polyunsaturated alcohols rarely constitute as much as 5% of the total (18,51; however, Lee et al., unpublished information). The 16:0 alcohol is usually the largest component, with 18:1 the next most abundant in fish and marine mammals. In *Allocyttus verrucosus*, however, the longer 20:1 and 22:1 homologs predominate. This bias toward longer chain lengths is also apparent in the fatty acids of this species, although the 18:1 fatty acid is still the main component, as it is everywhere except in the mullet roe lipids. The 16:1 fatty acid is more abundant than 16:0 in all species except *Allocyttus*, but polyunsaturated fatty acids predominate only in roe lipids and some copepods (Lee et al., unpublished information).

The analytical methods so far applied to the wax esters have not been precise enough to establish decisively whether the alcohol and acid moieties are combined randomly. Generally, compositions calculated from the observed alcohol and acid values on this basis are in reasonable agreement with compositions determined by direct GLC analyses of the native wax esters (18,43,89), although Iyengar & Schlenk (18) felt that for part of their data the differences exceeded the estimated experimental error.

TABLE IV

Alcohol and Fatty Acid Moieties From the Wax Esters of Marine Animals (Wt. %)

Species	*Gaussia princeps*		*Lampanyctus ritteri*		*Allocyttus verrucosus*		*Mugil cephalus*		*Latimeria chalumnae*		*Physeter catodon*	
Reference	80		46		38		18[a]		45		92	
Homolog	Alcohol	Acid	Alcohol	Acid	Alcohol	Acid	Alcohol	Acid	Alcohol	Acid	Alcohol	Acid
10:0					0.2							0.9
12:0	0.2	Trace			0.2	0.2					0.2	5.5
12:1					0.3	0.2						0.6
14:0	13.7	0.3	5.1	0.3	0.6	2.4	9.9	1.0	0.05	3.2	9.9	10.7
14:1		0.4	0.3		0.5	0.3					0.7	7.4
15:0	2.8	0.8	0.6	Trace	0.3	0.4	6.2	0.4	0.2	0.2	1.5	0.2
15:1-br	2.4	0.2	0.2									
16:0	56.3	0.7	81.5	2.4	8.7	8.0	54.3	4.1	45.0	3.1	27.2	4.5
16:1	2.6	19.2	1.1	4.2	1.8	6.7	14.4	23.3	0.04	13.9	10.1	21.4
16:2	1.0[b]	1.0[b]		0.6[b]	0.8	1.5		4.8		1.1[b]		
16:3					1.0			2.2				
17:0	0.8		0.5				1.7	0.1			1.0	0.2
17:1	0.2	1.1			0.2	1.1	1.5	2.2	0.08	1.2		1.1
18:0	2.5	Trace	0.4	0.7	5.7	1.8	6.4	0.8	11.2	0.7	2.8	1.3
18:1	4.3	59.5	6.8	71.5	18.7	35.4	5.3	12.8	40.4	70.6	38.6	35.0
18:2	1.5	1.5		2.7	0.7	0.6		3.8		0.5		
18:3				0.4	0.6	0.3		3.4[c]				
18:4						0.4		3.1				
20:1	5.8	5.2	0.4	14.0	26.4	16.5			2.7	4.1	6.9	9.2
20:4		1.5		d		0.7		3.7[c]				
20:5		0.7		0.1		2.2		8.8				
21:5		0.1						3.4				
22:1	2.3	3.4	0.3	1.8	26.4	9.1				0.6		
22:5		0.1				2.9		4.8[c]				
22:6		1.4		0.2		3.5		5.9				
24:1	0.9	0.2		0.6	1.9	0.8						

[a]Roe lipids.

[b]Identified as 17:1-branched.

[c]Two isomers.

[d]20:4 and 22:1 not resolved.

TABLE V

In Vivo Incorporation of Radioactive Substrates Into Wax Esters of Lantern Fishes (Nevenzel & Kayama, Ref. 42)

Substrate[a]	Per cent injected radioactivity recovered in lipid[b]	Per cent total lipid activity in		Per cent of wax ester radioactivity in	
		Wax esters	Polar lipid[c]	Acids	Alcohols
1-^{14}C Sodium acetate 100 μCi[d]	2.7	41	59	11	89
1-^{14}C-Palmitic acid, 80 μCi[e]	(90)	0.28	33.2	--	--
1-^{14}C-Palmitic acid, 60 μCi	(70)	5.6	9.4	51	49
Oleic-9,10-^{3}H acid, 60 μCi	(69)	7.5	11.8	95	5
1-^{14}C-Hexadecanol 60 μCi[f]	(47)	3.6	5.5	11	89

[a]The substrates, as bovine serum albumin complexes in isotonic saline (except where noted), were injected in two portions, one on either side dorsally, and the fish were returned to cold sea water in the dark for 0.5-3 hr, after which they were killed by freezing. The extracted lipids were separated by silicic acid column chromatography, and the radioactivity of the various fractions determined by liquid scintillation counting.

[b]Values in parentheses include unreacted substrate recovered, accounting for 26-66% of the injected radioactivity. That portion unaccounted for in lipids was metabolized to nonlipid products, or possibly a portion of the injected solution diffused out through the needle puncture into the sea water bath.

[c]The radioactivity was presumably in fatty acids esterified as mono- and diglycerides, phospholipids, etc.

[d]In isotonic saline.

[e]Dissolved in demethylsulfoxide.

[f]As sodium taurocholate-monopalmitin emulsion in isotonic saline.

Setting aside natural or man-made incidents involving petroleum, massive amounts of oily or waxy material contaminating the marine environment have been observed many times. In two such cases, an isolated incident at Wake Islane in June, 1965 (1), and the frequent reappearance in winter or early spring at Bute Inlet, British Columbia, of a solid material floating on the surface (71), chemical examination of the substances revealed that both were largely wax esters. Clearly, in light of the data assembled above, living organisms could be the immediate sources of these wax esters. It was concluded that the Wake Island material was primarily processed sperm whale oil. No specific organism has been suggested as the origin of Bute Inlet wax, although its composition is compatible with the invertebrate and fish data of Table III, consisting of C_{28}-C_{42} esters with a flat maximum at C_{34}-C_{36} (34% and 31%, respectively) (77; also, Nevenzel, unpublished data).

FUNCTION

Three possible functions for the wax esters present in some marine organisms are as a reserve energy store, as a buoyancy agent, and as a structural element. The wax esters in calanoid copepods and teleost roe and eggs are primarily a reserve food, but may have a secondary function as a buoyancy agent. As yet no studies have been published on lipid metabolism during the development of the embryo and the early larval stages of species whose eggs are rich in wax esters. In the pelagic copepod *Calanus helgolandicus* dietary fatty acids are rapidly incorporated into wax esters (among other lipid types); during starvation the wax esters decrease at least proportionately to the decrease in total fat, and their composition changes markedly (Lee et al., unbublished information). In the second category, the massive amounts of this lipid that are deposited in the muscles of some lantern fishes and gempylids, and in the coelacanth are probably a means of approaching neutral buoyancy; in these species the wax esters are only secondarily, if at all, an energy reserve. The evidence for this role is threefold. Theoretical calculations (summarized by Marshall, 32,33) show that marine fishes will be neutrally buoyant if they contain 21-24%, fresh weight of lipid, and the observed values in these species are 14-16%. Capen (4) demonstrated by direct measurement that mature *Triphoturus mexicanus* individuals

TABLE VI

Percentage of Initial Substrate Radioactivity Incorporated Into Wax Esters by Homogenates of Lantern Fish Tissues (42)

Labeled substrate[a]	Tissue	Nonradioactive substrate: None	Nonradioactive substrate: 1-Hexadecanol	Nonradioactive substrate: Palmitic acid
1-^{14}C-Palmitic acid	Liver	0.2	0.5	--
	Muscle	0.4	0.7	--
1-^{14}C-Palmitoyl CoA	Liver	--	-- (0.9)[b]	--
	Muscle	--	0.3 (1.5)[b]	--
Oleic-9,10-^{3}H acid	Liver	--	1.1	--
	Muscle	--	0.6	--
1-^{14}C-Hexadecanol	Liver	4.0	--	7.3
	Muscle	1.6	--	2.5

[a]150-200 mg of tissue were homogenized in 1.5-2.0 ml 0.1 M phosphate buffer of pH 7.8, and incubated with 5 mg Triton X-100 plus 1 μmole of each substrate at 19-21 C for 6 hr; total radioactivity used was 5 μCi.

[b]Without Triton X-100.

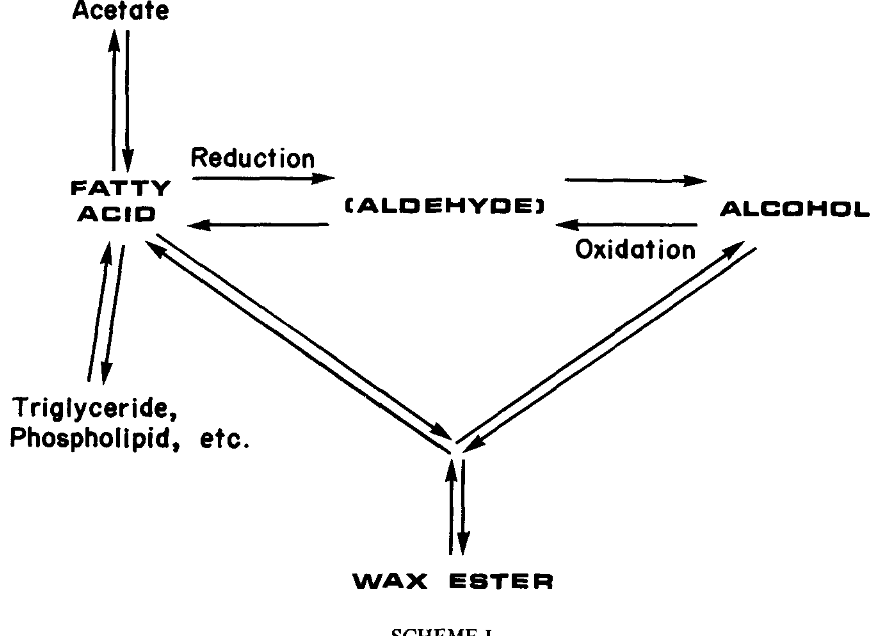
were in fact nearly neutrally buoyant. Finally, most of these species probably undertake daily vertical migrations of 300 m or more; during such movements pressure change of 30 atmospheres are experienced, and lipid, because of its dimensional stability under such changes, is greatly superior to a gas-filled swimbladder. The third function for large amounts of lipid is illustrated by the sperm whale, a warm-blooded mammal living in cold water: a thick external layer of blubber, which contains a high percentage of fat, serves as an insulating blanket. Buoyancy is probably a secondary function of the blubber, but may be the main function for the lipid of the spermaceti organ in the head.

The three functions discussed above are not unique to wax esters. The triglycerides are the usual form of energy reserve in most teleost fish eggs and in many pelagic crustaceans (83), and some mesopelagic fishes contain triglycerides in sufficient quantities to serve for buoyancy [i.e., *Diaphus theta* (45)]; even in the blubber of the sperm whale 23-25% of the lipid is triglyceride (55,89). The wax esters presumably were evolved by some marine animals as an alternative to triglycerides for meeting specific challenges of their environment. The following are possible advantages that the wax esters might have over triglycerides. There are indications that the wax esters have slightly lower densities than corresponding triglycerides (footnote 10, ref. 45), and thus provide more buoyancy per unit volume. Possibly the wax esters, in contrast to triglycerides, are not subject to hormone-controlled mobilization during periods of energy demand, thus stabilizing the buoyancy of the organism against short-term fluctuations; i.e., buoyancy control is divorced from energy demand. Since the glycerol for triglyceride synthesis would probably be made from carbohydrates, the deposition of wax esters rather than triglycerides could be a carbohydrate-sparing adaptation. Such a hypothesis would be consistent with the low carbohydrate diet of deep-sea organisms, but seems irrelevant to *Calanus*, which feeds on the primary producers of carbohydrates, photosynthetic organisms.

BIOSYNTHESIS

The evidence is conclusive in a few cases that the diet is not the source of the wax esters present in marine animals. For example, they are found in mullet lipids only after the ovaries mature, presumably without any change in diet (18); also, wax esters increase rapidly in the copepod *C. helgolandicus* when it is fed on diatoms containing no wax esters (Lee et al., unpublished information). Only for the stomach oils of the Procellariiform birds does the weight of the evidence suggest that the esters present are dietary residues (87). The conclusion, then, is that wax esters are biosynthesized by most of those marine species in which they occur.

A few investigations of this biosynthesis have been carried out in fish. In 1966 Malins (30), working with dogfish (*Squalus acanthias*) liver, showed that in vivo 1-^{14}C-palmitic acid was incorporated into free alcohols as well as into the alcohol and acid moieties of wax esters; the latter are only trace constituents in this species. In 1967 Friedberg & Greene (79) used dogfish liver preparations in vitro to show that 1-hexadecanol was incorporated into wax esters by homogenates of whole liver, by microsomal and supernatant fractions, and by a partially purified enzyme preparation; no cofactor



SCHEME I

requirements were found, and the presence of both long chain alcohol and fatty acid seemed sufficient for the reaction. They concluded that the alcohol and acid reacted directly (i.e., no activated intermediates were involved) to form the observed wax esters, and demonstrated that the synthetic enzyme also catalyzed the reverse reaction of hydrolysis. Nevenzel & Kayama (42), working with lantern fishes which contain large amounts of wax esters, found that the usual lipid precursors were incorporated in vivo into both the long chain alcohol and fatty acid portions of the wax esters (Table V). Tissue slice and homogenate studies, using muscle and liver of these fishes without added cofactors, gave similar results, although the incorporation of radioactivity from substrate into wax esters was poorer, as shown in Table VI. Kayama (Kayama, unpublished information) has extended these experiments to the carp and rainbow trout, neither of which contains more than traces of wax esters. Intestine, kidney, spleen, heart, hepatopancreas, and pyloric caecum were active in synthesizing wax esters; only muscle tissue was inactive.

Without attempting a comprehensive review of long chain alcohol metabolism in animals, we can note that rat and pig livers (79), other rat tissues (53), and mammalian tumor cells (53) all possess systems active in vitro in converting long chain alcohols to wax esters in the presence of acceptor fatty acids. Together these data suggest that the enzyme catalyzing the synthesis of wax ester from alcohol and acid is wide spread in those animal tissues having an active fat metabolism. By contrast, in vivo in at least some of these animals, exogenous long chain alcohols, whether administered free or esterified as wax esters, are oxidized to the corresponding fatty acids. Presumably this is also the fate of the alcohol moieties of the wax esters in copepod lipids eaten by herring: hydrolysis of the esters followed by oxidation of the liberated long chain alcohols to fatty acids. The pioneering work of Stetten and Schoenheimer (81) in feeding deuterated compounds to rats established the interconversion of 1-hexadecanol and palmitic acid and the conversion of 1-octadecanol to stearic acid. The oxidative pathway also is present in intact lantern fishes: in three fishes injected with $1\text{-}^{14}C$-hexadecanol the ratio of the radioactivity in total fatty acids compared to that in the wax ester alcohols was estimated to be >0.65, 2.8 and 2.9. In addition, a portion of the 53-62% of the injected carbon-14 which was not recovered in the total lipid probably was converted via palmitic acid to acetate and CO_2. The palmitic acid from the wax esters of one of these fish contained 93% of its carbon-14 in the carboxyl carbon, confirmation that the carbon chain of the alcohol was converted without degradation to the acid (42). The reverse conversion of palmitic acid to alcohols was

observed in vivo by Malins (30) for dogfish liver and by Nevenzel & Kayama in lantern fishes (Table V, Ref. 42). Sand & Schlenk (51) inferred from the structures of the polyunsaturated alcohols of roe wax esters and the good conversion of labelled fatty acids (including 18:2 and 18:3) into alcohols that the alcohols, including the polyunsaturated homologs, were biosynthesized from the corresponding fatty acids in the gourami in vivo.

These relationships are summarized in Scheme I. In most animals the net balance of the various reactions is toward the hydrolysis of wax esters and the oxidative catabolism of the alcohols. In most marine organisms, however, the balance is reversed; there is a net synthesis of alcohols, and these in turn are esterified with fatty acids to wax esters, which accumulate. If we assume that the same enzyme catalyzes both the synthesis and hydrolysis of wax esters, then changes in its activity or amount cannot determine the level of wax esters present at equilibrium. The true key to the occurrence of wax esters in marine animals should therefore be sought in the activity of the oxidative catabolism of long chain alcohols relative to their biosynthesis via reduction of fatty acids. No investigations specifically directed to determining the mechanisms and kinetics of these reactions have yet been reported for marine organisms.

A second mechanism of wax ester biosynthesis, different from the direct reaction of alcohol and acid discussed so far, was found in plants by Kolattukudy (90); it involved a transfer of acyl groups from phospholipids to the long chain alcohol. He also characterized a third mechanism which required the acyl-CoA ester in a purified enzyme system. Neither of these two mechanisms have been found in marine animals.

A final plea: In recent years the wide availability of GLC and the simplicity of transesterification techniques have resulted in the publication of useful fatty acid analyses for many exotic marine organisms, but application of the still simpler TLC to 100 μg of the lipid would have told us the lipid types in which these fatty acids occurred in situ. In addition, the use of fatty acid compositions in comparative biochemistry or to study the effect of temperature, pressure, etc. on lipid metabolism loses much of its edge when the comparison is made essentially between wax ester fatty acids in one species, triglyceride fatty acids in a second, and phospholipid fatty acids in a third. Not the least of the dividends from such TLC would be the probable discovery of additional organisms rich in wax esters.

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Bacterial Hydrocarbons: Occurrence, Structure and Metabolism[1]

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ABSTRACT

The chemical structures, general pathways of catabolism and the biosynthesis of bacterial, nonisoprenoid hydrocarbons are reviewed with emphasis on recent work on the chemistry and biosynthesis of the hydrocarbons of *Sarcina lutea*.

INTRODUCTION

This review considers only the nonisoprenoid, aliphatic hydrocarbons of bacteria and emphasizes recent work on the characterization and biosynthesis of these compounds. Reviews dealing primarily with the catabolism of a variety of hydrocarbons by bacteria are available (1-5).

OCCURRENCE AND CHEMICAL STRUCTURES

Janowski and ZoBell were first to report that bacteria produce aliphatic hydrocarbons other than gaseous ones (6). In this proceedings abstract, they describe the isolation of C_{10} to C_{25} paraffins from cultures of a species of *Desulfovibrio* in sea water with fatty acids as the only source of carbon. Subsequent reports of hydrocarbons in bacterial cultures followed, but Stone and ZoBell (7) pointed out that most of these were for cells grown with complex, uncharacterized culture media that could have contained hydrocarbons. They reported the presence of hydrocarbons (uncharacterized) in *Serratia marinorubrum* and *Vibrio ponticus* when grown in hydrocarbon free media.

Albro and Huston made the first relatively complete characterization of the hydrocarbons of a bacterium, *Sarcina lutea*, (8) and this was followed with reports from various laboratories of the hydrocarbons of *Vibrio marinus* (9), of additional studies of *S. lutea* (10-12) and an unidentified micrococcus (12). The distribution of the hydrocarbons of these bacteria on the basis of carbon number as well as the fatty acid composition of the lipids are summarized in Table I.

With *S. lutea*, complex mixtures of isomers and homologous series make difficult a completely unequivocal identification of all the components. However, Tornabene and his coworkers (10,11) resolved four individual isomers of each carbon number by gas liquid chromatography (GLC), and on the basis of mass spectra concluded that two of these had branched methyl groups in iso configuration (but see below) and two with anteiso branched methyl groups. All of the hydrocarbons were monounsaturated. With the strain of *S. lutea* we studied, approximately 90% of the hydrocarbons from early stationary phase cells were monounsaturated and the proportion of monounsaturated hydrocarbons decreased with older cells. Since over 94% of the fatty acids produced by oxidation of the monounsaturated hydrocarbons were either iso or anteiso branched chain acids, we concluded that most of the hydrocarbons had branched methyl groups on both ends of the molecule. We resolved the C_{29} hydrocarbons into three peaks by GLC that represented molecules the ends of which were anteiso-anteiso', iso-anteiso' and iso-iso'. With the C_{28} hydrocarbons, a fourth component which probably had a anteiso-normal configuration at the ends was resolved. Our identification of compounds with both iso and anteiso branch methyls would appear to distinguish the hydrocarbons of the strain of *S. lutea* we studied from that studied by Tornabene et al. Comparison of their published mass spectra with spectra recently obtained of the components resolved from the C_{29} hydrocarbons of the strain of *S. lutea* we studied leads us to believe, however, that one of the fractions that they resolved and identified as having methyl branches in only iso configuration has branch methyls in both iso and anteiso configuration. The culture of *S. lutea* which we obtained from the American Type Culture Collection (for which data are given in Table I) that was supposed to be identical to the one examined by Tornabene et al. contained an unidentified micrococcus which was clearly not *S. lutea*, and we were unable to make a direct comparison of the two strains.

These two strains of *S. lutea* and the unidentified micrococcus differ in the distribution of the hydrocarbons by carbon number,

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TABLE I

Hydrocarbon and Fatty Acid Composition of Bacteria[a]

	Vibrio marinus (9)	*Sarcina lutea* FD-533 (12)	*Sarcina lutea* ATCC-533 (11)	Unidentified micrococcus[b] (12)
Hydrocarbons				
C_{15}	6.6			
C_{16}	2.3			
C_{17}	80.1			
C_{18}	1.5			
C_{23}		0.3	0.7	1.3
C_{24}		0.1	4.4	3.4
C_{25}		0.9	13.4	36.3
C_{26}		1.6	28.6	7.8
C_{27}		18.4	24.8	46.3
C_{28}		12.7	23.7	1.8
C_{29}		65.3	2.5	2.9
C_{30}		0.4		
Other	9.5	0.3	1.9	0.2
Fatty Acids[c]				
br-C_{12}	1.1		0.1	
br-C_{13}	0.7	0.3	1.8	0.3
br-C_{14}	0	1.6	4.1	0.4
n-C_{14}	18.1[d]	0.2	6.4	0.9
br-C_{15}	0	94.1	70.1	89.5
n-C_{15}	0.7	0.7		
br-C_{16}	0	1.9	6.0	1.9
n-C_{16}	41.7[d]	0.5	5.4	2.0
br-C_{17}	1.5	0.8	4.1	2.8
n-C_{17}				0.1
br-C_{18}				1.6
n-C_{18}	22.8[e]		0.1	0.7

[a]Per cent distribution.
[b]Received from American Type Culture Collection as *S. lutea* strain 533.
[c]Abbreviations: br, branched and n, normal.
[d]Includes unsaturated fatty acids.
[e]All monounsaturated.

but in general they are in a range that is twice that of their lipid fatty acids. The implication of this with respect to their biosynthesis is discussed below. The hydrocarbons of *V. marinus* differ markedly from the three other bacteria in that the size range is similar to the fatty acids present. However, while most of the hydrocarbons have an odd number of carbon atoms, the fatty acids are primarily even-numbered. Saturated and unsaturated fatty acids and hydrocarbons were detected. The relationship between the fatty acids and hydrocarbons suggests that the latter may be synthesized by reductive decarboxylation of the fatty acids. No direct evidence for this pathway is available.

CATABOLISM OF EXOGENOUS HYDROCARBONS

The capability of a wide range of bacteria to grow with hydrocarbons as their sole source of carbon has been established. Two very recent and excellent reviews (4,5) on bacterial hydrocarbons discuss and evaluate the work that has been done on the catabolism of hydrocarbons thru 1964, and we will simply outline the present knowledge and briefly report on some more recent work. Aliphatic hydrocarbons (C_6-C_{20}) are oxidized by way of a primary alcohol and an aldehyde to yield the corresponding fatty acid. The exact intermediates in the formation of the primary alcohol are a point of contention not yet resolved. Pathways involving hydroperoxides or 1-alkenes and 1,2-epoxide derivatives have been proposed. In either case, molecular oxygen and reduced pyridine nucleotide (NADH) are required. Both the alcohol and aldehyde dehydrogenases involved in the conversion are dependent on NAD as cofactors. Oxidation may occur at both ends of the molecule to yield hydroxy fatty acids or the corresponding dioic acid. The capacity to produce dioic acids varies with dif-

ferent species and with the chain length of the alkanes.

An interesting by-product of the general mechanism occurs in *Micrococcus cerificans*. Both the alcohol and fatty acid produced from alkanes may appear as components of wax esters. More recently, this organism has been shown (13) to produce no wax ester when grown with nonacosane as sole carbon source. However, when both nonacosane and hexadecane were supplied, waxes with alcohols and acids with primarily 16, 17, or 18 carbons were produced. No nonacosanol or nonacosanoic acid were detected in either case. Of related interest, *M. cerificans* grew readily with alkyl chlorides (C_{16}, C_{18} and C_{20}) and bromides (C_{16} and C_{18}) but not with 1,16-dibromohexadecane as sole carbon source (14). When grown on *n*-hexadecyl chloride, the major esters produced were 16-chlorohexadecyl-16-chlorohexadecanoate and 16-chlorohexadecyl-hexadecanoate, and 80% of the fatty acids of the polar lipids were ω-chloro acids. The amount of wax ester produced decreased as the chain length of the alkyl halide increased and less ester was produced from the akyl bromide than from the analogous chloride.

Alk-1-enes were converted by *Pseudomonas aeruginosa* primarily to the corresponding ω-monounsaturated fatty acid by oxidation of the saturated end of the molecule (4,5). The double bond may also react to form the epoxide. The conversion to the acid has recently been confirmed in a study of the assimilation of 1-tetradecene by *P. aeruginosa* (15). It was also shown that α,ω-dienes (1,7-octadiene and 1,13-tetradecadiene) would not support growth of *P. aeruginosa*. *M. cerificans* assimilated 1-dodecene and 1-tetradecene with the production primarily of saturated fatty acids with an even number of carbon atoms, but the corresponding monounsaturated acid was not detected (16). However, with 1-pentadecene the corresponding 14-pentadecenoic acid was produced and with 1-hexadecene and 1-octadecene, the corresponding ω-monounsaturated acid, saturated acid and a saturated acid with one carbon atom less than the parent alkane were identified. The presence of the latter has been interpreted to indicate that oxidation of the double bond to yield a fatty acid occurred and the production of formaldehyde in cultures grown on 1-hexadecene was cited in support of this. These observations renew the argument on the involvement of alkenes in the oxidation of alkanes (4,5). The isolation of 1-hexadecene from five species of bacteria grown on hexadecane (17) also bears on the argument.

CONVERSION OF FATTY ACIDS TO HYDROCARBONS

Tornabene and Oro (18) studied the incorporation of radioactivity from ^{14}C-labeled leucine, isoleucine, acetate and palmitate into the hydrocarbons of *S. lutea*. Differences in distribution of radioactivity from these precursors between the hydrocarbons separated by GLC were noted. We have carried out more definitive experiments on the incorporation of various precursors into *S. lutea* hydrocarbons both in vivo and in vitro and both published (19-21) and unpublished observations are reviewed here.

A very large proportion of the hydrocarbons of the strain of *S. lutea* that we studied have a branch methyl on both ends of the molecule, a double bond near the center, and, on the average, the number of carbon atoms are equal to one less than two times the average number of carbon atoms in the fatty acids. These structural characteristics of the hydrocarbons and fatty acids are consistent with a biosynthetic mechanism by which two molecules of fatty acid condense as follows:

$$RCH_2COOH + R'COOH \rightarrow$$
$$RCH{=}CHR' + CO_2 + 2H_2O.$$

The distribution of the carbon chains of isoleucine, valine and acetate in the fatty acids and hydrocarbons synthesized by *S. lutea* in vivo is consistent with this general mechanism of head-to-head condensation (19). This can best be illustrated with the relationship between isoleucine and acetate, the anteiso C_{15} fatty acid (the major fatty acid) and the major hydrocarbon (C_{29} with anteiso branch methyls on both ends). Our data are most consistent with the pathway shown here in which the carbon from acetate are shown in italics and the carbon atoms from isoleucine in normal type. The distribution of the carbon atoms from these two precursors in fatty acids derived from monounsaturated hydrocarbons by oxidation of the double bond is also shown. The distributions determined were inconsistent with biosynthesis of the hydrocarbons by head-to-tail condensation of fatty acids or the elongation of fatty acids followed by reduction. Presumably the latter pathways would have to involve a methylation of the aliphatic chain to account for the branches on both termini, and the failure to find significant incorporation of methionine methyl groups into hydrocarbons was also inconsistent with these pathways.

$$CH_3CH_2CH(CH_3)CH(NH_2)COOH \longrightarrow CH_3CH_2CH(CH_3)COOH$$

$$\xrightarrow{CH_3COOH} CH_3CH_2CH(CH_3)CH_2(CH_2)_9COOH$$

$$\downarrow$$

$$CH_3CH_2CH(CH_3)CH_2(CH_2)_9CH{=}CH(CH_2)_8CH_2CH(CH_3)CH_2CH_3$$

$$\downarrow$$

$$CH_3CH_2CH(CH_3)CH_2(CH_2)_9COOH + CH_3CH_2CH(CH_3)CH_2(CH_2)_8COOH$$

Comparison of the distribution of the hydrocarbons and fatty acids in Table I suggests that, with the strain of *S. lutea* we studied, an essentially random pairing of fatty acids could account for the interrelationship of structures. More detailed analyses of the structure of both hydrocarbons and fatty acids (12) and studies on the incorporation of acetate, isoleucine and valine into fatty acids and hydrocarbons (19) showed that specificity for the incorporation of certain fatty acids existed. If the other strain of *S. lutea* and the unidentified micrococcus for which data are given in Table I incorporate fatty acids into hydrocarbons by the same mechanism described here, even greater specificity must occur to account for the preponderance of hydrocarbons with less than 29 carbon atoms, even though the major proportion of fatty acids have 15 carbon atoms. Adding fatty acids to the media of *S. lutea* (20) does cause changes in the hydrocarbon composition, and these changes are consistent with an increased synthesis of hydrocarbons by a head-to-head condensation of the fatty acids whose concentrations are altered with themselves and with the major C_{15} fatty acids.

In the head-to-head condensation mechanism, one molecule of fatty acid undergoes decarboxylation. A comparison of the extent to which ^{14}C of 1-^{14}C-palmitate and -16-^{14}C were incorporated into total hydrocarbons of *S. lutea* in vivo (20) showed that, when acetate was included in the growth medium, palmitate was incorporated without undergoing decarboxylation. In media with low acetate, approximately 70% of the palmitate incorporated was decarboxylated. The actual demonstration of this type of specificity has important implications in the interpretation of this type of data in other systems (22).

Yet additional specificity occurs. When the monounsaturated hydrocarbons isolated from *S. lutea* grown up with 1-^{14}C-palmitate were oxidized, the ^{14}C was found predominantly in fatty acid oxidation products other than palmitate (20). Since there was no redistribution of label from palmitate into other lipid fatty acids, the transfer of label must have occurred by the specific incorporation of the palmitate in a manner so that its carboxyl carbon atom ended up on the opposite side of the double bond from the remainder of the aliphatic chain derived from palmitate as follows:

$$CH_3(CH_2)_{13}CH_2{}^{14}COOH + RCOOH \rightarrow$$

$$CH_3(CH_2)_{13}CH{=}^{14}CHR.$$

The incorporation of palmitate into hydrocarbons by a cell free lysate has also been studied (21). Coenzyme A, Mg^{2+}, adenosine triphosphate (ATP), reduced nicotinamide-adenine dinucleotide phosphate (NADPH), and pyridoxal or pyridoxamine phosphate were required for optimum incorporation. NADH would not replace NADPH. The requirement for the first three cofactors was consistent with the participation of acyl CoA and this was confirmed by showing that in the absence of added coenzyme A, palmitoyl CoA was over 20 times better a precursor than the free acid. Pyridoxamine phosphate was twice as effective as pyridoxal phosphate in promoting the incorporation of palmitate. Whether pyridoxal phosphate or the amine were used, no difference in the incorporation of palmitoyl CoA or esterified palmitate was observed. For reasons that will be brought out below, this implicates pyridoxamine as a cofactor in the mode of entry of fatty acids into hydrocarbons without decarboxylation.

In further studies not yet published, it has been established that the same specificity in the location of the double bond in the final product occurs in vitro as in vivo. Whether palmitate added to the system undergoes decarboxylation during incorporation was found to be dependent on whether it is free or esterified. Approximately 30% of the free acid was decarboxylated while fatty acid supplied as the methyl ester or as triglyceride was essentially 100% decarboxylated. Also, the coenzyme A derivative of palmitate when added to the system was decarboxylated during incorporation into hydrocarbon.

Although it is clear that biosynthesis occurs by a head-to-head condensation mechanism, the intermediates of the pathway have not yet been fully elucidated. In the classical pathway (23) a ketone and secondary alcohol were proposed as intermediates. Both ketones and secondary alcohols which might appropriately fill this role were detected in the nonsaponifiable lipids of *S. lutea* (12). The secondary alcohols occurred in amounts too small to do anything more than

FIG. 1. Proposed mechanism for hydrocarbon biosynthesis in *S. lutea*.

establish their chemical identity. The ketone fraction was separated by GLC and shown to consist of a series of compounds with carbon-numbers in the same range and with a similar distribution as those in the hydrocarbons. In vivo heptacosane-14-one was not converted into hydrocarbons by *S. lutea*, and in vitro it was incorporated into a wide range of hydrocarbons with only 24% of the total in the expected C_{27} product. No incorporation of tricosan-12-ol into hydrocarbons in vitro was detected. Lack of accessibility of these precursors to the site of synthesis or their failure to equilibrate with enzyme bound intermediates could explain these results. It is also possible that fatty acid derivatives such as fatty aldehydes may participate in the condensation. In this case, the initial product of the condensation would be a secondary alcohol. Palmitaldehyde was incorporated into hydrocarbon, but its direct participation in the synthesis was inconsistent with the failure to find incorporation of secondary alcohols. The detection of the diacyl derivative of the alk-1-enyl glyceryl ether (neutral plasmalogen) in *S. lutea* suggested an indirect pathway by which fatty aldehydes could be incorporated without secondary alcohols as intermediates.

Neutral plasmalogen [synthesized from rat brain plasmalogen (24)] when added to cell free preparations of *S. lutea* decreased the incorporation of ^{14}C from 1-^{14}C-palmitate into hydrocarbon by 36%. Use of 1-^{14}C-palmitate meant that the swamping effect of neutral plasmalogen on the incorporation of fatty acids by the pathway not involving decarboxylation was being measured. When the effect of adding neutral plasmalogen on the incorporation of 16-^{14}C-palmitate was checked, an overall stimulation of 7% was measured, or it was calculated from the two sets of data that the incorporation of fatty acids into hydrocarbons by the pathway involving decarboxylation was stimulated 16%. That adding neutral plasmalogen in vitro did in fact stimulate the incorporation of fatty acids via the decarboxylation pathway was confirmed with tri-16-^{14}C-palmitin. In this experiment, adding the neutral plasmalogen stimulated incorporation of ^{14}C by 18%. It was also shown that the alk-1-enyl aliphatic group of neutral plasmalogens synthesized from the plasmalogens of *Clostridium butyricum* grown in the presence of ^{14}C-labeled palmitate served as a better precursor of hydrocarbons in vitro in *S. lutea* than did palmitate itself. These experiments are consistent with the direct participation of neutral plasmalogen in a head-to-head condensation pathway or as a precursor of an intermediate that does participate in the condensation. Condensation apparently occurs with a fatty acid derivative that is preferentially derived from esters or from acyl CoA or the coenzyme A derivative itself.

We propose a mechanism for the biosynthesis of hydrocarbons from fatty acids that takes these findings into consideration. As shown in Figure 1, a given fatty acid, RCH_2COOH, may be incorporated by two modes. It may be converted to the derivative $RCH_2C(=O)Y$ (the coenzyme A or acyl carrier protein derivative?) that, for some reason which is not clear, is in preferential equilibrium with acyl esters; or it becomes the aliphatic portion of a vinyl ether (either neutral plasmalogen or an as yet unidentified derivative RCH=CHOZ). The product of condensation is a monounsaturated hydrocarbon with its double bond in a position consistent with the established specificity of its formation.

DISCUSSION

Except for the fairly extensive studies that have been carried out on the catabolism of exogenous hydrocarbons, the biochemistry of bacterial hydrocarbons has only been barely touched. The composition of the hydrocarbons of a wider range of species needs to be explored. The biosynthesis has been examined in only one species and there is no reason to assume that the pathway in all bacteria is the same. Indeed, the structure of the hydrocarbons of *Vibrio marinus* (9) suggests an entirely different mechanism must be functioning. The amenability of bacteria to study in this field makes it clear that further work with them will also aid in answering some of the questions raised at this symposium in regard to the hydrocarbons of other organisms.

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Reactions of Biological Antioxidants: I. Fe(III)-Catalyzed Reactions of Lipid Hydroperoxides With α-Tocopherol

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ABSTRACT

Rates of α-tocopherol oxidation were measured for free-radical reactions produced by Fe(III)-catalyzed dissociations of hydroperoxides. The kinetics were treated as first-order in α-tocopherol. The hydroperoxides were preformed from methyl linoleate, methyl linolenate, ethyl arachidonate, methyl eicosapentaenoate, and a fraction of polyunsaturated fatty esters of menhaden oil. The degree of unsaturation of the lipid hydroperoxides had little effect on the rates of α-tocopherol oxidation. The rates of oxidation decrease with the concentration of water and increase with the acidity of the media. The pH data suggest a transition from one predominant mechanism to another, which may involve principally acid catalysis. A mechanism for α-tocopherol oxidation is suggested.

INTRODUCTION

Lipid autoxidation and peroxidation are involved in the oxidative deterioration of foods (1), biomembranes (2) and subcellular organelles (3). Free radical intermediates of oxidized lipids and secondary reactions of lipid peroxides contribute to the deteriorative reactions (4-6).

The primary products of lipid autoxidation are hydroperoxides, which can dissociate into free radicals. Dissociation mechanisms include irradiation, thermal homolysis and oxidation-reduction reactions (7). Iron-catalyzed decompositions of hydroperoxides proceed by oxidation-reduction mechanisms.

Biological antioxidants (2,8) may function as peroxide decomposers, metal-ion chelating agents, or hydrogen-atom donors. α-Tocopherol is an important natural hydrogen donor in free radical reactions.

More kinetic information is needed on reactions of α-tocopherol in order to define its mechanisms. The present investigation provides kinetic data of α-tocopherol oxidation in free-radical reactions with preformed hydroperoxides of polyunsaturated fatty acid esters. Fe(III)-catalyzed reactions of the hydroperoxides (7) were chosen to produce peroxy radicals, which are initial reactants of autoxidations. Such Fe(III) reactions permit greater control of initial reaction products, better reproducibility of kinetics, and faster reactions than noncatalyzed reactions.

EXPERIMENTAL PROCEDURES

Materials

Stock solutions of *d*-α-tocopherol (K & K Laboratories) and *dl*-α-tocopherol (Hoffman-LaRoche Co.; Nutritional Biochemicals Corp.) were made up in ethanol and stored at 2-4 C under nitrogen. Concentrations of α-tocopherol were determined spectrally (ϵ_m 3,260 at 292 nm in ethanol) (9). Adsorption chromatography on silica gel was used to remove impurities from α-tocopherol.

Lipid hydroperoxides were prepared, according to a published procedure (10), from methyl linoleate, methyl linolenate, ethyl arachidonate, methyl eicosapentaenoate, and a fraction of polyunsaturated fatty esters of menhaden (*Brevoortia tyrranus*) oil. Briefly, the procedure involved (1) autoxidations of the lipids in air at room temperature, (2) isolation of the hydroperoxides via preparative thin layer chromatography (TLC), and (3) preparation of ethanolic stock solutions of 0.0145 molar lipid hydroperoxides. The concentrations of methyl linoleate hydroperoxides were determined iodometrically and verified spectrophotometrically [ϵ_m 25,000 at 232 nm (11) in absolute ethanol]. The concentrations of other hydroperoxides were based on iodometric peroxide values. Peroxide values of 4,710 to 6,165 meq/kg were measured for the esters isolated by TLC prior to dilution in ethanol. The values represent purities of up to 95% monohydroperoxide.

TABLE I

Effect of Reactants on the Rates of *d*-α-Tocopherol Oxidation in 98.0% Ethanol at 37.0 C With Methyl Linoleate Hydroperoxides[a]

pH	Rate of reaction[b] Without $FeCl_3$, 10^2k, min^{-1}	Without LOOH(18:2), 10^2k, min^{-1}	Corrected rate, 10^2k, min^{-1}
7.0	0	0	3.3
5.0	0	0	4.9, 4.0, 3.9
4.0	<0.1	0	8.1, 8.2
3.0	3.7	0.1, 0.3	4.5, 4.5[c]

[a]2.0×10^{-4} M α-tocopherol, 2.35×10^{-4} M methyl linoleate hydroperoxide, and 1.28×10^{-5} M $FeCl_3$.

[b]First-order dependence on α-tocopherol. Computer calculated rates from 0.25 to 4.00 min reaction time span.

[c]$k_{obsd.} = 8.2 \times 10^{-2}$ min^{-1}.

TABLE II

Rates of *d*-α-Tocopherol Oxidation by Various Lipid Hydroperoxides in 98.0% Ethanol, pH 7.0, at 45.0 C

Lipid hydroperoxide	Rate[a], 10^2k, min^{-1}
LOOH(18:2)	3.68
LOOH(18:3)	3.86, 4.23
LOOH(20:4)	3.06, 3.12, 3.13, 3.10
LOOH(20:5)	3.08, 2.98, 3.10
LOOH(Fish PUFA)	4.80, 5.39

[a]Replicate values from separate reactions.

Kinetic Measurements

Oxidations of α-tocopherol were initiated by Fe(III)-catalyzed decompositions of the lipid hydroperoxides. The rates of oxidation were measured spectrophotometrically with a Beckman Model DB instrument which was calibrated periodically throughout the investigation. Temperatures of the kinetic reactions were controlled to ±0.02 C.

A typical procedure for kinetic measurements is as follows: 3.00 ml of 2.35×10^{-4} M methyl linoleate hydroperoxides in ethanol are placed in both sample and reference cuvettes in a thermostated cell holder of the spectrophotometer. Next, 30 μl of 2.00×10^{-2} M α-tocopherol from stock solution is added to the sample cuvette. After thermal equilibrium, the initial concentration of α-tocopherol is spectrally determined. The kinetic reaction is started by adding 4 μl of Fe(III) catalyst (1.00×10^{-2} M $FeCl_3$ in 95% ethanol). The rate measurements are begun 0.25 min after the reactants are mixed.

The rate of α-tocopherol oxidation was followed by continuously recording by differential spectrophotometry the change in absorbance at 292 nm. First-order rate constants were calculated by digital computer (IBM model 7044) for the reaction period between 0.25 min and 4.00 min (12).

RESULTS AND DISCUSSION

The course of hydroperoxide decomposition by iron, like other transition metals depends on the oxidation state of the metal. For example, the reactions depicted by Equations 1 and 2

$$ROOH + Fe(III) \longrightarrow ROO^{\bullet} + H^+ + Fe(II) \quad [1]$$

$$ROOH + Fe(II) \longrightarrow RO^{\bullet} + OH^- + Fe(III) \quad [2]$$

proceed at vastly different rates. Preliminary experiments demonstrated that a slower Fe(III)-catalysis is controlled easier than a faster Fe(II)-catalysis (12). A Fe(III) system has an advantage that it does not require the addition of a regenerating agent as would be required for a Fe(II) system (13). A system that uses only Fe(II) in the initial reaction will almost quantitatively produce alkoxy radicals (14). Fe(III) catalysis in the initial reaction produces equal amounts of peroxy and alkoxy radicals according to the summation of the above equations.

Examination of Reactants

Particular attention was given to reactions that occur in the initial 4 min period of α-tocopherol oxidation. An examination of possible interactions of reactants during this period was made (Table I).

Examination of possible thermal homolysis of methyl linoleate hydroperoxides, LOOH(18:2) [LOOH designates lipid hydroperoxide and (18:2) means carbon number:-number of double bonds of fatty ester hydroperoxides], to cause α-tocopherol oxidation revealed, in the second column of Table I, that this reaction is unimportant in the pH range of 7.0 to 4.0. The data, however, indicate an apparent acid catalysis at pH 3.0 in the absence of Fe(III). An examination of a reaction of α-tocopherol with Fe(III) in the absence of LOOH(18:2) indicated, as in Table I, that the reaction with Fe(III) is kinetically unimportant for systems at pH 7.0 to 4.0. At pH 3.0, corrections for a small Fe(III) reaction were made. Corrected rates for the complete reaction system are determined with a fair degree of confidence. The last column of Table I gives the rates when all reactants are present.

Because the method of differential spectra was used, a blank contained all reaction components other than α-tocopherol. An exami-

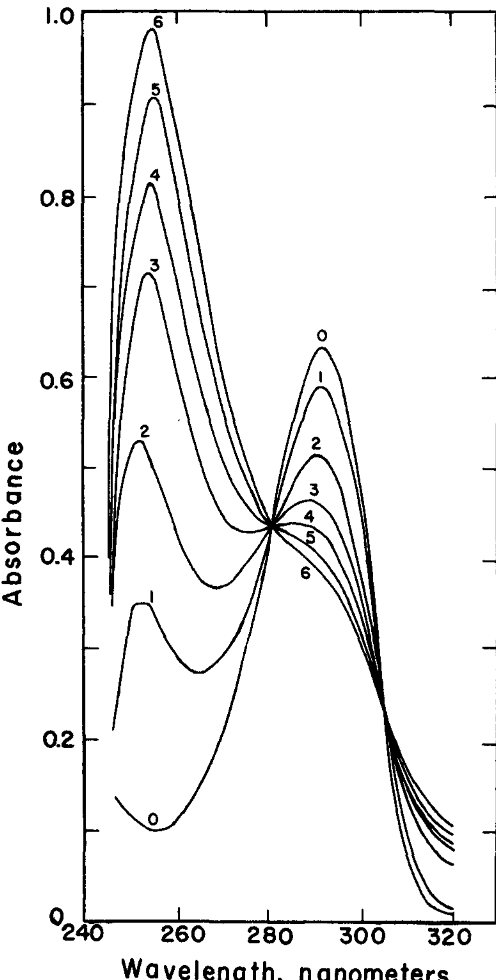

FIG. 1. Sequential spectra of a reaction of 1.94 x 10^{-4} M α-tocopherol, 1.28 x 10^{-5} M $FeCl_3$, 2.35 x 10^{-4} M methyl linoleate hydroperoxides, and 1.0 x 10^{-6} M HCl in 99.0% (v/v) (pH 6.0) ethanol at room temperature. Reaction times at start of each scan: 0, zero; 1, 0.25 min; 2, 2.25 min; 3, 5.25 min; 4, 7.25 min; 5, 9.50 min; and 6, 11.75 min.

nation of the blank revealed a negligible influence on the spectrum in the region of 292 nm. For example, spectrophotometric measurements of 2.35 x 10^{-4} M LOOH(18:2) with 1.28 x 10^{-5} $FeCl_3$ in ethanol, at 37 and 45 C, failed to show a significant reaction during the time period that rates of α-tocopherol oxidation were measured.

Evidence for the stability of the hydroperoxides was obtained from comparisons of rates of α-tocopherol oxidation on different days. For example, from an ethanolic stock solution of 1.27 x 10^{-2} M LOOH (20:5), aliquots were taken and used in Fe(III)-catalyzed reactions with $(2.04\pm0.01)10^{-4}$ M α-tocopherol. The reaction concentration of LOOH(20:5) was 2.34 x 10^{-4} M. A specific rate of 3.10 x 10^{-2} min^{-1} was observed on one day, while 48 hr later the rates of 3.08 x 10^{-2} and 2.98 x 10^{-2} min^{-1} were observed. If the LOOH(20:5) was unstable and decomposed in the stock solution, thereby decreasing the hydroperoxide concentration, then the rate would be less. Such instability was negligible compared to the effect of controls in the investigation.

Degree of Unsaturation of Lipid Hydroperoxides

Data in Table II summarize the results of α-tocopherol oxidations by hydroperoxides of lipids with various degrees of unsaturation. There are small but insignificant differences between reaction rates associated with the degree of unsaturation of lipid hydroperoxide (LOOH) esters of the same carbon atom chain length. There are apparent differences between reaction rates with LOOH(18:2) and LOOH(18:3) and those with LOOH(20:4) and LOOH(20:5). A slower reaction is associated with the longer chain length and greater degree of unsaturation compared to the shorter chain length and lesser degree of unsaturation.

The mixed hydroperoxides of the fish oil polyunsaturated fatty acid esters appear as an anomaly compared to the other more homogeneous hydroperoxides. The fatty acid composition of the esters used to prepare the LOOH(Fish PUFA) included 5.5% 20:4, 30.4% 20:5, 7.8% 22:5 and 29.6% 22:6, among others (10). The reaction of α-tocopherol with the LOOH(Fish PUFA) system gave rates that were about 50% faster than those associated with LOOH(18:2) and LOOH(18:3). This discrepancy is difficult to explain, considering the stability of the hydroperoxides.

The effect of decreasing the level of LOOH(18:2) in reactions with α-tocopherol was examined to determine the relative decrease in the corresponding rate. By changing the concentration of LOOH(18:2) from 2.35 x 10^{-4} M to 2.08 x 10^{-4} M, or 11.5 mole-%, in a reaction with 2.08 x 10^{-4} M α-tocopherol, the specific rate was lowered from 3.68 x 10^{-2} to 2.98 x 10^{-2} min^{-1} or 19.1%. Comparing these results to those in Table II, one finds that the 11.5% decrease in LOOH(18:2) concentration results in a decrease in rate that is less than the difference between the extreme values in the Table. Therefore, it appears that the relative differences in the rates shown in Table II are not due to decreased concentrations of LOOH that may result from peroxide instability as the degree of unsaturation increased.

It is possible that the rate of hydroperoxide

TABLE III

Rates of *d*-α-Tocopherol Oxidation With Methyl Linoleate Hydroperoxides[a]: Effect of Temperature, Acidity and Water Content of Ethanolic Media

pH	Aqueous ethanol medium, ethanol, %	Rate at 37.0 C, 10^2k, min^{-1}	Rate at 45.0 C, 10^2k, min^{-1}
7.0	100	13.6	21.3
	99.5	9.22	14.3
	99.0	7.94	10.7
	98.0	3.30	4.31
5.0	99.5	10.7	15.3
	99.0	9.3	13.0
	98.0	4.95	6.24

[a]Reactants concentrations: 2.0 x 10^{-4} M α-tocopherol, 2.3 x 10^{-4} M LOOH(18:2), and 1.3 x 10^{-5} M $FeCl_3$. Reaction time span, 0.25 to 4.00 min.

dissociation in an overall reaction sequence is nearly the same as the rate of α-tocopherol oxidation. In such a case, the differences in Table II may be due to competing rate-determining steps.

Examination of Reaction Intermediate

The spectra of α-tocopherol oxidation reactions with Fe(III) and LOOH(18:2) were recorded between 320 nm and 240 nm at various time intervals. An isosbestic point at 282 nm, illustrated in Figure 1, was determined from sequential overlapping spectra of the reaction in aqueous ethanol at pH 6.0 and 7.0, and in absolute ethanol. This isoabsorptive relationship in the spectra of the initial stage of α-tocopherol oxidation is indicative of a unimolecular transformation (15) and supports first order kinetics based on α-tocopherol concentration.

The initial product of α-tocopherol oxidation had a maximum absorbance near 255 nm. An isosbestic point at 283.5 nm was found for the spectra of various mixtures of 2.0 x 10^{-4} molar *d*-α-tocopherol and *d*-α-tocopherol quinone (Distillation Products Industries, Rochester) in ethanol. The spectrum of α-tocopherol quinone in ethanol shows a doublet peak with λ_{max} at 262 nm and 269 nm. The initial oxidation product is not the quinone, but some intermediate substance that is easily oxidized to the quinone, as the following will indicate.

The spectral properties of the substance contributing to the 255 nm peak were examined in two ways. First, a reaction in 99.0% ethanol at pH 4.0 after 6 min showed a concomitant spectral shift of the 255 nm peak to a doublet with a peak at 262 nm and strong shoulder peak at 269 nm. The doublet peaks correspond with those of α-tocopherol quinone.

Second, a reaction was run in neutral absolute ethanol; the 255 nm peak remained strong after 30 min of reaction. To the solutions in both cuvettes were added 20 μl aliquots of ethanol containing $NaBH_4$, and the reaction spectrum was recorded sequentially for another 30 min. The absorbance decreased during this period, but maintained the 255 nm λ_{max}. At this point, 5 μl of 6N-HCl was added to the reaction in the sample cuvette. A sequential recording of the spectrum showed a rapid (30 sec) shift from the 255 nm peak to a doublet with peaks at 262 nm and 269 nm (shoulder), which was identical to that of the first reaction.

We conclude that this spectral examination of α-tocopherol oxidizing with Fe(III) and LOOH(18:2) indicates a transition through an acid-unstable intermediate to form α-tocopherol quinone. The intermediate with λ_{max} 255 nm is not known, but α-tocopherol quinone methine (16) is a major suspect. Under the conditions of the reactions, 8a-ethoxy-α-tocopherone (17), which has an absorption maximum at 241 nm in ethanol (18), may possible form; however, the spectral data for tocopherone and other products of α-tocopherol oxidation show λ_{max} values well removed from 255 nm.

Effect of Water and Acid

Rates of α-tocopherol oxidation are compared for reactions in absolute ethanol and aqueous ethanol at pH 7.0 and 5.0 (Table III). The data in Table III show that as little as 0.5% H_2O in the solvent has a marked inhibitory effect on the oxidation of α-tocopherol. The greatest effect of H_2O is found between the reactions in anhydrous ethanol and in 99.5% ethanol. An increase in acidity from pH 7.0 to 5.0 increases the rate by as much as 50%. The percentage increase in rate is greater at the higher content of water in the solvent and at 37 C than at 45 C.

FIG. 2. A suggested mechanism for α-tocopherol oxidation.

Mechanistically, the data in Table III indicate that acid or hydronium ions are involved in the slow step of the overall reaction. This involvement may be to (a) facilitate an intramolecular transformation of an α-tocopherol oxidation intermediate, (b) increase available lipid free-radicals by catalyzing hydroperoxide dissociation (22), or (c) make Fe(III) more available by causing dissociation of less reactive $Fe(OH)^{2+}$ (14), which might form in neutral aqueous ethanol. Conversely, the involvement of water in the rate-determing step may be to inhibit hydroperoxide dissociation by combining with the Fe(III) catalyst to form the ion pair complex. Water may compete with α-tocopherol as a hydrogen-atom donor to free radicals. Water may also be a product of an intermediate step of the overall reaction, such that an increase in water content of the medium could inhibit the step by mass action effects.

Mechanism for α-Tocopherol Oxidation

α-Tocopherol can undergo oxidation to form a variety of products (17-19), including α-tocopherol quinone (20). Free-radical oxidation can yield α-tocopherol quinone methine (16). A complete mechanism for α-tocopherol quinone formation from α-tocopherol that includes the quinone methine structure has not been reported. A quinone methine has been essentially proven as an intermediate in coenzyme Q reactions (21) which are comparable to certain

vitamin E reactions. Based on arguments similar to those used for coenzyme Q, a mechanism that involves the quinone methine is suggested for α-tocopherol (Fig. 2).

This mechanism suggests acid-catalysis and hydration steps in addition to hydrogen-atom abstraction steps with an intermediate α-tocopherol quinone methine. The first two steps involve abstractions of two hydrogen atoms, as proposed by Knapp and Tappel (16). Next, the unstable quinone methine reacts with a proton to produce an intermediate oxonium ion, which rearranges to a carbonium ion. The reactive carbonium ion may then add a water molecule to form a second oxonium ion, which can expel a proton to produce α-tocopherol quinone.

The reaction of the quinone methine with a proton is assumed to be irreversible. This assumption is based partly on the present observation of a spectral isosbestic point. Also, no evidence is found for the conversion of α-tocopherol quinone to the quinone methine that would support a reversible mechanism.

The suggested mechanism incorporates the effect of pH, i.e., an increase in acidity of the reaction medium tends to accelerate the rate of α-tocopherol oxidation via the protonation of a principal intermediate, a quinone methine. The results of the present investigation, which demonstrate rate enhancement with decreasing pH, lend support to protonation steps of this mechanism.

This investigation indicates a need for further studies on the solvent dependence of reactions of α-tocopherol with lipid free-radicals. A rate law that takes into account solvent, hydrogen-ion concentration, and all reactants would be valuable to an understanding of the reactivities of vitamin E and similar biological antioxidants.

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Reactions of Biological Antioxidants: II. Fe(III)-Catalyzed Reactions of Methyl Linoleate Hydroperoxides With Derivatives of Coenzymes Q and Vitamin E

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ABSTRACT

Lipid free-radical oxidations of ubiquinol-6, ubichromenol-6, ubichromenol-10, and α-tocopherol hydroquinone were kinetically examined in the presence of Fe(III)-catalyzed dissociations of preformed methyl linoleate hydroperoxides. The rates of oxidation of the chromenols increased more than those of the hydroquinones as reaction acidity was increased. Differences in thermal effects upon rates were influenced by the levels of water in the reactions. The hydroquinones exhibited faster rates relative to α-tocopherol than the ubichromenols, while the rates for the latter varied markedly depending on the nature of the solvent.

INTRODUCTION

Hydroperoxides are the principal products in the autoxidation of lipids in biological materials. Free-radical chain processes, which result in polymerization of proteins (1) and damage to biological membranes and subcellular organelles (2,3), are induced by dissociations of lipid hydroperoxides (4).

Vitamin E, or α-tocopherol, inhibits free-radical reactions in the autoxidation of lipids (5). Vitamin E and the other natural compounds considered here are possible biological antioxidants because they can furnish hydrogen atoms (6) to free radicals and inhibit peroxidations. Such biological antioxidants may include the reduced and isomerized forms of coenzymes Q, namely, the ubiquinols and ubichromenols. Also, α-tocopherol hydroquinone, a reduced form of oxidized vitamin E, is considered an antioxidant.

This report is a continuation of a series that began with a kinetic investigation of α-tocopherol reactions with lipid peroxy radicals (7). The investigations reported here give information on the relative reactivities of ubiquinol, ubichromenols, α-tocopherol, and α-tocopherol hydroquinone in lipid free-radical reactions which involve preformed methyl linoleate hydroperoxides. Peroxy radicals from the hydroperoxides were generated by ferric-iron catalysis.

EXPERIMENTAL PROCEDURES

Materials

Ubiquinol-6. Ubiquinone-6 (coenzyme Q_6; Nutritional Biochemicals Corp. was reduced to ubiquinol-6, as follows. Approximately 100 mg of ubiquinone-6 was mixed in approximately 5 ml of 95% ethanol. Two drops of an aqueous 20% (w/v) $NaBH_4$ solution were added. The mixture was then well agitated on a vibration-type mixer, and allowed to stand for 2 min at room temperature. Next, the reaction was acidified with a few drops of 12 N H_2SO_4, diluted to 3X volume with distilled water, and extracted with *n*-hexane (Nanograde; Mallinckrodt Chemical Works). The mixture of reactants and the hexane extract were protected with nitrogen at all times. The *n*-hexane solution was dried over anhydrous Na_2SO_4, decanted into a pear-shaped flask, and evaporated with a rotary evaporator over a 50-60 C water bath. The ubiquinol-6, which was recovered from the hexane extract, was found to be 95 mole % pure by spectrophotometric analysis (8).

A stock solution of ubiquinol-6 was made up in 0.5 ml of 95% ethanol. The concentration of ubiquinol-6 was $(2.15 \pm 0.06)\ 10^{-2}$ M in the stock solution.

Spectral analysis of an autoxidation of ubiquinol-6 to ubiquinone-6 produced an isosbestic point at 293.5±0.3 nm in ethanol. A molar absorptivity (ϵ_m) of 3,615 at 293.5 nm was determined for ubiquinol-6 and ubiquinone-6 in ethanol. Analysis of ubiquinol-6 gave ϵ_m 3,860 at 289 nm (λ_{max}), ϵ_m 2,945 at 274.5 nm, and ϵ_m 569 at 255 nm (λ_{min}); while ubiquinone-6 gave ϵ_m 15,330 at 274.5 nm (λ_{max}) and ϵ_m 7,720 at 255 nm, in ethanol.

Ubichromenol-6 and Ubichromenol-10. Ubi-

chromenol-6 and ubichromenol-10 were prepared by the isomerization method of McHale and Green (9). The corresponding ubiquinones (Nutritional Biochemicals Corp.; Sigma Chemical Co.) were used in the isomerizations.

Ubichromenol-6 and ubichromenol-10 were separately purified by preparative TLC on chromatoplates coated with 2 mm layers of Silica Gel F_{254} (E. Merck, Darmstadt). The developing solvent was petroleum ether-ethyl acetate (80:20 v/v). The separated bands were revealed with a short wavelength UV light (2537 A). About 7 mm of one side of the chromatoplates were sprayed with a 5% (w/v) solution of phosphomolybdic acid in ethanol, and quickly heated at 110 C for 30 sec. A blue area that corresponded to ubichromenol reduction of phosphomolybdic acid corresponded to a very dark band revealed under the UV light. In each case, the adsorbent band was scraped from the plate, and the ubichromenol was recovered by extraction with peroxide-free diethyl ether. This procedure gave 81% (150 mg) yield of ubichromenol-6, which was highly pure as indicated by good agreement of molar absorptivities with the literature values (10).

Stock solutions of appropriate concentrations of ubichromenol-6 and ubichromenol-10 were prepared in ethanol. An isosbestic point at 253 nm was found in the spectra of free-radical oxidations of ubichromenol-6 and ubichromenol-10 in ethanol. These oxidations were carried out in the same manner as those for kinetic measurements (see below).

Spectrophotometric analysis of the ubichromenol-6 gave molar absorptivities as follows: (in isooctane) ϵ_m 19,300 at 231 nm, ϵ_m 8,720 at 274 nm, ϵ_m 8,030 at 281 nm, ϵ_m 3,500 at 330 nm; and (in absolute ethanol) ϵ_m 5,015 at 253 nm, ϵ_m 7,540 at 273 nm, and ϵ_m 1,100 at 299 nm. Spectral analysis of ubichromenol-10 gave molar absorptivities, as follows: (in isooctane) ϵ_m 16,510 at 230 nm, ϵ_m 7,960 at 274 nm, ϵ_m 2,910 at 330 nm; and (in absolute ethanol) ϵ_m 17,150 at 230 nm, ϵ_m 5,170 at 253 nm, ϵ_m 7,900 at 274 nm, and ϵ_m 1,645 at 300 nm.

α-Tocopherol Hydroquinone. A sample of *d*-α-tocopherol quinone (Distillation Products Industries) was reduced to α-tocopherol hydroquinone, as follows: 0.20 g of *d*-α-tocopherol quinone was dissolved in 50 ml of diethyl ether in a low-actinic glass separatory funnel, freshly prepared aqueous solution of 20% (w/v) sodium dithionite was added, and the mixture was shaken vigorously for at least 5 min. Nitrogen was used at all times to protect the reaction. The ethereal layer was recovered, washed three times with freshly boiled, but cool, distilled water, and dried over anhydrous K_2CO_3 in the separatory funnel. Precautions were taken to exclude adsorbed oxygen from the K_2CO_3. The α-tocopherol hydroquinone was recovered quantitatively by evaporation of the solvent in a stream and atmosphere of nitrogen at room temperature.

A stock solution of α-tocopherol hydroquinone was made up in a serum bottle containing nitrogen. The solvent, absolute ethanol, was purged with nitrogen. The concentration of the hydroquinone was determined spectrophotometrically. Quantitation was based on ϵ_m 2,925 at 287 nm λ_{max} in absolute ethanol.

Spectral analysis of an autoxidation of α-tocopherol hydroquinone to α-tocopherol quinone produced an isosbestic point at 282 nm (ϵ_m 2,800) in ethanol. α-Tocopherol hydroquinone gave the value of ϵ_m 1,540 in ethanol at the λ_{max} 269 nm for α-tocopherol quinone (ϵ_m 16,200).

α-Tocopherol. The α-tocopherol was the same as that used in the initial investigation reported in this series (7). Stock solutions of 2.0×10^{-2} M α-tocopherol in 95% ethanol were prepared. Concentrations were based on the spectrophotometric value ϵ_m 3,260 at 292 nm (λ_{max}). A spectral isosbestic point at 283.5 nm (ϵ_m 2,660) was determined for mixtures of 2.0 $\times 10^{-4}$ M α-tocopherol and 2.0×10^{-4} M α-tocopherol quinone.

Methyl Linoleate Hydroperoxides. The hydroperoxides of methyl linoleate were prepared by autoxidation of 99.9% methyl linoleate in air at room temperature and isolated by preparative thin layer chromatography, according to published methods (11). The methyl linoleate hydroperoxides, LOOH(18:2) [LOOH designates lipid hydroperoxide and (18:2) means carbon number:number of double bonds of fatty ester hydroperoxides], had a peroxide value of 5,750 meq/kg, which is 94% of the theoretical for monohydroperoxides. $E^{1\%}_{1cm}$ (232 nm) of 626 and $E^{1\%}_{1cm}$ (268 nm) of 29.6 were measured for the LOOH(18:2) in isooctane.

Stock solutions of $1.45 \cdot 10^{-2}$ M LOOH(18:2) in 95% ethanol were prepared. When solutions were stored under nitrogen at 4 C, the LOOH(18:2) was stable throughout the kinetic investigations.

Kinetic Measurements

The procedures for carrying out the oxidations and measuring the rates were essentially the same as those for Part I of the series (7). The only differences were in the actual spectral properties that were involved in the kinetic procedure. A Beckman model DB spectrophoto-

TABLE I

Rates of Biological Antioxidant Oxidation: Effect of Temperature, Acidity and Water Content of Ethanolic Media of Reactions With Methyl Linoleate Hydroperoxides

Compound	pH	Aqueous ethanol medium, ethanol, %	Rate at 37.0 C, 10^2k, min^{-1}	Rate at 45.0 C, 10^2k, min^{-1}
Ubiquinol-6	7.0	100	61.5	
		98.0	25.2	43.4[a]
	5.0	98.0	27.3	
Ubichromenol-6	7.0	100	14.3	24.2
		99.5	9.47	18.4
		99.0	6.63	8.89
		98.0	1.71	1.82
	5.0	99.5	12.1	20.0
		99.0	9.13	12.2
		98.0	2.93	2.87
Ubichromenol-10	7.0	100	14.5 ±0.3	26.8±0.2
		98.0	3.04±0.06	3.5±0.3
	5.0	98.0	3.76±0.04	
d-α-Tocopherol hydroquinone	7.0	100	38.6 ±0.2	51.6±1.5
		98.0		16.1±1.5
d-α-Tocopherol[b]	7.0	100	13.6	21.3
		98.0	3.30	4.31

[a]Rate of blank reaction without $FeCl_3$ added was 1.67 x 10^{-3} min^{-1} or 0.4% of 43.4 x 10^{-2} min^{-1}.
[b]See Reference 7.

meter, which was periodically calibrated, was used to measure absorbances.

The procedures for the oxidation and kinetic measurements for ubiquinol-6, for example, are as follows: 3.00 ml ethanol containing 2.35 x 10^{-4} M LOOH(18:2) and 2.00 x 10^{-4} M ubiquinol-6 are placed into a sample cuvette, while 3.00 ml of the 2.35 x 10^{-4} M LOOH(18:2) are placed into a reference cuvette. The cuvettes are then placed into a thermostated holder in the spectrophotometer and allowed to come to thermal equilibrium at 37.0±0.02 C (or 45.0±0.02 C). The kinetic reaction is started by adding 4.0 μl of 1.00 x 10^{-2} M $FeCl_3$ in ethanol to both cuvettes. After thoroughly shaking the cuvettes, the change in absorbance at 255 nm (near λ_{max} for ubiquinone-6) is measured. The absorbance at the isoabsorptive wavelength 293.5 nm (λ_i) is determined prior to the addition of Fe(III). Rate measurements are recorded between 0.25 and 1.75 min, and absorbances are read at 0.25 min intervals from the recordings. First-order specific rates are calculated by digital computer (IBM Model 7044) from the change in absorbance at 255 nm relative to the absorbance at λ_i.

For rates of oxidations of ubichromenol-6 and ubichromenol-10, the change in absorbances at 299 nm and 300 nm, respectively, were followed relative to absorbances at 253 nm (λ_i) for 4 min. The rates of α-tocopherol hydroquinone oxidations were based on changes in absorbance at 287 nm during the first 0.9 min. Rates of α-tocopherol oxidations were based on absorbances at 292 nm relative to absorbance at 283 nm for 4 min.

RESULTS

Ubiquinol-6 Oxidations

The results in Table I show that the addition of 2% (v/v) water has the effect of lowering the rate of ubiquinol-6 oxidation at 37 C by about two and one-half times. The rate is nearly unaffected by a 100-fold increase in the level of acid from pH 7 in 98.0% ethanol. A small blank reaction was observed at 45 C.

Ubichromenol-6 Oxidations

The results in Table I for ubichromenol-6 at 37.0 and 45.0 C show that the rates of oxidation decrease as the level of water in the reaction media increases. A comparison of pH-controlled reactions in 99.5%, 99.0% and 98.0% ethanol at 37.0 C indicates that the decrease in rate due to increased water content of the media is greater at pH 7.0 than at pH 5.0. By comparing 37 C reactions in 99.5% and 98.0% ethanol, values in the Table show 82% lower rate at pH 7.0 and 75% lower rate at pH 5.0. A 100-fold increase in acid levels from pH

7.0 slightly enhanced the rates when the solvent was unchanged.

In absolute ethanol and in 99.5% ethanol, the rates at 45 C were nearly double those at 37 C. In 98.0% ethanol, the oxidation appeared insensitive to thermal influence between 37 and 45 C. The temperature effected the rate more as the amount of water in the solvent decreases.

Ubichromenol-10 Oxidations

The results in Table I show that the rate for ubichromenol-10 in absolute ethanol at 45 C is about twice that at 37 C. At pH 7.0 and 98.0% ethanol, the rate was only slightly greater at 45 C than at 37 C. The addition of water to the anhydrous systems caused a substantial decrease in rate at both temperatures. At 37 C in 98.0% ethanol, an increase in the level of acid from pH 7.0 to pH 5.0 slightly enhanced the rate.

α-Tocopherol Hydroquinone Oxidations

Table I also shows that water effects the lipid free-radical oxidations of α-tocopherol hydroquinone. Under the kinetic conditions employed at 45 C, the addition of 2% water to the medium causes the rate to be reduced by about one third. In absolute ethanol, an increase from 37 C to 45 C causes about a 35% increase in the rate of oxidation.

DISCUSSION

Reaction Characteristics

The nature of the medium for reactions of ubiquinol-6, ubichromenol-6, ubichromenol-10, and α-tocopherol hydroquinone has the same effects as reported previously for reactions of α-tocopherol (7). The differences noted in the magnitude of the effects are related to molecular structures, bond energies, and enthalpies of specific reactions, the discussion of which is beyond the scope of this report.

Kinetic plots revealed an order of reaction greater than first order as the reactions proceeded to near completion. Only the first minute or several minutes of each reaction were kinetically measured and compared. The initial period of a reaction closely approximates a first-order reaction, consequently, the least number of complex oxidation products occur during this period.

The isosbestic points demonstrated in the spectra of the reaction mixtures are supportive evidence for reactions of the type A→B. Here, an isosbestic point for each oxidation reaction negates the transformation of antioxidants by dimerization.

Relative Rates

The type and structure of free radicals are important variables in reactions of hydrogen-atom donors or antioxidants. Relative reaction rates can be estimated by maintaining such variables constant for reactions under comparable conditions (12). Here, the reaction of each biological antioxidant was dependent on alkyl peroxy radicals generated from methyl linoleate hydroperoxides, while each set of reaction conditions were closely controlled.

The results show significant differences in reaction rates of the antioxidants. The relative rates for reactions in absolute ethanol at 37 C are in an order which is anticipated from differences in molecular structures, i.e., ubiquinol-6 and α-tocopherol hydroquinone oxidize faster than α-tocopherol and the ubichromenols. In this case, the order and relative rates were as follows: ubiquinol-6, 4.52; α-tocopherol hydroquinone, 2.86; ubichromenol-10, 1.07; ubichromenol-6, 1.05; and α-tocopherol, 1.00.

A greater difference in relative rates was found for reactions in 98% ethanol compared to those in absolute ethanol. For reactions at 45 C in aqueous 98.0% ethanol at pH 7.0, the order and relative rates were as follows: ubiquinol-6, 10; α-tocopherol hydroquinone, 3.7; α-tocopherol, 1.0; ubichromenol-10, 0.8; and ubichromenol-6, 0.4.

The order of relative rates of ubiquinol-6 and α-tocopherol hydroquinone correlates with differences between electronic inductive strengths of methoxy and methyl groups which are adjacent to phenolic groups in benzene ring positions. An increase in free-radical stability of the methyl-substituted hydroquinones is predicted from resonance structure formulas which include hyperconjugation (13). Thus, α-tocopherol hydroquinone is less reactive than ubiquinol-6, because of the added stability given to the former by hyperconjugation.

In the case of oxidations of the ubichromenols and α-tocopherol in absolute ethanol, the closeness of the relative rates suggests that the role of an inductive effect of side groups upon reactivities may be unimportant. On the other hand, the wide difference between their relative rates in 98.0% ethanol suggests that either solvent molecules are directly involved in the overall mechanisms or solvent polarity facilitates charge-transfer or charge-separation steps, or both, in the mechanisms. The chromenols and α-tocopherol (a chromanol) can undergo ring-opening reactions, which probably involve heterolytic cleavage of covalent bonds, rather than homolytic cleavage, in polar solvents (7).

The above interpretations help to explain some of the differences reported by others in

The above interpretations help to explain some of the differences reported by others in examinations of coenzymes Q, vitamins E, and their derivatives under a variety of reaction conditions (14-16). The work of Kaufmann and Garloff (14) demonstrated that solvent and emulsions contribute to different reactivities of these compounds. Reactions proceed at different relative rates in a homogenous system compared to a heterogeneous system. Additional differences are indicated in the present work due to oxidations by Fe(III)-catalyzed generation of free radicals rather than by autoxidation processes (16).

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Long Chain Fatty Alcohols in Normal and Neoplastic Tissues

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ABSTRACT

Small quantities of long chain fatty alcohols (esterified or free or both) were found in four normal tissues (about 0.01% of total neutral lipids) and three neoplasms (about 0.3% of total neutral lipids). The major chain lengths (16:0, 18:0 and 18:1) of the fatty alcohols in both normal and neoplastic cells qualitatively resemble the O-alkyl chain lengths of glyceryl ethers. Our data showing that long chain fatty alcohols occur in vivo support the biological significance of the metabolic pathway that uses fatty alcohols as a substrate for the alkyl chain in glyceryl ether biosynthesis.

INTRODUCTION

Long chain fatty alcohols in living cells have been found mainly in animals of the sea or in plants (1). Essentially nothing is known about their occurrence in mammalian tissues, except for the fatty alcohols present in wax esters of specialized glands (2) and pig liver (3). Metabolic studies (4-10) of long chain fatty alcohols have indicated that they are rapidly metabolized, and specific enzymic systems that use fatty alcohols to form waxes (11) and alkyl glyceryl ethers (12,13) have been discovered. The origin of fatty alcohols is obscure although it is known that they can result (14,15) from the enzymic cleavage (15,16) of glyceryl ether bonds. In view of the important role of fatty alcohols as precursors in the biosynthesis of glyceryl ethers, we believed that a study of their occurrence, especially in neoplastic cells that are rich in glyceryl ethers, was warranted.

METHODS AND MATERIALS

Animals, Tissues and Reference Compounds

Four normal tissues (beef brain, beef heart, rat liver and mouse preputial glands) and three transplantable neoplastic tissues (Walker 256 carcinosarcoma, Ehrlich ascites cells, and Morris hepatoma 7777) were used in these studies. The bovine tissues were obtained fresh from a local slaughter house. The livers were obtained from female Charles River rats weighing 150-170 g, and the preputial glands were obtained from 14 male ICR mice weighing 20-30 g. The Walker 256 carcinosarcoma, the Ehrlich ascites cells and the Morris hepatoma were obtained from our colony of tumor-bearing animals as previously described. All tissues were maintained in ice after their removal from the animals until the lipids were extracted.

The following compounds were used as reference standards during this study. Methyl esters of the fatty acids (pentadecanoic, palmitic, stearic, oleic, linoleic and arachidonic), dipalmitin, oleic acid, triolein and cholesterol palmitate (99% purity) were purchased from The Hormel Institute, Austin, Minn. The fatty alcohols were prepared from the methyl esters of the fatty acids by $LiAlH_4$ hydrogenolysis. Hexadecyl palmitate and 1,2-dipalmitoyl-3-hexadecyl glyceryl ether (99% purity) were purchased from Analabs, Inc., Hamden, Conn.

Extraction of Tissue Lipids and Their Resolution Into Neutral and Phospholipid Fractions

The tissues were first homogenized in methanol-chloroform (2:1 v/v) and then extracted of lipids by the method of Bligh and Dyer (17). The neutral lipids and phospholipids were separated on short silicic acid columns; only the neutral lipid fractions were used for subsequent analysis. All lipid samples were stored under nitrogen in chloroform at -23 C until analyzed.

Separation of Lipid Classes

Approximately 100 mg of the neutral lipid fractions isolated from the neoplasms were resolved into classes by preparative thin layer chromatography (TLC) on Silica Gel G layers (250 μ) in a solvent system of hexane-diethyl ether-acetic acid (80:20:1 v/v). The bands were located by spraying the plate with a solution of 0.1% 2,7-dichlorofluorescein in ethanol and viewing the chromatographic layer under ultraviolet light. The four different bands of components scraped from the plate were (a) cholesterol esters plus waxes, (b) glyceryl ether diesters plus triglycerides, (c) free fatty acids, and (d) diglycerides plus free cholesterol plus free alcohols. The lipids were eluted from the silica gel in a sintered glass funnel with 30 ml of 20% methanol in diethyl ether and then with 20 ml of chloroform. The solvents were removed by evaporation under vacuum.

The larger quantities of neutral lipids required from the normal tissues (0.5-2 g) were

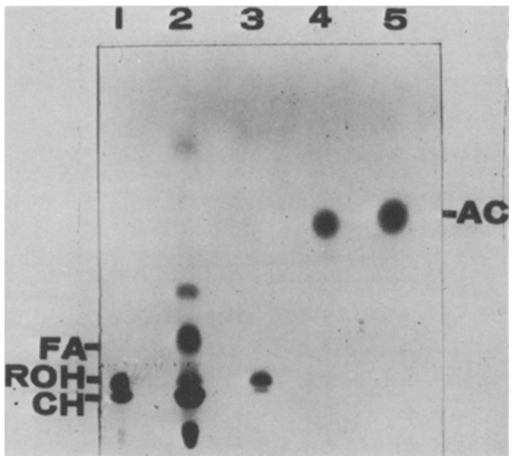


FIG. 1. Thin layer chromatogram of the nonsaponifiable fraction of the neutral lipids isolated from a Walker-256 carcinosarcoma. Chromatography was carried out on Silica Gel G layers in a solvent system of hexane-diethyl ether-acetic acid (80:20:1 v/v). Identification of lanes: 1, standard mixture of fatty alcohol (ROH) and cholesterol (CH); 2, total mixture of nonsaponifiable lipids from tissue; 3, fatty alcohols isolated from the mixture of nonsaponifiable lipids by preparative TLC; 4, acetate (AC) derivative of fatty alcohols purified from the mixture of nonsaponifiable lipids from tissue; and 5, standard-acetate (AC) derivative of hexadecyl alcohol.

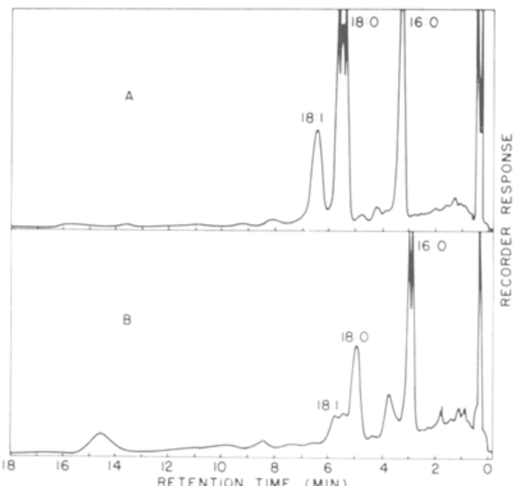


FIG. 2. Gas liquid chromatograms of the acetate derivatives of fatty alcohols isolated from the total lipids of Walker-256 carcinosarcoma (A) and beef heart (B). See Methods section of text for experimental conditions.

first fractionated by silicic acid column chromatography. A glass column, 2.2 cm in diameter, was packed in a hexane slurry to a height of 23 cm with silicic acid (Bio-Sil HA, Bio-Rad Laboratories); the total neutral lipids were applied to the column in 5 ml of hexane. The cholesterol ester plus wax Fraction (a) was collected by elution with 200 ml of 10% $CHCl_3$ in hexane (v/v); the triglycerides (b) were then eluted with 200 ml $CHCl_3$-hexane (1:1 v/v); the fraction containing cholesterol and free fatty alcohols (c) was collected by elution with 300 ml of chloroform. The minute quantities of free fatty acids (corresponding to Fraction c) in normal tissues were not analyzed. This type of column separation did not give pure lipid fractions, but it served to eliminate the major class, triglycerides, so that the smaller amounts of the other classes could be subsequently separated by TLC. Solvents were removed by evaporation under vacuum and the lipids were redissolved in chloroform; the lipid classes were isolated by preparative TLC in the same manner as that described for the lipids isolated from the neoplasms.

Isolation of Fatty Alcohols and O-Alkyl Glyceryl Ethers

Fraction a (cholesterol esters plus waxes) and, in most instances, Fraction d (cholesterol plus diglycerides plus free fatty alcohols) were saponified by addition of 2-3 ml of 2 N KOH in ethanol-water (75:25 v/v); this mixture was heated in a sealed tube at 100 C for 10-15 min. Three milliliters of water were added to the saponification mixture and the nonsaponifiables were extracted three times with 5 ml portions of hexane-diethyl ether (1:1). The pooled hexane-diethyl ether extracts were then washed five times with 10 ml portions of water, dried with anhydrous sodium sulfate, and the solvent was removed under vacuum.

The fatty alcohols were separated from the free cholesterol and traces of free fatty acids by preparative TLC on Silica Gel G with the solvent system of hexane-diethyl ether-acetic acid (75:25:2 v/v). When the ratio of free cholesterol to fatty alcohols was high, double development in this solvent system was necessary to obtain relatively pure samples of fatty alcohols. The fatty alcohols were eluted from the silica gel as described previously.

The glyceryl ether diester fraction, present only in the neoplasms, was contaminated with a significant amount of triglycerides. This fraction was reduced with $LiAlH_4$ and the O-alkyl glyceryl ethers were separated from the other reduction product (fatty alcohols) by TLC (18). The O-alkyl glyceryl ethers were eluted from the silica gel with 15% methanol in diethyl ether.

Preparation of Derivatives

Fatty alcohols were acetylated by heating for 1 hr at 100 C in the presence of 2 ml acetic anhydride and 0.5 ml pyridine. The free fatty acids were methylated by heating for 30 min at 100 C in the presence of 2 ml methanol containing 2% (v/v) sulfuric acid, and the O-alkyl glyceryl ethers were converted to their isopropylidene derivatives (19). The isopropylidene derivatives of the O-alkyl glyceryl ethers and the acetates of the fatty alcohols were purified by TLC on silica gel layers in a solvent system of hexane-diethyl ether (80:20 v/v). The bands were visualized under ultraviolet light after they had been sprayed with an ethanolic solution of dichlorofluorescein (0.1%). The derivatives were eluted from the silica gel with approximately 30 ml of diethyl ether.

Gas Liquid Chromatography

The chain length distribution was determined for each of the derivatives by gas liquid chromatography (GLC). A Victoreen Model 4000, dual hydrogen flame detector unit was fitted with two 6 ft x 1/8 in. columns packed with 10% EGSS-X on 100-120 mesh Gas Chrom P (Applied Science Labs., State College, Pa.). A carrier gas (He) flow of 30-35 ml/min and injection and detector temperatures of 260 C were used for all analyses. The methyl esters, alcohol acetates and isopropylidene derivatives of the alkyl glyceryl ethers were analyzed at column oven temperatures of 180, 190 and 200 C. Peak area measurements were used for calculating the weight percentage of the resolved components. Identification of peaks was made by comparing retention times to known standards.

In two experiments (Walker 256 tumor and beef heart samples), the amount of fatty alcohols was quantitated by using an internal standard. A known amount of 15:0 fatty alcohol, prepared by $LiAlH_4$ reduction of the corresponding methyl ester of the 15:0 fatty acid, was added to the total neutral lipid fraction. The lipid mixture was then saponified and the fatty alcohols were isolated from the nonsaponifiables by TLC. Acetate derivatives of the fatty alcohols were prepared for GLC. A comparison of the 15:0 alcohol acetate peak area with the peaks of the alcohol acetates in the samples provided a quantitative determination of the content of total alcohols present in these two tissues.

RESULTS AND DISCUSSION

The thin layer chromatogram pictured in Figure 1 shows the nonsaponifiables isolated from the neutral lipids of the Walker 256 tumor; it also serves to illustrate the TLC separations accomplished during these analyses. The chromatogram reveals that the fatty alcohol fraction isolated is still somewhat contaminated with cholesterol, but the sterol does not interfere in GLC analysis of fatty alcohol acetates because the acetate derivative of cholesterol is not eluted from the GLC column under these conditions. The fatty alcohols isolated were identified by TLC on the basis of their migration at the same R_f value as known fatty alcohols; moreover, after acetylation, their R_f value corresponds with that of the acetate derivatives of known fatty alcohols. Gas liquid chromatography (Fig. 2) of the fatty alcohol acetates isolated by TLC provided further proof for their identification since cochromatography of the fatty alcohols from the tissues and a standard mixture of fatty alcohol acetates revealed identical retention times.

We also determined whether saponification of other compounds that migrate with or near the fatty alcohols on TLC, e.g., diglycerides, fatty acids or cholesterol, might contribute any artifactual component that could be confused with fatty alcohols. Only alk-1-enyl glyceryl ethers, which can exist as a diglyceride type compound, gave any indication of possible interference with our TLC analyses of fatty alcohols. Therefore, free alk-1-enyl glyceryl ethers were isolated by TLC after $LiAlH_4$ reduction of phosphatidyl ethanolamine that was purified from pig kidney. The products formed by $LiAlH_4$ reduction were acetylated and analyzed by TLC and GLC to see which resembled the fatty alcohol acetates. TLC of the acetylated products showed only a single spot with a much lower R_f value than that for a known alcohol acetate; we believe that it is the diacetate derivative of alk-1-enyl glyceryl ethers. GLC failed to show any peaks under the conditions used for the analysis of the acetates of fatty alcohols.

The neutral lipids from the four normal tissues and three neoplasms investigated were found to contain small but significant amounts of fatty alcohols; they exist as wax esters and what we believe to be in the free form. The free fatty alcohols were obtained from Fraction d which also contained diglycerides and cholesterol. This fraction was usually saponified to remove the diglycerides that had a TLC R_f similar to that of free fatty alcohols. Since the fatty alcohols isolated after saponification of Fraction d could have been derived from wax esters of monohydroxy fatty acids or some other unknown class of lipids, we also carried out a GLC analysis of Fraction d (isolated from

TABLE I

Chain Length Distribution of Fatty Acids, Fatty Alcohols and O-Alkyl Side Chains of Glyceryl Ethers (wt %) in Neoplastic Tissues[a]

Tissue	Chain lengths	Free fatty acids	O-Alkyl glyceryl ether	Free fatty alcohols	Wax alcohol
	16:0	22	39	30	23
	18:0	28	19	51	44
Walker 256	18:1	18	38	19	26
carcinosarcoma	18:2	6	2	Trace	1
	20:4	13	---	---	---
	16:0	23	46	33	35
	18:0	21	20	46	37
Ehrlich	18:1	20	28	7	16
ascites cells	18:2	14	2	1	1
	20:4	5	---	---	---
	16:0	16	37	49	31
Morris	18:0	14	27	22	29
hepatoma 7777	18:1	23	33	15	27
	18:2	12	1	Trace	2
	20:4	15	---	---	---

[a]Only major chain lengths are reported in this Table. Significant quantities (< 5%) of other chain lengths were present in some samples; however, these consisted of only saturated and monoenoic chains for the fatty alcohols.

the Walker 256 carcinosarcoma) that had been directly acetylated; no saponification or purification of the fatty alcohols were done before or after acetylation. This analysis gave the same GLC pattern and quantitation (based on the internal standard) of mass and composition of fatty alcohols that were obtained after saponification. These data indicate that the fatty alcohols of Fraction d are undoubtedly present in the free form of the Walker 256 carcinosarcoma.

On the basis of GLC, the amounts of fatty alcohols appeared to be in the same range for all three neoplasms and about equally divided between wax esters and free fatty alcohols. The normal tissue lipids contained much lower quantities of the fatty alcohols; the fatty alcohols in bovine brain and heart existed entirely in the free form, whereas the small quantities of fatty alcohols in rat liver were present entirely as wax esters. The preputial gland, a rich source of waxes (2), also contained free fatty alcohols.

The neutral lipids of the Walker 256 tumor and beef heart were analyzed quantitatively for total alcohols by addition of an odd chain fatty alcohol as an internal standard. These measurements indicated that the neutral lipids of the tumor contained 0.3% (w/w) fatty alcohols. Since five to ten times more neutral lipid had to be used to even demonstrate the presence of alcohols in the normal tissues, only 0.1% of the odd chain alcohol was added as the internal standard to the neutral lipids of beef heart. The GLC analyses of the isolated alcohol acetates revealed that even this small amount of internal standard made up more than 90% of the total peak areas detected. These data indicate that the beef heart lipids contained approximately 0.01% fatty alcohols in the neutral fraction.

The extremely low levels of fatty alcohols in the normal tissues precluded any quantitative determinations of their chain length distribution, although it was possible to identify the presence of the three main peaks, 16:0, 18:0 and 18:1 (Fig. 2). Although the Walker 256 tumor contained only trace quantities of other chain lengths, the Ehrlich ascites cells and the Morris hepatoma 7777 did contain small amounts (< 5%) of longer chain saturated and monoenoic fatty alcohols. The chain length distribution patterns of the larger amounts of fatty alcohols found in the tumor lipids were quantitated and compared to the O-alkyl glyceryl ether side chains and the free fatty acids from the same tumors. The results of these analyses are summarized in Table I. The fatty alcohols and the alkyl chains of the glyceryl ethers consisted of the same three major chain lengths, 16:0, 18:0 and 18:1, whereas the free fatty acids also contained significant quantities of 18:2 and 20:4 carbon chains. Essentially no polyunsaturated chains

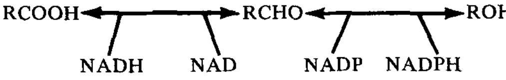
were found in the fatty alcohol or the O-alkyl glyceryl ether fractions.

The possibility that the polyunsaturated fatty alcohols were not detected by TLC because of their lower R_f value was also investigated. Unsaturated fatty alcohols (obtained after $LiAlH_4$ reduction of highly unsaturated phosphatidyl ethanolamine from pig kidney) were combined with 10 times the amount of cholesterol and separated as previously described. Fatty alcohols present in the mixture were resolved from the cholesterol by TLC. The acetate derivatives were prepared, and GLC analysis demonstrated that even though the added fatty alcohols contained >40% of the 20:4 chain length, they were separated from the cholesterol because the same chain length distribution was found in the fatty alcohols isolated after TLC as that found in the original mixture.

Our results are in agreement with the known precursor-product relation (13) between the fatty alcohols and the O-alkyl glyceryl ether side chains. This relation was first suggested because of the similarity of double bond locations in the alkyl chains and the fatty alcohols (2).

In spite of the qualitative similarity in the distribution pattern of the three major chain lengths found in the fatty alcohols and the O-alkyl glyceryl ethers, quantitative differences do exist. The fatty alcohols contained a somewhat higher ratio of 18:0 to 18:1 or 16:0 than did the glyceryl ethers (Table I). These data imply some selectivity in the use of fatty alcohols for alkyl glyceryl ether biosynthesis. This is borne out by our recent enzymic studies on the incorporation of fatty alcohols of different chain lengths into the O-alkyl moieties of glyceryl ethers, e.g., octadecyl alcohol was not incorporated as rapidly as hexadecyl alcohol (20).

The origin of fatty alcohols is not yet known, although the following interconversions have been postulated (12,15) on the basis of earlier investigations in rat liver (14-16) on the cleavage of O-alkyl glyceryl ethers to fatty aldehydes and glycerol.



The fatty alcohols, particularly those found in liver, could originate from the cleavage of the O-alkyl glyceryl ethers. However, since activities of the cleavage enzyme are very low or absent (21) in the neoplasms that we have investigated, fatty alcohols in these cells are probably derived via a mechanism other than cleavage of the ether bond.

ACKNOWLEDGMENT

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Gas Liquid Radiochromatography of Intact Natural Triglycerides[1]

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ABSTRACT

Gas liquid radiochromatography was successfully applied to the simultaneous analysis of mass and radioactivity of intact standard and natural triglycerides labeled with ^{14}C in their fatty acid or glycerol moieties. Quantitative measurements of mass and radioactivity were made with a gas chromatograph equipped with dual glass columns, stream splitters, combustion train, and a proportional gas counter. Under optimum conditions, as little as 500 cpm/peak of tristearin could be detected with a relative error of 10%. At higher counting rates the error was less than 5% and the achieved resolution of radioactivity was only slightly inferior to that of the mass. The counting efficiency of the system was better than 90%. The study showed that the detected radioactivity was proportional to the mass and not the moles of the labeled triglyceride measured in the hydrogen flame ionization detector. Practical application of the system is limited by the need for samples of relatively high specific activity (500-1000 dpm/50 μg/peak).

INTRODUCTION

Over the last few years gas chromatography of intact natural triglycerides has emerged as a useful tool for lipid research (1). The original conditions of separation have since been modified and improved by several laboratories (2-8) and the technique has now been extended to include triglycerides with molecular weights exceeding 1000. The true recovery of the long chain triglycerides from the gas chromatographic columns, however, has not been established. Indeed, it has been suggested (4,7) that the response of the flame ionization detector to the long chain glycerides (C_{54}-C_{66}) approximates the mole rather than the weight proportions commonly observed for the short and medium chain length glycerides (C_{36}-C_{54}). Furthermore, poorly conditioned columns or good columns under improper operating conditions have given incomplete recoveries of most glycerides and it has been impossible to determine the fate of the unrecovered lipid or the reason for the low detector response.

In the present study comparisons were made of the proportions of mass and radioactivity in the starting materials and of the peak area and radioactivity in the effluents of the gas chromatograph using ^{14}C-labeled triglycerides. The results confirmed earlier claims (9) that the response of the hydrogen flame ionization detector to triglycerides is related directly to their mass or combustible carbon content. For this demonstration we employed a radio gas chromatograph similar to that described by Swell (10).

MATERIALS AND METHODS

Reagents, Standards and Samples

All solvents and chemicals were of reagent grade quality and were used without further purification. High purity (99%) monoacid triglycerides, monoglycerides and free fatty acids were purchased from Applied Science Laboratories, Inc., State College, Pa. Radioactive monoacid triglycerides labeled with ^{14}C in the carboxyl carbon of the fatty acid and 1-^{14}C-fatty acids were obtained from New England Nuclear Corporation, Boston, Mass.

Radioactive tridecanoin to be used as internal standard was made by reacting the chloride of 1-^{14}C-decanoic acid with glycerol (11). A similar method was used for the preparation of trierucin from the chloride of erucic acid and 2-^{14}C-glycerol. Radioactive mixed triglycerides were formed by incubating evert sacs of rat small intestine with micellar solutions containing 1-^{14}C-fatty acids, 1-monoolein and taurocholic acid (12). The lipids were extracted by the method of Bligh and Dyer (13) and were purified or resolved, or both, by standard methods of thin layer chromatography (TLC) (14). The triglycerides were isolated on thin layers of Silica Gel G (Merck & Co.) using heptane-isopropyl ether-acetic acid (60:40:4). The glycerides were further resolved on the basis of unsaturation on thin layers of Silica Gel G impregnated with silver nitrate (20%) using chloroform-methanol (99.5:0.5) as the

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[1]Presented at the AOCS-AACC Joint Meeting, Washington, D.C., April 1968.

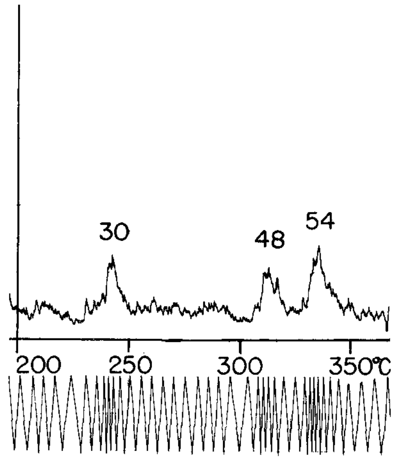

FIG. 1. Radio gas chromatogram of standard triglycerides at signal-noise ratio of 2. About 0.5 μg containing 300 dpm of each triglyceride was injected. Triglycerides identified by the total number of acyl carbons. The chromatographic system and operating conditions were as given in the text.

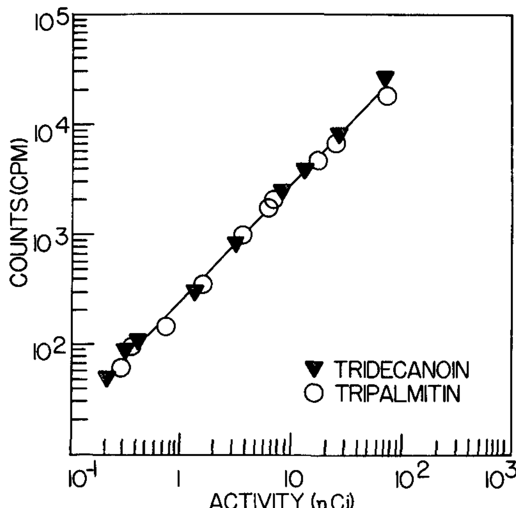

FIG. 2. Linearity of the radio gas chromatographic system: ○, tripalmitin; ▲, tridecanoin. Chromatographic conditions as described in the text.

developing solvent. The triglycerides were recovered from the gel by elution with diethyl ether. The extracts were suitably diluted with internal standard and taken to dryness. For gas chromatography the residues were dissolved in CS_2.

Gas Liquid Radiochromatography

A barber-Colman 5000 Series Radio Gas Chromatograph (Barber-Colman Co., Rockford, Ill.) equipped with dual glass columns, dual hydrogen flame ionization detectors, a differential electrometer, stream splitter, combustion train and a proportional radioactive gas counter was modified as follows. The red rubber stoppers at the effluent end of the glass columns and in the front of the quartz combustion tube were replaced with glass to metal Kovar seals (15). A Swagelok fitting was placed on the metal tube at the end of the column and this was attached by means of a reducer to the 1/16 in. transfer line. At the front of the combustion tube a 1/16 in. stainless steel tube was silver-soldered to the metal part of the Kovar seal and this was attached to the stream splitter by means of a Swagelok coupler. A similar splitter system was attached to the end of the reference column by means of another Kovar seal in order to compensate for the column bleed in the hydrogen flame ionization detector. All the connections were made so as to reduce to a minimum any dead volumes or sharp elbows in the line. Finally the length of the transfer line leading from the end of the combustion tube to the counter was cut to an absolute minimum to reduce the diffusion of $^{14}CO_2$, which would have otherwise led to poor peak resolution.

The chromatographic separations were made with glass columns (2 ft x 1/8 in. i.d.) packed with 3% JXR on Gas Chrom Q (100-120 mesh) supplied by the Applied Science Laboratories, Inc. The columns were conditioned at 350 C for 2 hr using argon flow of 150 ml/min. At the end of this time the columns were tested for recoveries of the mass of standard triglycerides (C_{30}-C_{54}). If the recovery of tristearin with respect to tridecanoin was less than 90% the columns were conditioned for an additional 2 hr at 350 C' If after this or one more conditioning the columns still failed to give better than 90% recovery of the mass of tristearin they were discarded. Other operating conditions were as follows: injector, 310 C; detector, 340 C; transfer line, 325 C; combustion tube, 700 C; column flow, 130 ml/min of argon; flame detector flow, 12-15 ml/min of argon; counter tube flow, 115 ml/min of argon; propane flow, 10-11 ml/min. The high voltage source was operated at 1750 volts. The columns were temperature programmed from 200-325 C at 5 C/min. The flame detector response was recorded with an Electronik 15 1 mv recorder (Minneapolis-Honeywell Regulator Co., Philadelphia, Pa.) and a Barber-Colman PR 125 recorder served to monitor the response of the radioactivity counter. Peak areas were measured

TABLE I

Operating Efficiency of Counting of the Complete Radio Gas Chromatographic System

Radioactivity combusted[a] (dpm)	Radioactivity detected: Theoretical[b] (dpm)	Radioactivity detected: Experimental[c] (cpm)	Efficiency (E)
8920	1510	1330	88.1
6750	1140	1040	91.2
5110	860	780	90.7
3530	550	500	90.9

[a]The amount of tripalmitin injected ranged from 10-40 μg. The mass and radioactivity of the combusted triglyceride was calculated from the known total radioactivity injected and the splitter ratio, which was 10:1 in favor of the combustion train.

[b]The theoretical rate of counting was calculated from the total radioactivity combusted, the flow rate of the carrier (130 ml/min) and the detector volume (22 ml).

[c]The experimental value was obtained from the disc integrator record of the detected area of the radioactive peak.

by Disc integration or by triangulation to an accuracy of ±4% or better.

Limit of Detection

A practical limit of detection of radioactive triglycerides was determined for the entire system. This was accomplished by injecting progressively decreasing amounts of the triglyceride and estimating the activity that gave a signal-noise ratio of 2 (16).

Repeatability

Replicate injections of tripalmitin and tristearin were made in the presence of internal standard, and standard deviations were calculated from the ratios of the areas of the radioactive peaks. The amounts and activity of the triglycerides injected ranged from 10,000-100,000 dpm/10-100 μg in 1-5 μl of CS_2.

Linearity of Counting

Stock solutions of tridecanoin (spec. act. 133,000 dpm/mg) and tripalmitin (spec. act. 550,000 dpm/mg) were evaporated to dryness and dissolved in sufficient carbon disulfide to give solutions containing 300-150,000 dpm/1-5 μl of CS_2. Duplicate injections of each sample were made and the average values of radioactivity detected were plotted against the amounts injected. Identical aliquots were transferred to counting vials and the radioactivity determined by scintillation counting. All tests were done under optimum working conditions, which required no correction for losses of triglyceride on the column.

Efficiency of Counting

The efficiency of the proportional gas counter was determined by injecting a known amount of radioactive triglyceride (N) and recording the detector response (A) under optimum conditions of operation of the entire gas chromatographic system. The operational efficiency (E) was calculated on the basis of the split ratio (S), the flow rate through the detector (F) and the volume of the detector (V), as well as the recovery (R) of the triglycerides from the chromatographic column. The calculation may be summarized as follows: $E = A \times F/V \times R \times S \times N$. Under the test conditions the flow rate was 130 ml/min, the detector volume was 22 ml and the recovery of the triglyceride mass was 100%. The amounts of total triglyceride injected ranged from 10-50 μg and the total number of counts varied from 3000-10,000 dpm.

Contamination

The extent of radioactive contamination of the gas chromatographic column, the oxidation chamber and the counting system was determined by comparison of background count rates taken before and after elution of various loads of labeled triglyceride.

Scintillation Counting

Radioactivity was assayed in a Nuclear Chicago Liquid Scintillation System (720 Series). The lipid residue was dissolved in 0.5 ml of toluene and 10 ml of the scintillation solution containing 0.5% PPO and 0.03% POPPO in toluene added and the solutions counted. The counts were corrected for quenching using callibration standard.

RESULTS AND DISCUSSION

Operating Conditions

The general layout of the analytical system

TABLE II

Determination of Specific Activity of Standard Triglycerides by Radio Gas Chromatography

	Relative recoveries[b]		Specific activities	
Triglycerides[a] tested	Mass (%)	Radioactivity (%)	Calculated (dpm/mg)	Determined (cpm/mg)
48	98.8	100.2	199,900	201,900
54	100.7	102.4	185,800	189,700
48	99.5	97.8	199,900	197,300
54	99.7	98.4	185,800	185,200
48	99.1	97.7	70,300	69,500
54	97.7	99.0	118,500	120,700
48	100.1	97.4	70,300	69,100
54	100.8	101.6	118,500	118,900

[a]Equal weight mixtures of tridecanoin (30), tripalmitin (48) and tristearin (54) were injected each time at two different levels of concentration.

[b]Relative recoveries of mass and radioactivity of test triglycerides were determined in relation to the tridecanoin used as internal standard.

has been discussed by Swell (10). For continuous high temperature operation it was essential to replace with Kovar seals the rubber septae at the effluent end of the columns and at the front of the combustion tube. The resolution of the triglyceride peaks was best when the diffusion of $^{14}CO_2$ was minimized by reducing as far as possible the length of the transfer line between the end of the combustion tube and the counter. Erroneous radioactive responses a few seconds after injection were eliminated by substituting carbon disulfide or chloroform for petroleum ether as the solvent of triglycerides. The false response was believed to be due to the rather large flash volume of petroleum in the combustion tube which would cause a surge of carrier gas and a change in the ratio of carrier gas to quench gas with concomitant increased ionization in the counter. Chloroform did not cause this but since copper chloride was formed in the combustion tube it tended to clog the 1/16 in. tube leading from the combustor to the counter tube. Carbon disulfide was best since it was converted to CO_2 and SO_2. As a result of these alterations in instrumentation and sample handling, it was possible to obtain gas chromatograms of triglycerides which differed little in the tracings of their mass and radioactivity. The inclusion of the splitter in the effluent stream, however, resulted in some tailing which made both the mass and the radioactivity patterns of the glycerides slightly inferior to those recorded without the splitter.

Limit of Detection

The lowest limit of detection of radioactivity depends upon the level of signal required to barely discern the peak from the background noise. As a realistic practical limit, Penton and Hartman (16) have suggested that signal which produces a signal-noise ratio of 2. In our experiments this required about 300 dpm/peak of triglyceride. Figure 1 shows a radio gas chromatogram obtained for tridecanoin, tripalmitin and tristearin at this level of activity. The random noise is about 5 chart units above the electrical zero, while the tops of the peaks project to about 10-13 units. At this level the detection of the radioactive peaks does not depend on the observer's experience or the prior knowledge of the approximate time of elution. These peaks, however, are characterized by large base widths and a relatively poor resolution that is due to an instrument setting at low probable error, which is necessary in order to get a reliable detection. In certain instances the sensitivity of the detection can be further increased by lowering the carrier flow, but this alteration in the working conditions is not compatible with an effective recovery of the majority of the triglycerides.

Repeatability and Linearity

The repeatability or reproducibility of the system was determined by injecting various quantities of tripalmitin and tristearin and assessing the recoveries of the radioactivity in relation to that of tridecanoin used as internal standard. At levels of 300-500 dpm/1-2 μg/peak of triglyceride the relative error of measurement was about 20%. At levels of 1000 dpm/5 μg/peak of triglyceride, the error was reduced to about 5%, with a further increase in accuracy and reproducibility at higher counting rates (3% error at 4500 dpm/5 μg/peak).

TABLE III

Radio Gas Chromatographic Analysis of Natural Mixtures of Triglycerides[a]

Triglycerides[b]	Saturates[c]	Monoenes[c]	Dienes[c]	Reconstituted[d]	Original[e]
42	33.6			16.2	16.8
44	45.0			21.7	18.7
46	17.8	46.8	22.4	30.1	28.0
48	3.6	40.6	24.1	20.8	21.2
50		12.6	30.9	8.6	10.9
52			22.5	2.5	4.4
Total	48.3	40.5	11.2	100.0	100.0

[a]Total cpm, %.

[b]Triglycerides identified by total number of acyl carbons.

[c]Saturates, monoenes and dienes represent triglyceride groups with 0, 1 and 2 double bonds, respectively, per triglyceride molecule.

[d]Values obtained by proportional summation of the estimates of triglycerides in the various fractions.

[e]Values obtained by analysis of the total sample before fractionation.

Figure 2 shows a plot of the amount of radioactivity injected versus the amount of activity detected for tridecanoin and tripalmitin under optimum conditions of operation. Excellent linearity is seen for both triglycerides in the range 3000-150,000 dpm/peak. Since the dead-time of the counter (counter tube and rate indicating circuitry) was about 1 microsecond, the coincidence loss was negligible even at the highest counting volumes. Mixed triglycerides of varying specific activities gave similar plots which passed through the origin in the 0-100 μg mass range. The slope of the line varied somewhat with the column and the operating conditions, which suggested that the recoveries of the mass also varied.

In the course of the studies of the reproducibility and linearity, it became apparent that the radioactive monitoring system was not subject to any significant radioactive contamination by any incompletely combusted carbon residues. Observation of the background count rate made at the completion of the linearity study indicated that the base line had risen to no more than 80 cpm. Under the usual working loads with essentially complete recoveries of mass, the background count rate was 30-50 cpm.

Efficiency of Detection of Radioactivity

The absolute counting efficiency of the radio-detector may be defined as the ratio of the count rate in counts per minute observed within the sensitive volume of the detector over the radioactivity of the sample in disintegrations per minute times 100. For gas counters this has been estimated (17) to be close to the theoretical efficiency of 95%. For the present purposes an operational efficiency of the detection system was assessed by calculating the ratio of radioactivity in counts per minute, as determined after correcting for the flow rate and the split ratio, over the radioactivity of the sample admitted to the chromatographic system. Table I gives the results of four injections of tripalmitin over a threefold range of concentration. The calculated average efficiency is slightly over 90%. Identical results were obtained with ^{14}C-labeled tridecanoin. Assuming that the detector was operating at its maximum theoretical efficiency (95%) this data would indicate that less than 5% of the radioactive triglyceride remained unaccounted for. These results are in good agreement with those of Swell (10) who observed 90-95% efficiencies for cholesterol esters under comparable working conditions.

Relation of Peak Area to Mass and Radioactivity

For this purpose we compared the mass and radioactivity tracings obtained in the radio gas chromatograph for a mixture of simple triglycerides labeled with ^{14}C in the carboxyl carbon of the fatty acids. Each peak represented approximately 1000 dpm and 5 μg of triglyceride. Table II gives the recoveries of mass and radioactivity calculated from several such chromatograms obtained using different solute proportions. The calculations were based on comparisons of the peak areas recorded in the mass and radioactivity detectors. In relation to tridecanoin, which was assumed to be quantitatively recovered, the recoveries of the mass of tripalmitin and tristearin varied from 97.8% to 100.8%, while the recoveries of their radioactivities ranged from 97.4% to 102.4%. Table II also compares the specific activities (dpm/mg) of the tripalmitin and tristearin as determined by radio gas chromatography and by the method of preparation of the labeled compounds. In all cases there was an excellent

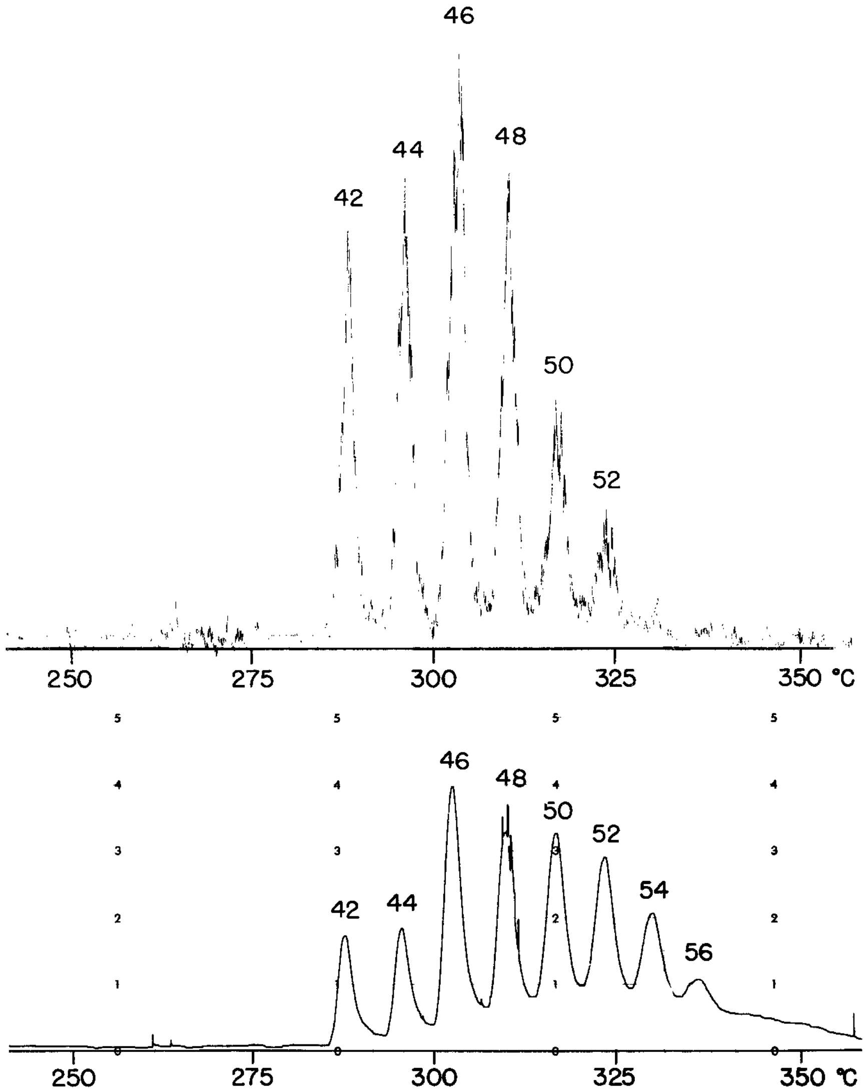

FIG. 3. Comparative recoveries of mass and radioactivity of a natural mixture of triglycerides in the radio gas chromatograph. Peak identification and gas chromatographic conditions as in Figure 1. Sample: triglycerides synthesized by rat intestine from ^{14}C-labeled myristic and palmitic acids and 1-monoolein; 10,000 dpm of total radioactivity injected.

agreement between the two sets of data. These results indicate that essentially all of the triglycerides were recovered from the column, and that the response of the flame ionization detector to these compounds is very close to their mass.

Table III relates the recovery of radioactivity in the total triglyceride sample to that estimated by means of a radioactive internal standard for the subfractions derived from argentation TLC. The triglyceride sample was derived from an incubation of 1-^{14}C-myristic and 1-^{14}C-palmitic acids and 1-monoolein with evert sacs of rat intestine (12). Figure 3 gives the mass and radioactivity patterns recorded for the total. The mass peaks range from 42-56 acyl carbon atoms, while those of radioactivity cover peaks with 42-52 acyl carbons. The mass pattern shows considerable base line elevation, which is due to a lack of reference column in the mass detector side in this particular run. The total radioactivity injected was about 10,000 dpm, and the pattern of elution of the activity was easily reproduced within a relative

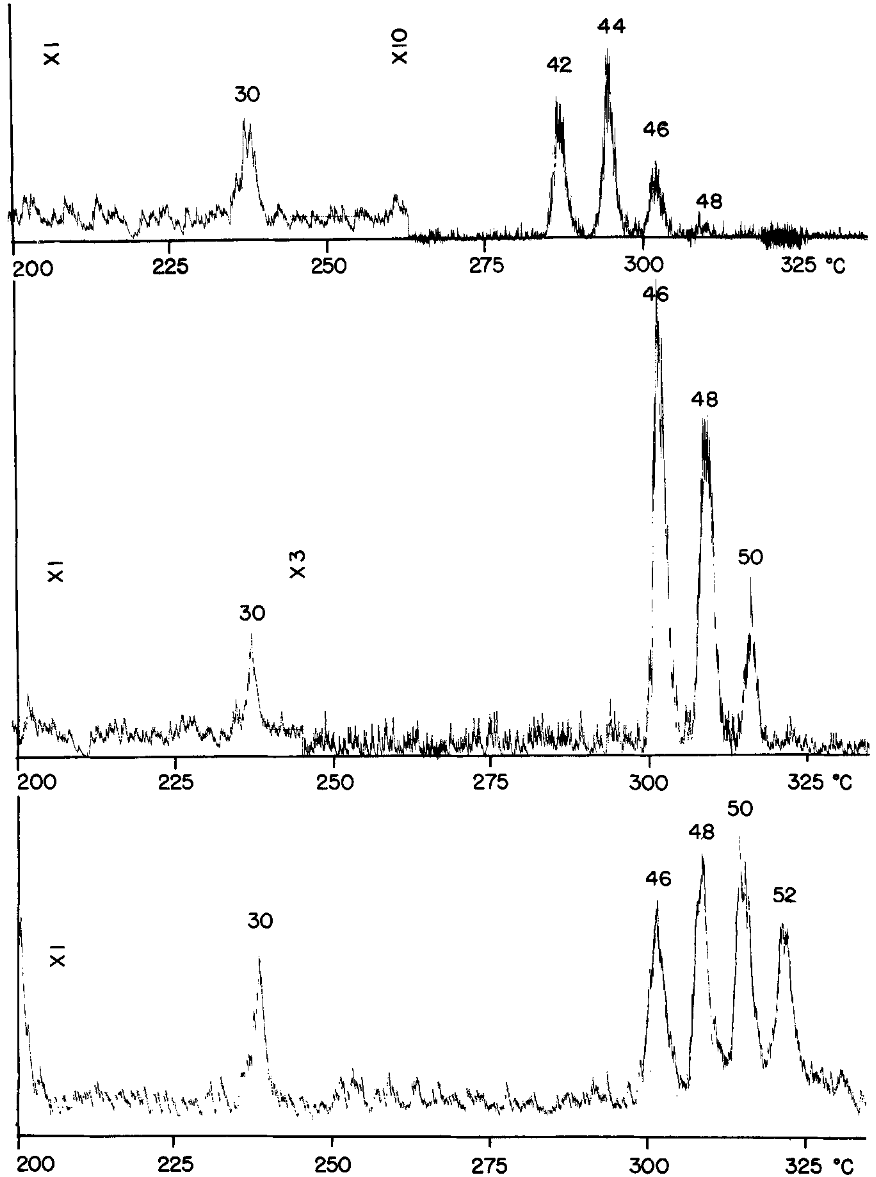

FIG. 4. Radio gas chromatographic analysis of a natural mixture of triglycerides. Peak identification and gas chromatographic conditions as in Figure 1. A, saturates; B, monoenes; C, dienes. The total radioactivity injected ranged from 8,000 to 15,000 dpm.

error of 5%, which was the working range. Following fractionation on silver nitrate plates, the radioactivity associated with each triglyceride fraction was determined by gas chromatography in the presence of a known amount of radioactive internal standard. The tracings of the radioactivity obtained are shown in Figure 4. All the radioactive peaks present in the original sample are recovered and completely resolved. As seen from Table III, the saturates made up 48.3%, the monoenes 40.5% and the dienes 11.2% of the total. These estimates compared very favorably with those derived from scintillation counting of the appropriate thin layer bands. The reconstitution values in Table III were obtained by proportional summation and normalization of the radioactivity data recorded for the individual fractions. Although imperfect, the agreement between the radioactivities of the various molecular weight

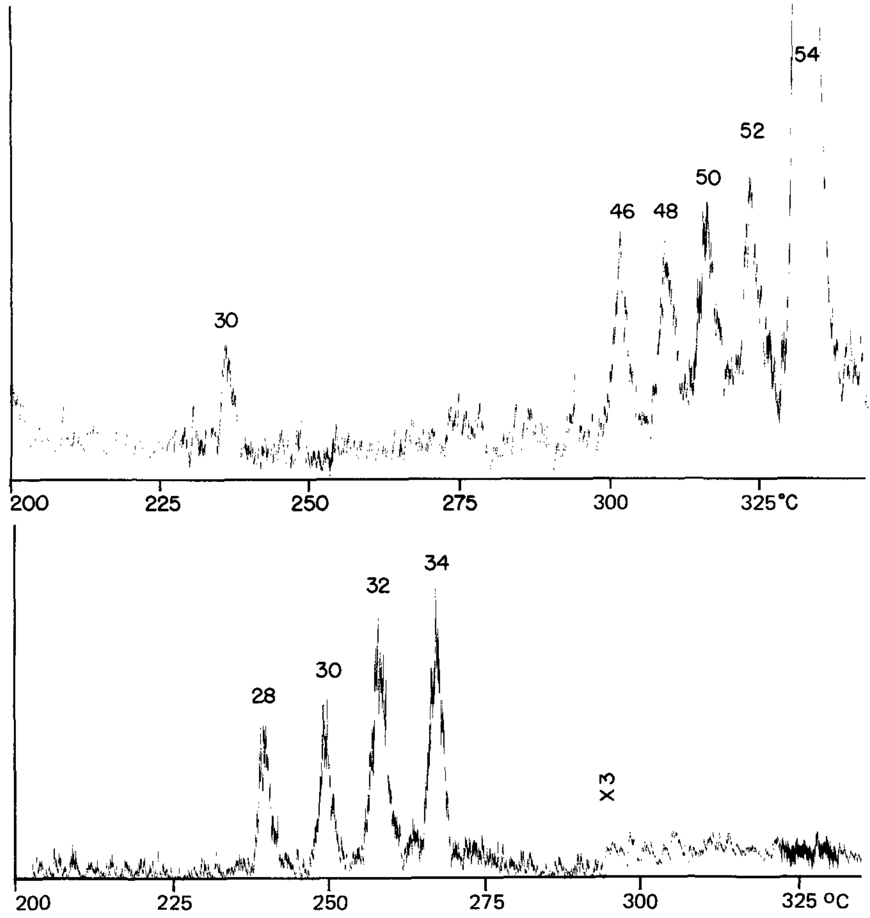

FIG. 5. Radio gas chromatographic analysis of natural mixtures of glycerides containing tristearin and free diglycerides. Peak identification and gas chromatographic conditions as in Figure 1. A, triglycerides of rat mucosa plus excess of tristearin; B, free diglycerides of rat mucosa.

groups in the original and reconstituted mixture is of the order commonly observed for mass analyses (1). Part of the discrepancy in these estimates could be due to the rather low proportion of the internal standard added to the monoene and diene bands, which could be increased.

Figure 5 shows that the system is capable of equally effective resolution and quantitation of natural triglyceride mixtures containing components with a total of 54 acyl carbon atoms, as well as of free diglycerides. The upper radio gas chromatogram was obtained with an incubation mixture similar to that shown in Figure 3, except that a known excess of ^{14}C-labeled tristearin was added. Determination of the peak areas of suitably attenuated runs showed that the fivefold excess of this glyceride not only was correctly recovered but that it had no effect on the recovery of the radioactivities in the other glyceride peaks. The lower chromatogram shows the resolution obtained for the free diglycerides isolated during triglyceride biosynthesis from 1-^{14}C-fatty acids in the rat intestine. Similar patterns have been recorded for the diglycerides released from glycerophosphatides, labeled in the fatty acid moieties, following digestion with phospholipase C (Kuksis et al., in preparation). A simultaneous determination of both mass and radioactivity for each diglyceride peak has allowed the estimation of their specific activity, and has thus provided an indication of the turnover of individual molecular species of these phosphatides, which has been lacking in previous analyses (18). The acetates and silyl ethers of diglycerides are also satisfactory for quantitative radio gas chromatography. These derivatives may be preferred for columns which give low recoveries of free diglycerides. The free diglycerides, their acetates

TABLE IV

Recovery of Mass and Radioactivity During Incomplete Elution of Triglycerides From Gas Chromatographic Columns[a]

Triglycerides	Mass (total %)	Radioactivity (total %)	Specific activity of triglycerides	
			Original	Recovered
Tripalmitin	79.3	79.8	199,900	201,200
	85.1	84.8	199,900	198,300
	89.8	90.8	199,900	202,120
Tristearin	71.6	68.0	185,800	176,500
	71.7	67.8	185,800	175,700
	74.9	74.5	185,800	184,800
Trierucin	29.5	28.1	112,500	107,200
	23.3	24.4	112,500	117,800

[a]The total amounts of radioactivity injected ranged from 4,000-10,000 dpm/peak. Recoveries of mass and radioactivity were computed by reference to labeled tridecanoin used as internal standard of mass and radioactivity. It was assumed that the whole sample was injected each time and that the tridecanoin was completely recovered.

and silyl ethers all have nearly identical retention temperatures on the short silicone columns.

The resolution of these complex mixtures of radioactive triglycerides into their components and a satisfactory subsequent reconstitution indicates clearly that the response of the radioactivity monitoring system is additive over a wide range of carbon numbers and unsaturation, as well as the mass and total activity. With increasing molecular weight of the triglyceride the recovery of the radioactivity decreases in proportion to the decrease in the recovery of the mass, as shown in Table IV. In all cases examined the recoveries of the mass and radioactivity corresponded closely giving specific activities which were identical to those calculated for these compounds on the basis of the method of preparation. The response of the hydrogen flame ionization detector is therefore closely related to the mass of all triglycerides. These observations are in agreement with the results of preparative gas chromatography (19), where it has been shown that the weight of the collected triglyceride is proportional to the area response in the flame detector. The apparent molar proportions of the peak area response noted for mixtures of very long chain triglycerides (4,7) must therefore represent incomplete recoveries of the mass from the column and not some change in the combustibility of these glycerides in the flame ionization detector.

Fate of Unrecovered Triglyceride

In order to determine the fate of the triglycerides which failed to appear in the column effluent in the proportion anticipitated on the basis of their weight in the injection mixture, a series of experiments was conducted under a variety of operating conditions, and the column packing extracted with the usual lipid solvents. The recovered radioactivity was measured by scintillation counting and the compounds responsible for it were identified by TLC and GLC. Table V gives the recoveries of radioactivity from different sections of various columns. On Column A, a single run with ^{14}C-labeled tristearin was made at a suboptimal flow rate (60 ml/min). As judged from the detector response only about 70% of the injected activity passed through the column as a peak. The bulk of the missing 30% was found in the first 6 in. of the column packing. There were traces of activity found in all segments of the column with a significant enrichment in the final 2 in. which also included the siliconized glass wool plug. An identical distribution of radioactivity was found for another column which gave poor recoveries (75%) of tristearin due to improper packing or conditioning, or both. The distribution pattern of the trierucin not completely recovered from Column B was only slightly different. Despite the well conditioned column and the high flow rates of the carrier gas (150 ml/min), much of this triglyceride was retained at the front of the column, with less than 50% of the injected activity appearing in the effluent as a symmetrical peak. Column C was subjected to a total of 10 injections of trierucin each of which was followed by the usual temperature programming until the trierucin peak was recovered. Under these conditions a considerable build-up of radioactivity occurred with the characteristic distribution pattern observed previously. Following a single injection of trierucin in Column D after 6 hr of conditioning, the mass recovery was only 20%, which suggested that 80% of the

TABLE V

Distribution of the Radioactivity Remaining on the Column[a]

Section of column (in inches)	Type of column and tests[b]					
	A	B	C	D	E	F
Glass wool plug	950		1510		30	
0 - 2		380	520	1560		
2 - 4	480	185	480	650		
4 - 6	490	100	355	390	30	
6 - 8	150	70	350	320		
8 - 10	150	70	250	280		
10 - 12	120	72	240	370	15	
12 - 14	150	50	200	220		
14 - 16	140	60	200	200		
16 - 18	130	65	210	170		
18 - 20	390	60	200	140	30	
Glass wool plug		0	100	80		

[a]Radioactivity was determined by scintillation counting of lipid extracts of various sections of the chromatographic packing. Rechromatography of the extracts indicated that the activity was associated with undegraded triglycerides.

[b]Column A, well conditioned packing, 70% of injected tristearin recovered due to low flow rate of the carrier gas (60 ml/min); Column B, newly conditioned column, 30% of injected trierucin recovered at optimum flow rates; Column C, well conditioned column, 10 injections of trierucin giving less than 50% recovery each; Column D, newly conditioned column, maintained at 350 C for 20 min following an initial recovery of 20% of the injected trierucin; Column E, thoroughly conditioned column giving complete recoveries of tridecanoin, tripalmitin and tristearin after more than 100 separate injections of radioactive triglycerides of 2000-10,000 dpm/injection.

activity was retained on the column. In order to displace this activity, the column was heated at 350 C for 20 min before extracting the packing. As can be seen from Table V, much of the radioactivity still remained on the column, even though the time of additional heating exceeded more than four times the retention time of trierucin at this temperature. On the other hand, a thoroughly conditioned Column E, which had given complete recoveries of triglycerides including tristearin and which had been used for over 100 injections of radioactive glycerides each containing 2000-10,000 counts, gave a background of about 30 counts per 2 in. of packing. In all cases tested the recovered radioactivity was due to undegraded triglycerides.

In the past a variety of explanations have been advanced to account for the incomplete recoveries of high molecular weight triglycerides from gas chromatographic columns. Several of these can now be eliminated as a result of the above observations. It had been suggested (4,20) that as the molecular weight of the triglycerides increases at some point the molecules become so large and so nonvolatile that they enter the column as a stable aerosol which is not delayed by the liquid phase. Since in the present study the missing triglyceride was found mainly at the front of the column the aerosol must have become quickly precipitated if indeed the glycerides entered the column in this form at all. The above data also exclude the possibility that the glycerides were degraded either in the injector or on the column. Degradation during injection should have led to the appearance of radioactivity in the solvent front, which was not the case. Should the degradation have taken place on the column little or no residual activity would have been anticipated on the packing since the degradation products would have been immediately volatilized and lost.

Watts and Dils (7) have recently favored differential adsorption of triglycerides as a possible explanation of the losses on the column. It is not immediately obvious, however, why simple adsorption would produce the pattern of retained activity seen in Table V. Furthermore, the greatest losses are experienced with the higher molecular weight triglycerides, which are the least polar ones, as demonstrated by TLC (21). It would seem more likely that at the temperatures of gas chromatography all triglycerides are initially detained on the packing to about the same extent, but that those with the higher molecular weights are partitioned more slowly. Under improper operating conditions not all of the material admitted to the column would appear with the rapidly migrating front of the main component. Such an effect would explain both the peculiar distribution of the

triglyceride remaining on the column and the recovery of each triglyceride as a function of the uniformity and thickness of the coating of the liquid phase and of the flow rate of the carrier gas.

Practical Consequences

Since columns which give complete recoveries of all glycerides in a sample are only infrequently prepared it is customary to apply small correction factors to adjust the peak areas for losses on column. In a few instances (4,7,20), however, the applied correction factors have been large enough to suggest losses of up to 50% of the material. According to the present study, this missing material is still on the column by the end of the run. While appropriately spaced injections could still yield valid results, closely sequenced runs with rapid cooling of the column will lead to build-up of triglyceride on the column and an eventual appearance of false peaks. In preparative gas chromatography this would result in serious contamination of the collected peaks. With radioactive triglycerides there would be a gradual increase in the background count-rate of the column. It is also possible that as a result of the accumulation of the glyceride in the column, there would be a change in the recovery of the peaks, requiring a change in the peak area correction factors. It would therefore seem to be a good practice to allow extra time for venting of the trailing glycerides through the detector at the maximum column temperature to avoid contamination of the subsequent samples.

Since the resolution of the column increases as the square root of the column length (22), while the recovery decreases proportionally, there is little to be gained by increasing the column length beyond 2 ft which is the length necessary to effect adequate resolutions based on carbon number.

Effective gas chromatography of high molecular weight triglycerides requires thin and extremely uniform coatings of liquid phase. Lack of uniformity in the coating due to improper application or poor conditioning of the column leads to distortion and incomplete recovery of the solute. The larger the proportion of the more heavily coated particles in a packing the greater is the loss of triglyceride at any given flow rate.

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Changes in the Structure of Soybean Triglycerides During Maturation

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ABSTRACT

Soybeans of the Hawkeye variety were picked at eleven periods from 30 to 111 days after flowering and extracted with chloroform-methanol. The triglyceride fraction of five pickings, selected 35 to 91 days after flowering (when synthesis of lipid was most active), were isolated by silicic acid thin layer chromatography (TLC) and species composition determined using argentation TLC and lipase hydrolysis. The triglyceride content of the total lipid increased from 6.5% at 30 days after flowering to 85% in the mature bean (111 days). The major changes in fatty acid composition of the triglycerides occurred during the first 52 days after flowering. During this period linolenic acid decreased from 34.2% to 11.7%, the percentages of linoleic and oleic acids increased, stearic remained fairly constant and palmitic decreased slightly. Large quantitative changes occurred in the molecular species of the triglycerides of the bean during maturation; some triglycerides containing linolenic acid could not be detected approximately 66 days after flowering. Although changes occurred in the percentage and amount of each triglyceride species, the positional distribution of fatty acids remained virtually unchanged throughout maturation. Linolenic acid was distributed fairly uniformly between the β-position and the α-positions, linoleate favored esterification in the β-position, and oleate the α-positions. Most of the stearic and palmitic acids were esterified in the α-positions. The consistency of the positional arrangement of the fatty acids indicated that the mode of glyceride synthesis was established very early during maturation and molecular species composition was controlled by the fatty acids available for synthesis.

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INTRODUCTION

The lipid of soybeans undergoes virtually a complete transformation in composition during maturation (1,2). Recent studies by Hirayama and Hujii (2) suggest that triglycerides may be synthesized via monoglyceride intermediates as well as by the Kennedy pathway (3). The former mechanism was based mainly on the observation that relatively large amounts of monoglycerides were present during the early stage of maturation and that these compounds may be acylated (4) or phosphorylated (5,6) by plant enzymes. However, in a recent study (1) we could not confirm the presence of monoglycerides as normal constituents of immature soybeans. The purpose of this study was to gain an insight into the mode of synthesis of triglycerides from changes in their structures and molecular species composition during maturation of the beans.

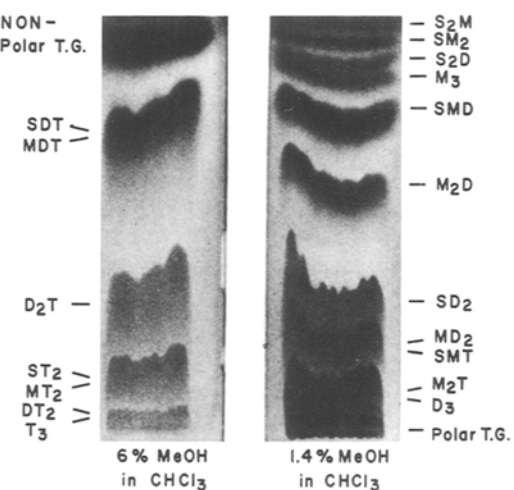

FIG. 1. Argentation TLC of soybean triglycerides with chloroform-methanol on plates coated with Silica Gel G. TG, triglyceride; S, saturated; M, monoenoic; D, dienoic; T, trienoic acid moieties of triglycerides, e.g., SMD, a triglyceride containing one saturated, one monoenoic and one dienoic fatty acid; T_3, trilinolenin.

TABLE I

Analysis of Hawkeye Beans During Maturation

Picking	Age[a]	Lipid[b] (wt %)	Residue (wt %)	Triglyceride of total lipid (wt %)
1	30	6.0	20.6	6.5
2	35	5.4	14.0	17.5
3	40	6.1	15.5	27.0
4	46	6.5	16.2	44.6
5	52	7.5	19.5	50.0
6	56	8.2	20.4	53.7
8	66	10.0	23.8	76.0
9	89	15.2	32.4	83.0
10	91	16.0	33.0	83.5
11	111	22.7	37.2	85.0

[a]Days after flowering

[b]Based on weight of wet tissue.

MATERIALS AND METHODS

Soybeans of the Hawkeye variety were grown at the University of Minnesota Experimental Station, Waseca, Minnesota. Beans were picked from the second and third nodes of plants at 11 intervals from 30 to 111 days after flowering, stored at -20 C, and the lipid extracted as previously described (1).

Lipid Analyses

Triglyceride content of the total lipid was determined by quantitative thin layer chromatography (TLC) by the charring-densitometry technique (1,7,8) using triolein (Lipids Preparation Laboratory, The Hormel Institute, Austin, Minnesota) as a standard.

Isolation of Triglycerides

Triglycerides were separated from the total lipids by TLC on plates of Silica Gel G. The plates were developed in 15% diethyl ether in petroleum ether (30-60 C). The triglyceride band was detected by viewing the plate under ultraviolet light after spraying with 0.2% 2,7-dichlorofluoroscein in methanol. The appropriate band was then scraped from the plate and the triglycerides recovered from the adsorbent by extraction with 10% methanol in diethyl ether.

Triglyceride Species Analysis

Each sample was fractionated by argentation TLC with solvent systems consisting of 1.4% to 6.0% methanol in chloroform, depending on the polarity of the triglycerides (9). Individual bands were visualized under ultraviolet light after spraying with 2,7-dichlorofluoroscein and recovered from the plate by extraction with 10% methanol in diethyl ether in a Buchner funnel, followed by acidification of the adsorbent with 10% aqueous HCl in a separatory funnel and extraction with diethyl ether. Quantitative determination of the amount, as well as composition of each band, was carried out by the addition of a known amount (10 to 100 μg) of methyl pentadecanoate as an internal standard, converting the triglycerides to

TABLE II

Triglyceride Fatty Acids of Maturing Hawkeye Soybeans, (Wt %)

Fatty acids	Age[a] 30	35	40	52	66	91
16:0[b]	13.9	12.3	12.9	11.8	10.6	10.6
18:0	3.8	4.4	4.1	4.2	4.7	3.9
18:1	11.4	17.0	25.2	25.2	26.3	25.5
18:2	37.7	37.5	42.2	47.1	49.6	52.4
18:3	34.2	28.7	15.6	11.7	8.8	7.6

[a]Days after flowering.

[b]Short-hand designation for fatty acids: number before colon, chain length; number after colon, number of double bonds.

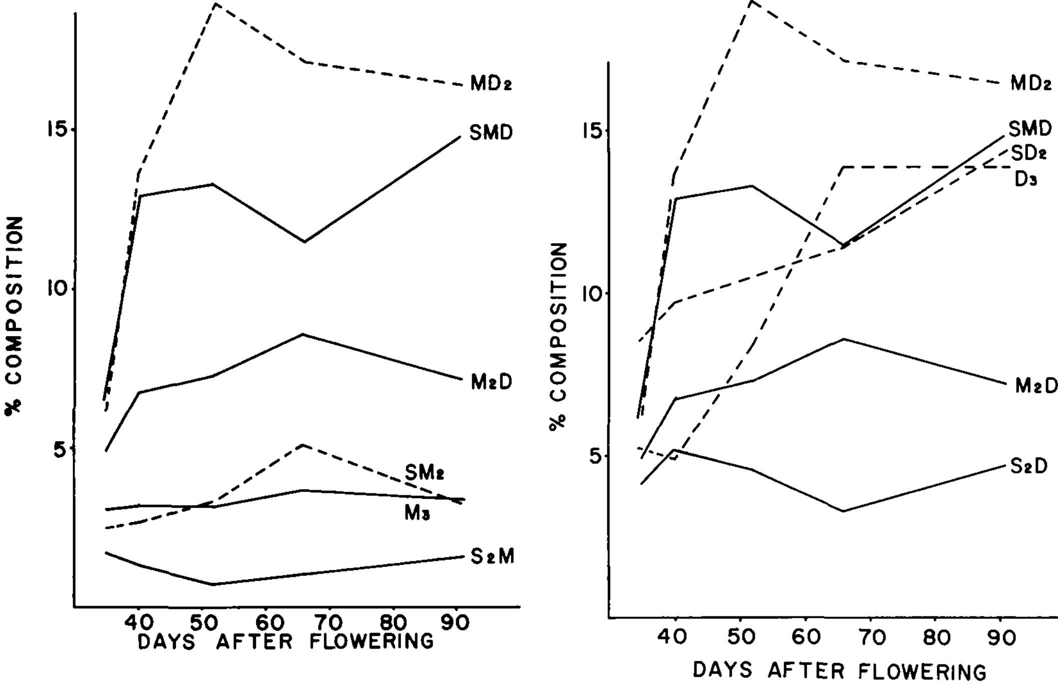

FIG. 2. Changes in molecular species composition of major monoene containing triglycerides. S, saturated; M, monoene; D, diene component fatty acids.

FIG. 3. Changes in molecular species composition of major diene containing triglycerides during maturation. S, saturated; M, monoene; D, diene component fatty acids.

TABLE III

Triglyceride Analyses of Maturing Hawkeye Soybeans, (Wt %)

TG Species[b]	Age[a]				
	35	40	52	66	91
S_2M	1.65	1.30	0.70	1.00	1.54
SM_2	2.45	2.62	3.24	5.08	3.24
S_2D	4.10	5.18	4.58	3.23	4.62
M_3	3.06	3.17	3.15	3.68	3.35
SMD	6.55	12.83	13.28	11.40	14.74
M_2D	4.89	6.70	7.23	8.55	7.12
SD_2	8.57	9.69	10.48	11.36	14.33
MD_2	6.19	13.51	18.93	17.10	16.34
SMT	3.31	3.22	3.37	1.90	1.62
M_2T	3.12	2.21	1.84	1.56	1.24
D_3	5.20	4.84	8.34	13.80	13.75
SDT	11.50	8.33	5.15	4.37	4.38
MDT	6.68	12.20	7.88	6.30	5.31
D_2T	11.77	5.20	7.27	7.47	6.75
ST_2	5.19	1.61	0.49	---	---
MT_2	2.60	1.99	1.54	---	---
DT_2	8.50	4.24	2.61	2.48	1.77
T_3	4.67				

[a]Days after flowering.

[b]TG, triglyceride; S, M, D and T, saturated, monoenoic, dienoic and trienoic component fatty acids, respectively.

TABLE IV

Lipase Hydrolysis of Triglycerides From Hawkeye Soybeans
Fatty Acid Analyses, Per Cent Esterified in β-Position[a,b]

	Age[c]				
	35	40	52	66	91
16:0	8.6±0.9	5.3±0.4	4.4±0.4	3.5±0.4	5.8±0.6
18:0	8.1±1.5	6.5±0.5	4.4±0.4	3.0±0.2	6.6±1.0
18:1	27.5±0.3	19.9±0.4	21.1±0.3	27.2±0.4	26.9±1.8
18:2	47.0±0.6	53.8±1.0	50.0±0.1	46.0±0.1	45.1±0.5
18:3	33.7±0.4	31.9±0.1	31.9±1.4	31.7±0.9	34.5±0.8

[a]Per cent esterified in β-position = $\frac{\% \text{ in } \beta\text{-monoglyceride}}{3 \times \% \text{ in triglyceride}}$.

[b]Figures represent the average of triplicate lipase hydrolysates of the same triglyceride sample.

[c]Days after flowering.

methyl esters by heating with 6% HCl in methanol (9,10) and analysis by gas liquid chromatography (GLC).

GLC of methyl esters was carried out with a Barber-Colman series 5000 gas chromatograph equipped with a hydrogen flame detector. The column was 6 ft x 1/4 in. glass, packed with 12% EGSS-X on 60-100 mesh Chromosorb P, with an operating temperature of 185 C and a flow rate of 60 ml per min of nitrogen.

Lipase Hydrolysis

The general technique described by Mattson et al. (11), as modified in previous studies (10), was used. Briefly, at least 2 mg of triglyceride was suspended in 1 ml of tris buffer, pH 8, then 20 µl of bile salts (1% aqueous solution), 50 µl saturated aqueous $CaCl_2$ and 8 mg lipase were added. The reaction was carried out in a water bath at 40 C with vigorous stirring for 15 min. The reaction mixture was poured into ethyl ether over anhydrous sodium sulfate; the ethyl ether solution was allowed to dry, and concentrated to a small volume under a stream of nitrogen after filtration. The β-monoglycerides were separated by TLC on Silica Gel G with diethyl ether-petroleum ether (b.p. 30-60)-acetic acid (30:70:2). The monoglyceride band was scraped directly into an ampule and esterified by heating it with 6% HCl in methanol. Fatty acid composition was determined by GLC of the methyl esters.

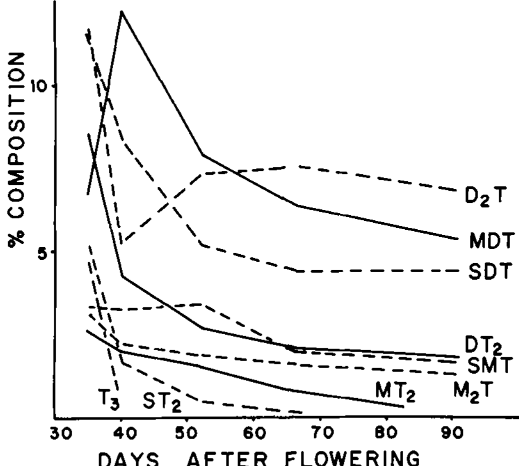

FIG. 4. Changes in molecular species composition of triene containing triglyceride. S, saturated; M, monoenoic; D, dienoic; T, trienoic component fatty acids.

RESULTS

Data on the beans at various intervals during maturation are shown in Table I. Soybeans of the Hawkeye variety are slow maturing, and thus, pickings were made over a period of 111 days after flowering. However, the major changes in triglyceride and fatty acid composition had occurred by approximately 66 days after flowering. Fatty acid composition of the triglycerides at various stages during maturation is shown in Table II. The major changes occurred in the relative amounts of linolenic, linoleic and oleic acids. The percentage of linolenic acid decreased, that of oleic and linoleic increased. However, there was an increase in the absolute amounts of all fatty acids as the beans matured.

Some 18 different groups of molecular species of triglycerides were detected in the beans during maturation; most of these were separated in individual bands by argentation-TLC as illustrated in Figure 1. The composition of those bands that contained mixtures of species were calculated on the basis of their fatty acid composition (9,10). Quantitative analyses of species composition of the trigly-

TABLE V

Lipase Hydrolysis of Individual Triglyceride Species Hawkeye Beans, 66 Days After Flowering, Wt % of Fatty Acids Esterified in β-Position

TG Species[a]	16:0	18:0	18:1	18:2	18:3
S_2M	11.0	4.6	84.4		
SM_2	3.1	Trace	96.9		
S_2D	7.9	10.0		82.2	
SMD	2.6	Trace	28.8	68.6	
MD_2			13.3	86.7	
D_2T				83.4	16.6

[a]TG, triglycerides: S, M, D and T, saturated monoenoic, dienoic and trienoic component fatty acids.

cerides at various stages during maturation of the bean are summarized in Table III. Changes in triglyceride species were generally quantitative but several species containing linolenic acid, particularly trilinolenin, disappeared completely as the beans matured. The pattern of the changes are illustrated in Figures 2, 3 and 4. In general, there was a decrease in the percentage of all species containing linolenic acid, and an increase in all species containing linoleic and oleic acid (except those species containing linolenic acid).

In order to obtain information on isomer composition, the distribution of fatty acids in the primary (α) and secondary (β) positions were determined via lipase hydrolysis. Results on total triglyceride fractions are summarized in Table IV. For comparison, individual groups of species isolated from the eighth picking, 66 days after flowering, are shown in Table V. The pattern of the distribution of fatty acids was highly consistent in all samples. Linolenic acid was distributed fairly uniformly between the β- and α-positions of the glycerol moiety. Linoleic acid favored the β-position, and oleic acid favored the α-positions. Most of the palmitic and stearic acids were distributed in the α-positions. Results on the individually separated species of the eighth picking showed that linoleic acid took precedence over linolenic acid as well as oleic and the saturated acids for the β-position.

DISCUSSION

The present study shows that extensive synthesis of triglycerides occurs in soybeans during maturation. The increase in triglyceride is accompanied by extensive changes in molecular species composition. These changes appeared to be directly related to changes in fatty acid composition inasmuch as the percentages of the molecular species containing linoleic and oleic acids increased and that of linolenic decreased in accord with changes in the percentages of these acids.

The constancy of the distribution of the fatty acids between the β- and α-positions of the molecule during large changes in molecular species composition indicates that the mode of synthesis is established at the beginning of the development of the bean. The decrease in the percentages of linolenic containing triglycerides does not appear to be due entirely to a dilution effect because some species containing this acid were completely absent in the lipid of the mature bean. Thus, it appears that a redistribution or transformation of acids occurs during maturation in accordance with the fatty acids available for synthesis and their relative affinity for esterification in the β- or α-positions. The mechanism of this transformation is not known. Desaturation of fatty acids apparently may occur in the lipid carrier (12), however, chain elongation probably requires a direct association of the free acid with the enzyme. Extensive morphological changes also occur in the bean during maturation and may involve translocation of lipid. Thus, although the triglycerides of the bean serve mainly for storage of energy, the precise relationship of their synthesis to that of the constituent fatty acids appears to be highly complex.

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SHORT COMMUNICATIONS

Fatty Acid Composition of Tissue Lipids From Miniature Swine: Influence of Dietary Sucrose and Starch

ABSTRACT

The fatty acid composition of serum, liver and adipose tissue from Pitman-Moore miniature swine was determined following their consumption of starch- or sucrose-containing diets for a period of one year. Among the tissues studied there were no significant differences in the fatty acid composition due to the type of dietary carbohydrate (starch or sucrose). The cholesteryl ester fatty acid composition of all samples studied remained quite constant. There were minor fluctuations in fatty acid composition of phospholipids and triglycerides from serum collected at different intervals following initiation of the diets.

Miniature swine have become increasingly popular in recent years as research animals and a considerable body of data has accumulated regarding various anatomic, physiologic and biochemical characteristics of these animals. The literature contains several reports on the total fatty acid composition of swine tissues (1-3), but there is a paucity of information on fatty acid composition of individual lipids from miniature swine consuming defined diets. In this report we present the fatty acid composition of lipids of serum, liver and adipose tissue of Pitman-Moore miniature swine fed a starch- or sucrose-containing diet for a period of one year.

Twenty-two male animals, approximately 2 months of age, were purchased from Vita-Vet Laboratories, Marion, Indiana. Upon receipt, the animals were placed on a diet of commercial hog chow. At the end of one month, blood samples were collected for baseline analyses and the swine were immediately placed on Diet A (Table I). Twelve animals received the sugar-containing diet and 10, the starch-containing diet. After eight months all animals were switched to Diet B (Table I). After one year, one-half of the animals were killed by electrocution and samples of liver and retroperitoneal adipose tissue were removed for analysis. Blood was collected at 7 and 12 months following initiation of Diet A. Lipids were extracted with chloroform and methanol (2:1) and from the lipid extract, phospholipids, triglycerides and cholesteryl esters were separated by thin layer chromatography in Skellysolve B-diethyl ether-acetic acid (146:50:4). Methyl esters of the component fatty acids were prepared and separation was carried out by gas liquid chromatography as described previously (4), with the exception that separations were temperature programmed at 2 C/min from 150-185 C. Relative concentrations of fatty acids were determined by means of an infotronics, CRS-108 digital integrator. Quantitative results with fatty acid standards (quantitative standards KA,KB, and KD from Applied Science Laboratories) agreed with the stated composition with a relative error of $<2\%$ for major components (those comprising more than 10% of the total mixture) and $<10\%$ for minor components (those comprising less than 10% of the total mixture).

The fatty acid composition of the diets is shown in Table I and the fatty acid composition of the serum lipids, in Table II. At no time were there significant differences between the starch or sucrose groups in the fatty acid composition of the serum lipids. The cholesteryl ester fatty acid composition remained quite constant in serum and tissues throughout the entire period of study.

There were, however, significant differences in the fatty acid composition of the phospholipids and triglycerides over the various collection periods. In the phospholipid fraction there was a substantial increase in serum levels of arachidonic acid from baseline to seven months. Serum linoleic acid levels were similar for baseline and seven-month samples but had increased significantly by 12 months. Slight reductions in stearic acid levels were also seen at 12 months. In the triglyceride fraction the major serum change was a reduction in the relative concentration of linoleic acid at seven months compared with the baseline and a slight

TABLE I

Composition of the Diet by Ingredients and Overall Fatty Acid Composition

Dietary constituents (per cent by weight)			Fatty acid composition (per cent of total)		
	Diet A	Diet B	Fatty acids[d]	Diet A	Diet B
Commercial hog chow[a]	40	34.5	14:0	0.2	0.6
Dry egg yolk	16	16	16:0	22.6	24.3
Hegsted salt mixture	2	2	16:1	2.3	1.7
Vitamin supplement[b]	1	1	18:0	9.9	6.2
Sucrose or cornstarch[c]	41	41	18:1	41.9	42.0
Peanut oil	---	5	18:2	20.1	23.4
Crystalline cholesterol	---	0.5	18:3	1.0	0.6
			20:4	<1.0	<1.0

[a]Security Pig Starter (18% protein, 3% fat, 5% crude fiber), Security Mills, Inc., Knoxville, Tenn.

[b]Per 100 lb. feed: vitamin A, 4.5×10^7 units; vitamin D_2, 6.25×10^5 units, DL α-tocopherol, 6250 units; ascorbic acid, 225 g; choline chloride 375 g; menadione, 11.25 g; para-aminobenzoic acid, 25 g; niacin, 22.5 g; riboflavin, 5 g; pyridoxine HCl, 5 g; thiamin HCl 5 g; calcium pantothenate, 15 g; vitamin B-12, 6.75 g (0.1% in gelatin); biotin, 100 mg; folic acid, 450 mg.

[c]The cornstarch was provided by Corn Products Co., Argo, Ill.

[d]Fatty acids are designated by chain length:number of double bonds.

TABLE II

Fatty Acid Composition of Serum Lipid From Swine Consuming a Starch- or Sugar-Containing Diet for up to One Year[a]

		7 months' diet		12 months' diet	
Fatty acids[b]	Baseline (N=21)	Sugar (N=11)	Starch (N=10)	Sugar (N=6)	Starch (N=5)
Phospholipids					
14:0	0.5 0.1[c]	---	0.3 0.1	---	---
16:0	19.0±0.7	17.3±1.1	17.3±1.4	17.2±0.9	16.4±1.6
16:1	1.6±0.1	0.9±0.5	0.9±0.5	1.2±0.2	1.9±0.6
18:0	39.9±2.4	42.5±2.2	39.9±2.5	32.1±1.0	32.9±0.3
18:1	18.4±1.3	15.6±1.4	15.7±0.8	15.2±0.8	14.2±0.6
18:2	9.1±0.7	8.2±0.6	9.6±1.0	15.4±1.1	16.0±1.4
20:4	3.3±0.4	12.7±1.1	11.6±1.3	14.9±1.7	13.1±1.5
Triglycerides					
14:0	0.7±0.1	---	---	---	---
16:0	20.0±0.6	20.4±0.8	17.8±1.2	23.5±1.1	22.4±0.8
16:1	3.8±0.4	1.6±0.3	1.2±0.5	2.5±0.8	---
18:0	10.0±0.9	16.5±1.3	15.6±1.7	12.0±1.4	10.3±0.9
18:1	38.5±1.0	46.0±1.6	50.0±2.6	40.9±2.2	42.0±0.7
18:2	19.4±1.0	10.0±1.0	10.3±0.9	13.4±1.3	15.0±1.6
20:4	3.1±0.4	1.3±0.2	5.3±3.1	---	---
Cholesteryl esters					
14:0	0.3±0.1	---	0.2 0.1	---	---
16:0	13.8±0.5	14.3±1.1	13.5±0.9	13.3±1.0	11.4±1.1
16:1	2.7±0.2	1.7±0.4	1.7±0.2	2.4±0.3	1.8±0.5
18:0	5.2±0.3	9.7±1.0	7.7±0.7	6.2±0.9	6.0±1.2
18:1	33.9±0.8	36.0±1.2	39.1±1.4	36.3±2.1	34.9±1.6
18:2	34.8±0.7	30.5±1.3	31.3±2.3	33.5±1.5	39.2±1.7
20:4	6.2±0.4	6.6±0.8	4.7±1.1	5.4±1.4	5.2±0.5

[a]Values expressed as the percentage of total fatty acids.

[b]Fatty acids are designated by chain length:number of double bonds.

[c]Mean ± the standard error of the mean.

TABLE III

Fatty Acid Composition of Liver and Adipose Tissue From Swine Consuming a Starch- or Sugar-Containing Diet for One Year[a]

Fatty acid[b]	Liver						Adipose tissue	
	Phospholipids		Triglycerides		Cholesteryl esters		Triglycerides	
	Sugar (N=6)	Starch (N=5)	Sugar (N=6)	Starch (N=5)	Sugar (N=6)	Starch (N=5)	Sugar (N=3)	Starch (N=4)
14:0	---	---	0.5±0.1	0.3±0.1	---	---	2.3±0.5	2.4±0.4
16:0	12.1±0.5[c]	11.1±0.4	19.8±0.7	18.6±0.6	16.6±2.7	25.0±2.3	30.5±2.1	30.9±2.4
16:1	<1.0	<1.0	2.9±0.3	1.7±0.3	1.6±0.5	---	4.3±0.7	4.6±0.5
18:0	33.0±0.7	35.5±1.0	10.4±0.4	11.2±0.4	12.7±2.2	14.0±1.6	10.5±1.6	8.4±0.7
18:1	15.3±0.9	13.1±1.1	45.0±1.1	44.4±0.7	49.5±4.3	40.3±2.4	40.6±2.1	41.0±2.3
18:2	16.3±1.4	17.2±1.0	14.4±0.5	15.5±0.3	13.7±1.7	15.9±1.1	10.9±1.0	11.7±0.9
20:4	18.8±1.0	19.5±1.0	3.4±0.7	2.9±0.3	1.8±0.6	0.7±0.3	0.2±0.1	0.3±0.1

[a]Values expressed as the percentage of the total fatty acids.

[b]Fatty acids are designated by chain length:number of double bonds.

[c]Mean ± the standard error of the mean.

increase again at 12 months. Minor fluctuations were also noted in levels of oleic acid over the period of study.

The fatty acid composition of liver and adipose tissue is shown in Table III. Only the triglyceride fatty acid composition of adipose tissue is shown since it comprises more than 95% of the lipids in this fraction. In liver and adipose tissue, as in serum, there were no significant differences between the starch or sucrose groups in the fatty acid composition of the lipids studied.

Whether the differences in fatty acid composition of the serum collected at various periods throughout the experiment are the result of developmental changes (rapid growth of the animals was observed to continue over the entire 12 months), or due to dietary manipulations is not known. Fatty acid composition of swine tissues can be altered by changes in dietary fatty acid composition (2,3). However, the fatty acid composition of the two diets in this study (Table I) is quite similar and it would appear unlikely that differences in serum fatty acid composition are attributable to differences in composition of the diets. On the other hand, changes in fatty acid composition have been noted by other workers to accompany growth and maturation, and would appear to be the more likely explanation for the relatively minor changes that were seen in this study.

In no case were significant differences apparent in fatty acid composition of the two dietary groups (starch or sucrose). This is of interest in view of the reports of differences in lipogenesis in animals consuming different types of carbohydrates (7).

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The Effect of Sonication on Lipase Activity[1]

ABSTRACT

Studies were made to evaluate the effect of sonication on activity of porcine pancreatic lipase and on activity of this lipase when in contact with substrate. Lipase was rapidly inactivated by sonication at 50 C, but was stable at 30 C or lower temperatures. Sonication for 4.5 min of lipase and olive oil at 38 C produced 2.7 times as much free fatty acid as without sonication. Hydrolysis of tripalmitin was achieved by sonication of lipase and tripalmitin dissolved in methyl myristate.

Although numerous papers have been published regarding the effects of sonication on various enzymes, only two papers have been published regarding the sonication of lipases. Kasahara and Yoshinare (1) sonicated milk lipase and Buonsanto et al. (2) sonicated plasma lipase. Since hog pancreatic lipase is commonly used as a tool for triglyceride and phospholipid analysis, it was of interest to determine the effects of sonication on this enzyme since lipid systems frequently are prepared by sonication.

The objectives of the studies reported here were: (a) to establish the effects of sonication on the hydrolysis rate of triglycerides by the crude enzyme using various time and temperature treatments; (b) to sonicate combined enzyme and substrate, and to compare the relative rates of hydrolysis with that in a nonsonicated system; and (c) to establish whether sonication could be used as a means of increasing the rate of lipase hydrolysis of the higher melting triglycerides, such as tripalmitin, without increasing the temperature used during the hydrolysis.

Heating 0.2 g of dried pancreatin in 10 ml H_2O, or sonicating it for 1 min at various temperatures produced the same type of inactivation curve (Fig. 1) for both treatments. However, the lipase was more rapidly inactivated when sonicated. The latter was attributed to the intense cavitation produced during the sonication treatment.

Crude pork pancreatic lipase (0.2 g dried pancreatin in 10 ml H_2O) was subjected to sonication for 1-30 min at temperatures of 10, 30, 40 and 50 C using a Branson model S-75 Sonifier at a frequency of 20,000 cps and intensity of 23 w/cm^2. The sonicated enzyme was used for the subsequent hydrolysis of an olive oil emulsion at 38 C (3). There was no activation of the enzymes during the first 5 min of sonication at 10, 30 or 50 C. At 40 C there was what appeared to be a slight activation of the enzyme during the first 5 min of treatment (Fig. 2). However, the enzyme lost activity as the sonication time was increased. At 50 C, the combination of heat and intense cavitation rapidly inactivated the enzyme.

When 10 ml of a semi-purified lipase preparation having a specific activity of 1,795 [prepared according to Sarda et al. (4)] was sonicated at 30 C for 1, 2, 5 and 10 min, slight activation of the enzyme during the first 5 min was again noticed. It appears that the degree of lipase purity may be related to the temperature at which activation occurs, i.e., crude lipase is activated at a higher temperature than a semi-purified lipase. Since the experiments with the semi-purified lipase were not performed at temperatures other than 30 C, the above statement cannot be further amplified.

It has been reported by other workers (2,5-12) that various enzymes are activated when sonicated for short-time periods. There is some indication that, in cases where increased enzyme activity was reported during the first few minutes of sonication, the enzymes were not pure. Such a situation exists with milk xanthine oxidase where the enzyme is attached to the surface of the fat globules as lipoprotein cenapses. Upon sonication, these cenapses

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[1]Journal Paper No. 4181 of the Michigan Agricultural Experiment Station.

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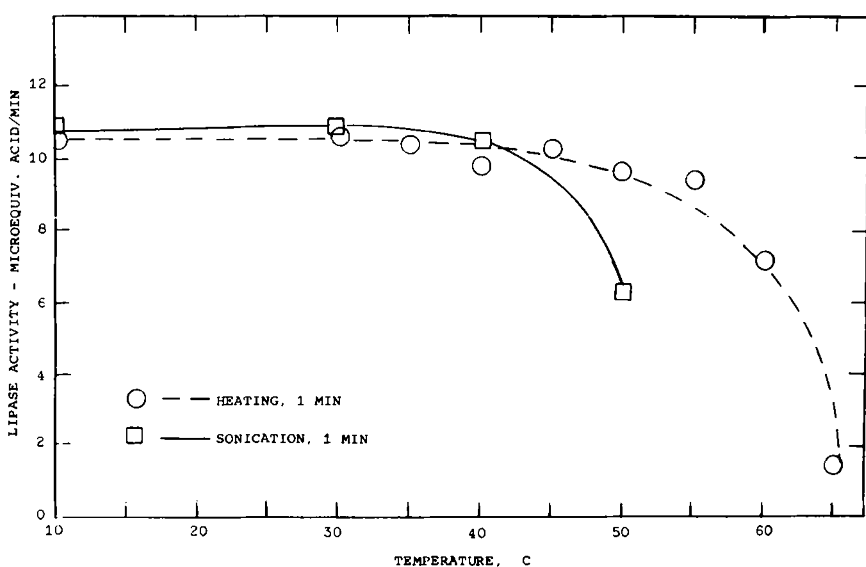

FIG. 1. Effect of heating and sonicating for 1 min at various temperatures on lipase activity.

break and the enzyme is intensely activated before loss of activity finally occurs (12). Since both the crude and semi-purified lipase in this study existed as a lipoprotein, it is postulated that the observed activation with lipase is similar to that observed with milk xanthine oxidase. Therefore, the observed increase in activity during the first few minutes of sonication may be only an apparent increase, i.e., the sonication treatment may break the lipoprotein complex to release the enzyme or active groups on the enzyme.

Many workers have reported that N_2, H_2 or other gases decrease enzyme inactivation during sonication. Similar results may be obtained with lipase by lowering the sonicating temperature. At 10 C, nearly all of the original lipase activity was retained after 30 min sonication

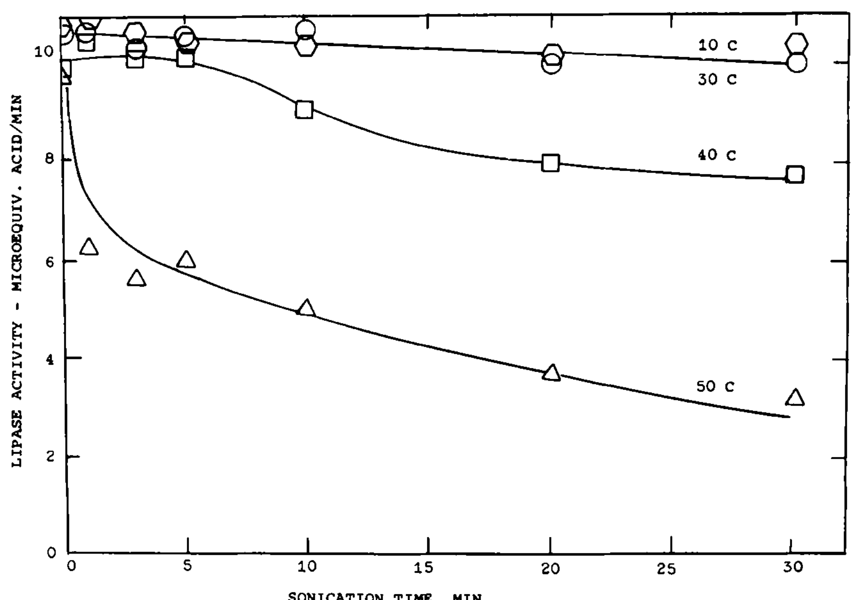

FIG. 2. Effect of sonication time and temperature on lipase activity.

TABLE I

Effect of Sonication and Stirring of Enzyme and Olive Oil-Gum Arabic Emulsion for 4.5 Min at 38 C

Run No.	M Equiv. of total fatty acids liberated	
	Sonication	Stirring
1	144	44
2	134	58
3	141	58
4	134	48
5	129	49
Average	138	51

treatment in air. It is evident from Figure 2 that any temperature between 10 and 30 C will give nearly the same rate of lipase activity for a given sonication treatment. As the temperature was increased above 30 C, there was a progressive inactivation of the enzyme. Therefore, 30 C may be considered to be the approximate critical temperature of sonication. This means that there was practically no alteration in lipase activity during sonication at any temperature below 30 C for periods of time up to 30 min.

To determine whether sonication would increase the rate of triglyceride hydrolysis by the enzyme, lipase (5 mg pancreatin in 0.25 ml H_2O) and an olive oil emulsion were sonicated at 38 C for 4.5 min. The degree of lipolysis was then compared to that of another sample which was stirred at 600 rpm for 4.5 minutes (Table I). The simultaneous sonication of lipase and olive oil liberated 2.7 times as much fatty acid as the stirring system. This significant increase in rate was attributed to three facts: (a) a better emulsion of olive oil and gum arabic was obtained; (b) there was a greater turnover rate of substrate at the oil-water interface for the enzyme to act upon; and (c) the lipase activity increased during the first 5 min of sonication. Mechanical agitation (stirring at 600 rpm) was evidently not sufficient to provide the conditions necessary to promote hydrolysis equivalent to that when sonication was used.

Since sonication produced a 2.7 fold increase in the amount of fatty acids liberated in the lipase-substrate experiment, it was of interest to see whether sonication would increase the hydrolysis rate of a high melting point triglyceride such as tripalmitin. Preliminary experiments showed that tripalmitin would not hydrolyze even with sonication unless the temperature of hydrolysis was raised to 64 C, the melting point of the tripalmitin. This temperature of course would soon inactivate the lipase (Fig. 1). However, with the addition of methyl myristate, which acted as a carrier for the tri-

TABLE II

Effect of Sonication and Stirring of Enzyme and Tripalmitin[a] for 9.5 Min at 45 C

Run No.	M Equiv. of total fatty acids liberated[b]	
	Sonication	Stirring
1	11	0
2	13	0
3	19	0
Average	14	0

[a]The tripalmitin contained methyl myristate as a carrier.

[b]The methyl myristate blanks have been subtracted in the above figures.

palmitin (13), the hydrolysis temperature could be lowered to 45 C. Therefore, a tripalmitin emulsion, containing methyl myristate as a carrier, was sonicated with lipase for 9.5 min at 45 C. The degree of hydrolysis was compared to that in a duplicate sample stirred at 600 rpm. Even at 45 C, the hydrolysis rates were very slow (Table II). No fatty acids were liberated during the experiment involving only stirring of lipase and tripalmitin. Under these conditions, sonication of substrate and lipase did not increase the hydrolysis rates of the higher melting point triglycerides unless a carrier was present to lower the melting point.

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Oxidation of Fatty Acids by Heat Treated Hemoproteins

ABSTRACT

The hemoproteins catalase and peroxidase, after heat treatment which decreased their enzyme activities, became more efficient as heme catalysts of linoleic acid oxidation. These effects might be of importance for preservation and storage of food.

Hemoproteins are known to catalyze the oxidation of unsaturated fatty acids by oxygen (1). It has been shown that cooking of meat results in a conversion of ferrohemochrome to ferrihemochrome which might increase the oxidation of tissue fat (2). Other food products which have been heat treated to minimize enzyme action during storage, often develop rancid compounds formed by oxidation of fatty acids. In the case of food from plant sources, this can be ascribed to remaining activities of lipoxygenase, or to small amounts of hemoproteins always present. In order to get information of the role played by hemoproteins in this respect, we investigated the oxidation of linoleic acid by catalase (E.C.1.11.1.6.) and peroxidase (E.C.1.11.1.7.), both ferric hemoproteins, that were subjected to heat treatment or acidification followed by neutralization. At the same time, the variations in enzyme activity on hydrogen peroxide were measured.

In these experiments ox liver catalase, 39,000 units/mg, (Boehringer) and two preparations of horseradish peroxidase, RZ 1.73 and 3.0 (Worthington) served as sources of the hemoproteins. Concentrations of $0.4 \cdot 10^{-2}$ μmole/ml for catalase and $1.3 \cdot 10^{-2}$ μmole/ml for peroxidase in a 0.01 M sodium potassium phosphate buffer at pH 6.5 were used. These concentrations correspond to $1.6 \cdot 10^{-2}$ and $1.3 \cdot 10^{-2}$ μmole heme/ml, respectively.

Heat treatment in increments of 5-10 C was performed by two procedures. For the first one, covering the temperature range 25-95 C a specially designed apparatus was used to pump the hemoprotein solutions through thin walled narrow glass tubes heated in a water bath. In the second procedure the heat treatment was carried out over the range 25-140 C on hemoprotein solutions in sealed thin walled glass ampoules which were immersed in a glycerol bath and rotated around the vertical axis at such a speed that the solution was distributed in a thin film over the inner wall of the ampoule. In both procedures the hemoproteins were held at 0 C both before and after heat treatment. The heating lasted 52 sec in the first, and for 120 sec in the second at each temperature studied. In another experiment, a catalase solution at 25 C was brought to pH 3 with hydrochloric acid and immediately returned to pH 6.5 with sodium hydroxide before the heat treatment for 120 sec.

After the different treatments, the hemoproteins were assayed by a polarographic procedure for their effects on the oxidation of linoleic acid at 25 C and pH 6.5 (3). After suitable dilution the enzyme activities were measured by well-known spectrophotometric methods. The oxidation states of the treated and untreated hemoproteins were compared from spectra (360-600 nm) that were run both before and after reduction of the heme groups to pyridine ferrohemochrome (4).

The results (Table I) show that the linoleic acid oxidation power per molecule catalase at 25 C is higher than that of peroxidase. Provided that the four hemes per molecule of catalase and the one of peroxidase can be considered equivalent, the values for untreated catalase may be divided by four to compare the effect per mole heme. By doing so, it can be seen that the same order of effect is obtained. On heat treatment, both of the hemoproteins gave expected losses in catalase and peroxidase enzyme activities, whereas the linoleic acid oxidation rate rose as much as 11 times that for the untreated controls. The two different preparations of peroxidase behaved similarly. Catalase subjected to pH treatment, gave a

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Oxidation of Fatty Acids by Heat Treated Hemoproteins

ABSTRACT

The hemoproteins catalase and peroxidase, after heat treatment which decreased their enzyme activities, became more efficient as heme catalysts of linoleic acid oxidation. These effects might be of importance for preservation and storage of food.

Hemoproteins are known to catalyze the oxidation of unsaturated fatty acids by oxygen (1). It has been shown that cooking of meat results in a conversion of ferrohemochrome to ferrihemochrome which might increase the oxidation of tissue fat (2). Other food products which have been heat treated to minimize enzyme action during storage, often develop rancid compounds formed by oxidation of fatty acids. In the case of food from plant sources, this can be ascribed to remaining activities of lipoxygenase, or to small amounts of hemoproteins always present. In order to get information of the role played by hemoproteins in this respect, we investigated the oxidation of linoleic acid by catalase (E.C.1.11.1.6.) and peroxidase (E.C.1.11.1.7.), both ferric hemoproteins, that were subjected to heat treatment or acidification followed by neutralization. At the same time, the variations in enzyme activity on hydrogen peroxide were measured.

In these experiments ox liver catalase, 39,000 units/mg, (Boehringer) and two preparations of horseradish peroxidase, RZ 1.73 and 3.0 (Worthington) served as sources of the hemoproteins. Concentrations of $0.4 \cdot 10^{-2}$ μmole/ml for catalase and $1.3 \cdot 10^{-2}$ μmole/ml for peroxidase in a 0.01 M sodium potassium phosphate buffer at pH 6.5 were used. These concentrations correspond to $1.6 \cdot 10^{-2}$ and $1.3 \cdot 10^{-2}$ μmole heme/ml, respectively.

Heat treatment in increments of 5-10 C was performed by two procedures. For the first one, covering the temperature range 25-95 C a specially designed apparatus was used to pump the hemoprotein solutions through thin walled narrow glass tubes heated in a water bath. In the second procedure the heat treatment was carried out over the range 25-140 C on hemoprotein solutions in sealed thin walled glass ampoules which were immersed in a glycerol bath and rotated around the vertical axis at such a speed that the solution was distributed in a thin film over the inner wall of the ampoule. In both procedures the hemoproteins were held at 0 C both before and after heat treatment. The heating lasted 52 sec in the first, and for 120 sec in the second at each temperature studied. In another experiment, a catalase solution at 25 C was brought to pH 3 with hydrochloric acid and immediately returned to pH 6.5 with sodium hydroxide before the heat treatment for 120 sec.

After the different treatments, the hemoproteins were assayed by a polarographic procedure for their effects on the oxidation of linoleic acid at 25 C and pH 6.5 (3). After suitable dilution the enzyme activities were measured by well-known spectrophotometric methods. The oxidation states of the treated and untreated hemoproteins were compared from spectra (360-600 nm) that were run both before and after reduction of the heme groups to pyridine ferrohemochrome (4).

The results (Table I) show that the linoleic acid oxidation power per molecule catalase at 25 C is higher than that of peroxidase. Provided that the four hemes per molecule of catalase and the one of peroxidase can be considered equivalent, the values for untreated catalase may be divided by four to compare the effect per mole heme. By doing so, it can be seen that the same order of effect is obtained. On heat treatment, both of the hemoproteins gave expected losses in catalase and peroxidase enzyme activities, whereas the linoleic acid oxidation rate rose as much as 11 times that for the untreated controls. The two different preparations of peroxidase behaved similarly. Catalase subjected to pH treatment, gave a

TABLE I

Rate of Linoleic Acid Oxidation by pH- and Heat-Treated Catalase and Peroxidase, Compared With the Change in the Enzyme Activity

Heat treatment at temperature C[a]	Catalase			Peroxidase	
	Linoleic acid oxidation rate[b]		Enzyme activity %, untreated[c]	Linoleic acid oxidation rate[b], untreated	Enzyme activity %, untreated
	pH-treated	untreated			
25	458	20	100	8.7	100
53	460	25	---	10.5	100
61	350	70	92	15.7	---
71	290	95	5	23.5	---
81	235	100	0	35.7	---
90	205	105		47.5	80
100	195	95		58.6	---
109	185	98		68.5	---
120	167	83		79.5	50
130	---	75		88.0	20
140	---	---		96.0	14

[a]Treated 120 sec at each temperature.

[b]Micromole O_2/min μmole protein.

[c]The enzyme activity was completely lost by pH-treatment.

23-fold increase which was followed by a decrease on subsequent heat treatment. Of practical interest is the fact that the increased ability of the hemoproteins to oxidize linoleic acid was retained on storage at 2 C for at least four months.

Since the results from the spectral analysis showed no changes in the oxidation state of the hemoprotein iron, the observed effects can be considered to result from a possible increased unfolding of the protein to cause a greater exposure of the heme groups to the substrate, or a shift towards higher spin state of the ferric iron (5). These questions are presently being studied in our laboratory.

These observations on the effects of heat and pH treatment on heme catalyzed oxidation of unsaturated fatty acids may be of concern also in the preservation and storage of food from plant sources, where in fact the inactivation of catalase and peroxidase enzymes often is taken as a criterion of sufficient heat treatment.

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The Removal of Water and Nonlipid Contaminants From Lipid Extracts

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ABSTRACT

A technique is presented for the removal of water and water-soluble nonlipid contaminants from lipid extracts. The process is quicker and simpler than existing techniques and lipid recoveries of greater than 97% have been obtained.

INTRODUCTION

The removal of water and nonlipid contaminants from lipid extracts are problems that have plagued lipid biochemists for some time. A number of methods have been used in the past (1-16) to remove nonlipid contaminants, but none have proved completely satisfactory. In a previously published technique (16) using Sephadex LH-20, many of the difficulties of removing nonlipid contaminants were overcome, but the technique required the prior removal of water, and involved the use of columns which take time to make and run. Another technique has been developed, and is reported here, in which not only nonlipid contaminants, but also water, are removed in one process. The method utilizes the ability of Sephadex G-25 to swell in water, and at the same time to absorb water-soluble substances. The lipid, which is not absorbed by the Sephadex, is washed off with a solvent (e.g., chloroform) that is not miscible with water. In this method it is not necessary to form a column except to ensure complete washing of the Sephadex in the final stages.

METHODS

Radioactivity was measured in a Packard Tri-Carb Liquid Scintillation Spectrometer.

Chlorophyll was determined by the method of Arnon (17).

All chromatograms were run on Silica Gel G (Merck) thin layer plates in chloroform-methanol-water (65:25:4 v/v/v). The plates were sprayed with 50% sulfuric acid and heated at 105 C for 10 min to detect the lipid spots.

PROCEDURE

The essential procedures of the technique as applied to leaf lipid extracts are outlined in Figure 1. The preliminary homogenization, as used by us, is not essential to the technique, but has been found to extract most, if not all, of the lipid from this leaf tissue. After filtering off the insoluble residue, Sephadex G-25 (Pharmacia, Uppsala, Sweden) is added to the filtrate in the approximate proportion of 1:1 w/w, Sephadex to water in tissue. Since Sephadex G-25 can take up more than twice its own weight in water, this is more than sufficient to absorb all the water in the extract. In the presence of chloroform and methanol, the Sephadex takes up no water, but as these sol-

TABLE I

Recovery of Lipid, Nonlipid and Chlorophyll Components in Chloroform and Methanol-Water Washings After Sephadex Treatment

Material	Total recovery	Per cent recovered in: Chloroform washing	Methanol-water washings
^{14}C-Lipid[b]	98.0±6.2	97.2 ±5.5	0.84±0.7
^{14}C-Nonlipid[c]	92.2±3.5	1.02±0.8	91.2 ±4.2
Chlorophyll[d]	96.2±3.3	96.2 ±3.3	0±

[a]Lipid and nonlipid materials were determined as radioactivity; chlorophyll was determined spectrophotometrically.

[b]Mean of five determinations.

[c]Means of three determinations. Recovery was low because the solubility of some components in methanol-water was affected by drying, prohibiting complete resuspension and counting.

[d]Mean of 10 determinations.

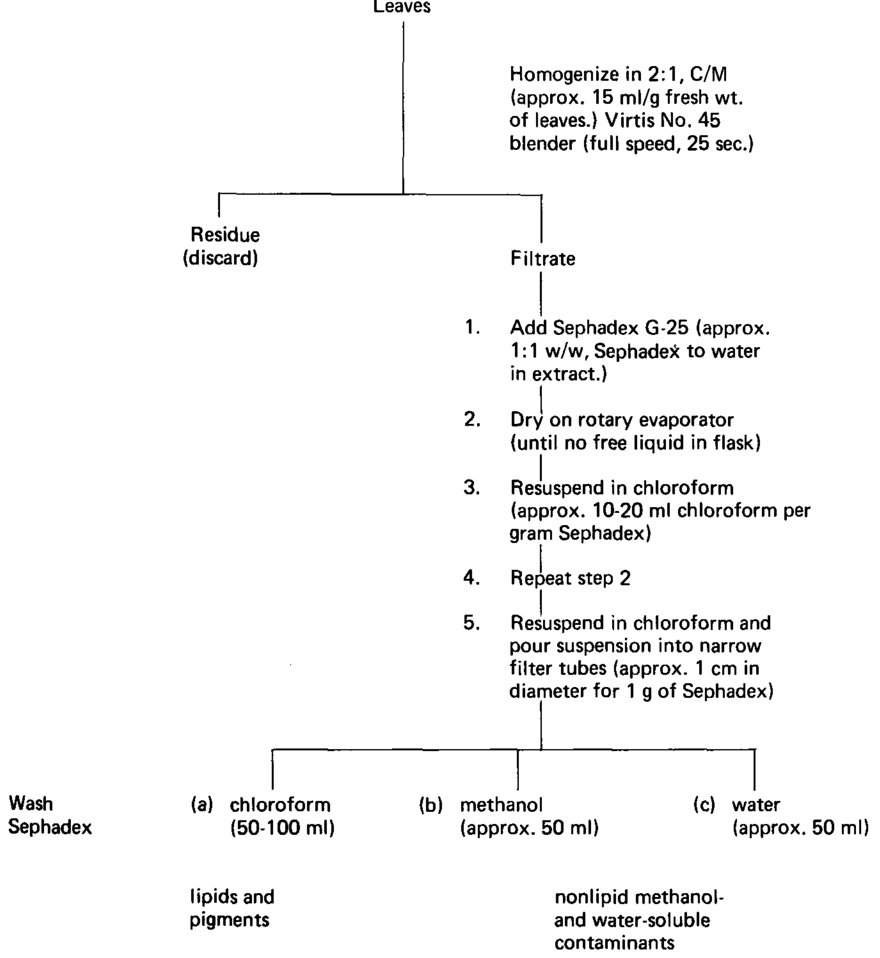

FIG. 1. Outline of procedure used to separate lipid-soluble and nonlipid contaminants from extracts of *Vicia faba* leaves.

vents are evaporated off, the Sephadex begins to adsorb water until no free liquid remains in the flask. It is not necessary to dry the Sephadex past the point at which it clings to the walls of the flask. To ensure the complete adosrption of water, the Sephadex is resuspended in chloroform and dried down once more, after which it is again suspended in chloroform and washed with the same solvent to elute the lipid.

Problems were encountered in the preliminary experiments in which some of the more polar lipids were adsorbed onto the Sephadex. These could be removed by washing with polar solvents but this results in removal of nonlipid contaminants as well. By pouring the chloroform suspension of Sephadex into narrow filter tubes (or chromatographic columns) and washing the Sephadex thoroughly with chloroform, most, if not all, of the lipid can be eluted with no significant nonlipid contamination. Nonlipid methanol- and water-soluble components can be recovered by washing the Sephadex in the filter tube with first methanol, and then water.

RESULTS

To test the technique, samples of pure, radioactive (^{14}C) plant lipids were added back to nonradioactive homogenates, and the separation procedure using Sephadex followed. Similarly, in a separate set of experiments samples of radioactive nonlipid contaminants were added to nonradioactive homogenates. The chloroform and the methanol-water washings from each experiment were chromatogrammed and autoradiographed. The resulting thin layer plates (Fig. 2) indicate little or no

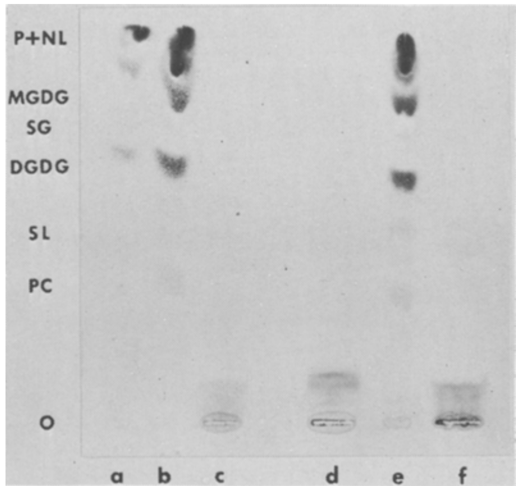

FIG. 2. Thin layer chromatogram of chloroform and methanol-water washings, using technique outlined in Figure 1. (a) Radioactive lipid extract, as added to homogenate (see text); (b) chloroform washing and (c) methanol-water washing after separation of lipid and nonlipid material using Sephadex G-25; (d) radioactive nonlipid fraction as added to homogenate; (e) chloroform washing; and (f) methanol-water washing after similar separation. Extracts a and d were prepared from ^{14}C-acetate fed leaf discs using the technique outlined in Figure 1. Lipid abbreviations: MGDG, monogalactosyl diglyceride; DGDG, di-galactosyl diglyceride; SL, sulfolipid; PC, phosphatidyl choline; PI, phosphatidyl inositol; SG, steryl glycoside; UPL, unidentified phospholipid; and P + NL, pigments and neutral lipids. Fractions c, d and f contain only unidentified nonlipid material spots.

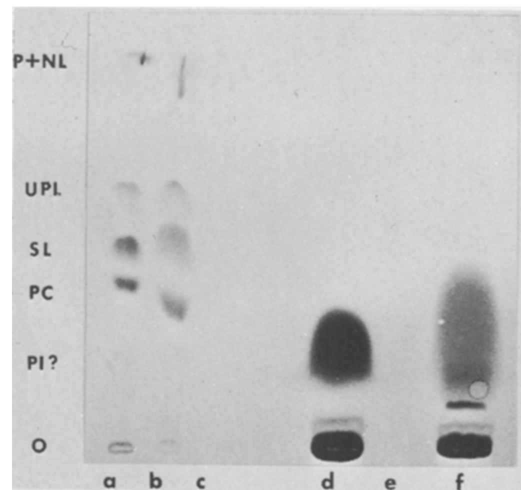

FIG. 3. Autoradiograph of thin layer plate in Figure 2. Legend as Figure 2.

contamination of the lipid fractions with nonlipid material, or vice versa. Figure 3, an autoradiograph of the same plate, shows the radioactive compounds in each fraction; in each case the radioactivity is almost entirely confined to the expected lipid or nonlipid fraction.

Quantitative determinations of similar experiments (Table I), indicate that when radioactive lipid is added to the homogenate more than 95% of the activity can be recovered in the lipid fraction. Similarly, more than 90% of radioactive nonlipid material added can be recovered in the nonlipid fraction.

Estimates of chlorophyll indicate that this pigment is recovered in quantities greater than 96% of that in the original homogenate.

DISCUSSION

The principle behind the method outlined is that Sephadex G-25 swells in aqueous solution absorbing up to 2.5 times its own weight in water. At the same time water-soluble nonlipid materials are dissolved in the water in the swollen beads. The lipid materials do not enter the beads and may be removed by washing with a water-immiscible lipid solvent.

Problems were encountered during the studies with adsorption of some lipids onto the Sephadex. These difficulties were largely overcome by thorough washing in the manner described. However, trial runs should be made to determine recovery of lipid before this method is used routinely.

The method outlined has been designed for the extraction and purification of lipids from the leaves of *Vicia faba*. It is thought, however, that this technique could be adapted to almost any type of plant or animal tissue, and used with a wide variety of different solvents and solvent combinations, in large or small scale separations. The technique may also prove useful in removing lipid contaminants from nonlipid extracts, as the water-soluble materials are easily recovered from the Sephadex.

ACKNOWLEDGMENTS

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Studies on the in Vivo Metabolism of Retinoic Acid in the Rat

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ABSTRACT

A time study of the metabolism of 6,7-^{14}C-retinoic acid after intraperitoneal injection of physiological levels (17 μg, 0.39 μc) into vitamin A deficient rats, which had been repleted with retinoic acid for two weeks up to two days before injection, resulted in a rapid metabolism to more polar compounds in the small intestine and its contents and a slower metabolism to primarily different materials in the liver and kidney. The major route of metabolism resulted in the urinary excretion of 60% of the injected dose in 24 to 27 hr. Urinary metabolites of 15-^{14}C-retinoic acid were eluted from silicic acid at a similar concentration of solvents as the ring labeled metabolites although only 32% of the injected dose was recovered in 24 hr. Compounds chromatographically similar to the urinary metabolites were observed at various times in the liver, kidney and small intestine plus contents in addition to retinoic acid and other metabolites. The relative amounts of the metabolites in the different tissues studied varied as a function of the tissue and the time of analysis after injection. Most of the radioactivity from all tissues was extractable into methanol. A liver subcellular distribution of the radioactivity derived from the intraperitoneal injection of 650 μg of 6,7-^{14}C-retinoic acid (25.9 μc) after 3 hr indicated a minimal level of association of radioactivity (150-250 dpm/mg protein) with all fractions and a greater association of radioactivity with the lysosomal-microsomal fraction (300-350 dpm/mg protein) and the 60-100% ammonium sulfate precipitable (750-800 dpm/mg protein) and 100% ammonium sulfate soluble fractions (422 dpm/mg protein) of the soluble supernatant.

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INTRODUCTION

Since the discovery of the biological activity of retinoic acid by Arens and van Dorp (1-3) in 1946, and the demonstration of the oxidation of retinal to retinoic acid both in vitro (4-8) and in vivo (4,9-12), the question has arisen whether retinoic acid is metabolized still further to a biologically active form. Recent studies suggest that retinoic acid undergoes isomerization (13,14), decarboxylation (15-17), and glucuronic acid conjugation (18) mechanisms. Hopefully, chemical structures can be eventually drawn for all the metabolites of retinoic acid and the pathways of metabolism understood. Knowledge of the biological activity of the metabolites of retinoic acid should then greatly aid the efforts which have been devoted to determining a biochemical function of the vitamin apart from its role in vision. The present study has attempted to follow the tissue distribution of metabolites after small physiological doses.

EXPERIMENTAL PROCEDURES

Reagents

All-*trans* retinoic acid, 6,7-^{14}C-retinoic acid and 15-^{14}C-retinoic acid were a gift from Hoffmann-LaRoche and Co., Nutley, N.J. One molar hydroxide of Hyamine 10X, 2,5-diphenyloxazole (PPO) and 1,4-bis-2-(5-Phenyloxazolyl)-benzene (POPOP) were obtained from the Packard Instrument Co. Silicic acid especially prepared for the chromatography of lipids by the method of Hirsch and Ahrens was obtained from BioRad Laboratories (Calbiochem Corp., Los Angeles, Calif.) and used for column chromatography after activation at 150 C for 24 to 48 hr. Organic solvents and reagents were analytical grade. Isooctane was redistilled before use. Bovine serum albumin was obtained from Sigma Chemical Co., St. Louis, Mo.

Preparation of Rats

For the retinoic acid metabolism studies, vitamin A deficient rats prepared essentially as described previously (19), which were losing weight, were repleted with retinoic acid for two weeks by feeding a retinoic acid supplemented diet (10 μg of retinoic acid per 10 g of vitamin

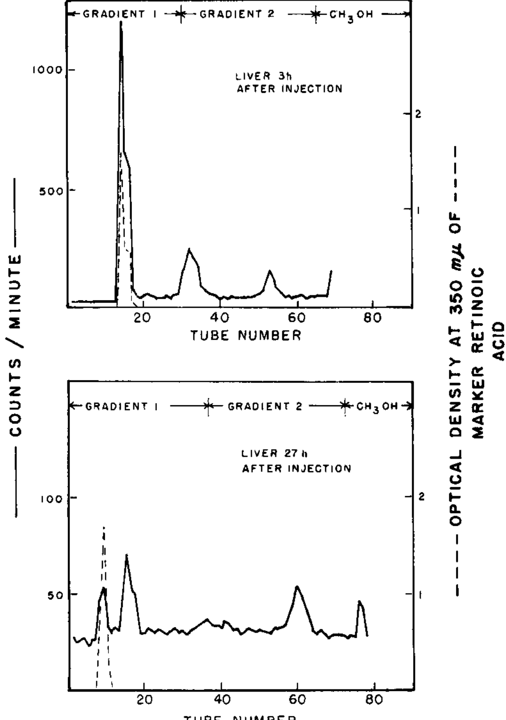


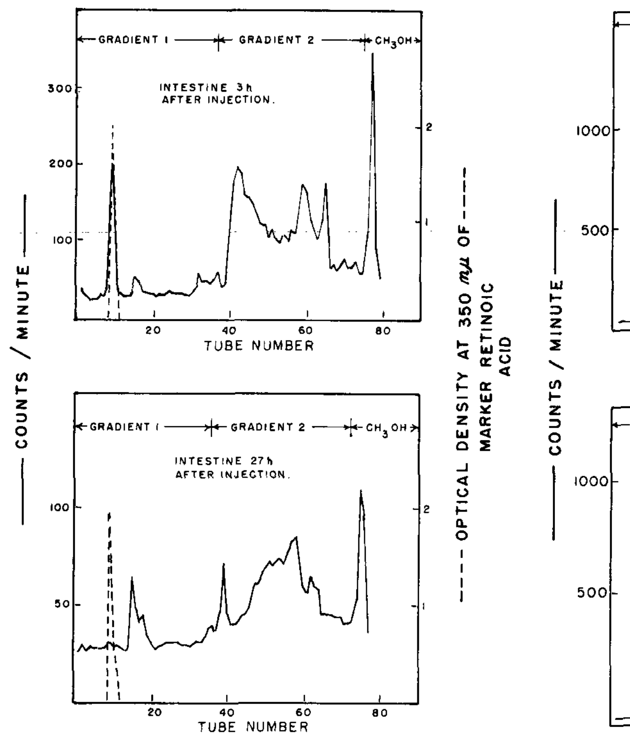
FIG. 1. Silicic acid chromatography of methanol extracts of liver at time indicated after intraperitoneal injection of 17 μg of 6,7-^{14}C-retinoic acid.

FIG. 2. Silicic acid chromatography of methanol extracts of kidney at time indicated after intraperitoneal injection of 17 μg of 6,7-^{14}C-retinoic acid.

A free diet). This diet was replaced with the vitamin A free diet for two days preceding the injection of the labeled compounds. The endogenous retinoic acid should be rapidly lost from these animals and after several days low endogenous amounts of vitamin A materials should be present. This should optimize formation of radioactive metabolites due to a minimal dilution of injected retinoic acid and its metabolites by endogenous vitamin A materials. For the liver ^{14}C-retinoic acid distribution study vitamin A deficient rats were similarly nutritionally prepared, except that they were repleted with intraperitoneal injections of 250 μg of retinoic acid in 50 μl of absolute ethanol every three days. For both these studies the actual experimental animals were selected from a group of rats on the repletion regimen.

In Vivo Metabolism of 6,7-^{14}C-Retinoic Acid

Four rats were injected intraperitoneally with 17 μg (0.39 μc) of 6,7-^{14}C-retinoic acid in 0.1 ml of absolute ethanol-Tween 80-H_2O (2:1:7 v/v/v). They were killed at 1, 3, 9 and 27 hr, respectively, after injection. Water was supplied ad lib. The rats killed at 1, 3 and 9 hr were not given food but the rat killed at 27 hr was allowed access to the vitamin A deficient diet. Blood plasma was obtained from oxalated (0.01 M potassium oxalate) blood collected after decapitation. Urine and feces were collected and frozen until analysis. The liver, kidneys, small intestine plus contents and adrenals were removed after death, weighed and immediately extracted. Intestinal contents were included due to the reports of a predominant excretion of vitamin A metabolites in rat bile (9,18,20-22). The tissues were chopped thoroughly with scissors and homogenized in a loose-fitting Potter-Elvejhem homogenizer. After homogenizing, 6 vol (w/v) of methanol (containing 1 mg of propyl gallate per liter) were added and the tissues were further homogenized. The resulting homogenate was centrifuged at 1000 x g x 10 min and the clear supernatant (methanol extract) was removed. The residual material was air dried for three days before it was weighed, sampled, and the radioactivity analyzed. Duplicate aliquots of the methanol extracts and residues, the fecal



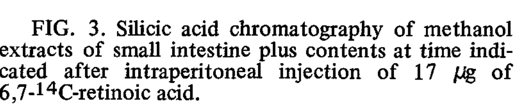


FIG. 3. Silicic acid chromatography of methanol extracts of small intestine plus contents at time indicated after intraperitoneal injection of 17 μg of 6,7-^{14}C-retinoic acid.

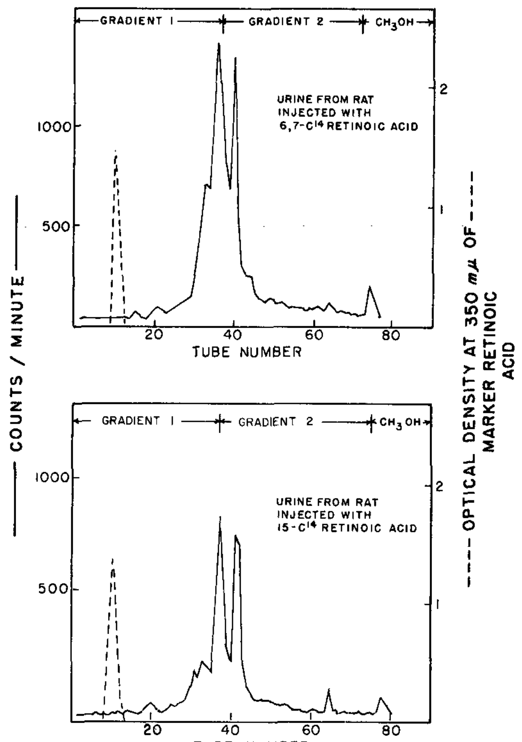


FIG. 4. Silicic acid chromatography of urinary metabolites of retinoic acid collected for a 24 hr period after injection of the labeled compounds.

samples, and duplicate aliquots of the blood plasma and urine samples were also analyzed for radioactivity by a modification of the methods described by Herberg (23). Approximately 10 mg duplicate samples of residues from the methanol extracts were dissolved in 1 ml of hydroxide of Hyamine by heating at 60 C for 24 hr in glass scintillation vials. In some instances it did not appear that the residue had completely solvated, but upon addition of scintillation solution, solvation was achieved. Fifteen milliliters of 0.5% PPO-0.01% POPOP in toluene were added and the samples counted in a Packard Tri-Carb scintillation counter, model 3214. Aliquots of urine and 0.1 and 0.5 ml aliquots of blood plasma were evaporated to dryness by warming under a N_2 stream, 1 ml of hydroxide of Hyamine was added and after heating at 60 C for 20 hr, 15 ml of 0.5% PPO-0.01% POPOP in toluene were added and the samples were counted. Fecal samples were put in scintillation vials and heated with 1 ml of hydroxide of Hyamine at 60 C for 20 hr. Fifteen milliliters of 0.5% PPO-0.01% POPOP in toluene were added. Solvation was not complete but samples were counted as such. Considerable quenching was observed (less than 1% counting efficiency) with the fecal samples. An internal standard (^{14}C-toluene, Packard Instrument Co.) was used in all cases to determine quenching.

Column chromatography of the methanol extracts was achieved as follows. One half to 5.5 ml aliquots of the methanol extracts combined with 1 mg of propyl gallate and 200 μg of nonradioactive retinoic acid were mixed into 2 g of activated silicic acid. This resulted in a slurry which was subjected to evacuation in a vacuum dessicator until a free-flowing powder resulted (up to 4 hr depending on the amount of extract added). This powder was then added to an isooctane head on the top of a column of 6 g of activated silicic acid prepared in isooctane by adding the dry silicic acid to isooctane and allowing it to settle by gravity (usually overnight). The addition of the methanol extract to the silicic acid before application to the column apparently resulted in a deactivation of the silicic acid. This was indicated by an accumulation of the marker reti-

noic acid at the junction of the activated silicic acid and the silicic acid with the adsorbed extract when the initial isooctane solution was run through. The retinoic acid remained at this junction until the proper concentration of diethyl ether was achieved to cause its elution. This resulted in sharp elution patterns similar to what would be obtained if the material had been applied to the column in a small amount of solvent. A gradient elution device adapted from the method of Bock and Ling (24) was used to develop the columns. A concave gradient was achieved by use of two cylindrical chambers of 6.4 cm i.d. for the reservoir and 7.6 cm for the mixing chamber. This concave gradient was used to maximize resolution in the first part of the column in the region retinoic acid is eluted. The mixing chamber was filled with 250 ml of solvent and the reservoir was filled with 190 ml of solvent. The first gradient (gradient 1 in Fig. 1, 2, 3 and 4) was an isooctane-diethyl ether gradient and the second (gradient 2 in Fig. 1, 2, 3 and 4) was a diethyl ether-absolute ethanol gradient. The columns were stripped with methanol. The flow rate was adjusted to 2 ml a minute using a slight N_2 pressure. The columns were run at 25 C in diminished light with 10.4 ml fractions being collected. The final column dimensions were 1.4 x 13 cm. The presence of the marker retinoic acid was determined by taking a total ultraviolet spectrum of the column eluates on a Perkin-Elmer Model 202 recording spectrophotometer. Radioactivity was determined by evaporating the whole fraction or an appropriate aliquot to a residue in a scintillation vial with the aid of a stream of warm air. The fraction residues were then dissolved in 15 ml of 0.3% PPO in toluene and counted. An average of 50% of the applied radioactivity was recovered in the total eluates from the columns.

Study of the Urinary Metabolites Derived From 6,7-^{14}C-Retinoic Acid and 15-^{14}C-Retinoic Acid

For the urinary metabolite study two rats, weighing approximately 225 g, prepared the same as the rats described under the in vivo metabolism experiment, were injected intraperitoneally either with 17 μg (0.39 μc) of 6,7-^{14}C-retinoic acid or 9 μg (0.33 μc) of 15-^{14}C-retinoic acid dissolved in 0.1 ml of absolute ethanol-Tween 80-H_2O (2:1:7 v/v/v). The rats were allowed access to the vitamin A free diet during the 24 hr in which the urine was collected separately from the feces, in test tubes immersed in a dry ice-acetone bath. At the end of 24 hr 5 ml of methanol containing 1 mg of propyl gallate per liter were added and the tubes allowed to warm up until the methanol could be mixed into the urine. Aliquots were taken for radioactivity analysis and subjected to column chromatography as described previously.

Liver Subcellular Distribution of Radioactivity Derived From 6,7-^{14}C-Retinoic Acid

For the liver subcellular distribution study, 1.3 mg (25.9 μc) of 6,7-^{14}C-retinoic acid dissolved in 0.2 ml of absolute ethanol was injected intraperitoneally into two rats (244 and 223 g) nutritionally prepared as described previously. After 3 hr the rats were killed and the livers removed. A subcellular fractionation scheme was employed for the preparation of the particulate fractions. All work was performed at 4 C or on ice. The fresh livers were homogenized in 3.5 vol (w/v) of 0.25 M sucrose with three strokes of a motor driven Potter-Elvejhem homogenizer. The homogenate was centrifuged at 700 x g x 10 min, the supernatant removed, saved, and the precipitate resuspended in 25% of the original volume with 0.25 M sucrose and centrifuged at 700 x g x 10 min. The supernatant was removed and the precipitate washed a second time in a similar manner. A mitochondrial fraction was prepared from the three combined supernatants by centrifugation at 5,000 x g x 10 min with two washes with 25% of the original volume. A lysosomal fraction was prepared by centrifugation of the three mitochondrial supernatants at 15,000 x g x 25 min with two washes with 0.25 M sucrose using 25% of the original volume of the three mitochondrial supernatants. A microsomal fraction was prepared by centrifugation of the three lysosomal supernatants at 105,000 x g x 60 min with one wash in approximately 20 ml of 0.25 M sucrose. The fluffy lipid layer on top of the first 105,000 x g x 60 min supernatant was removed and saved, separate from the clear middle supernatant layer. The lipid layer (plus supernatant contamination) was centrifuged at 105,000 x g x 60 min, the lipid layer removed, resuspended with about 20 ml of 0.25 M sucrose and centrifuged at 105,000 x g x 60 min. After a similar second washing, a lipid fraction was obtained. The clear supernatant fraction was subjected to ammonium sulfate fractionation at 4 C. A concentration of 40% (242 g/liter) ammonium sulfate was achieved by slowly adding the required amount with stirring. The precipitate was collected by centrifugation and washed by resuspension and stirring twice with 30 ml of 40% ammonium sulfate in 0.25 M sucrose. The 40% ammonium sulfate supernatant was concentrated to 60% (131 g/liter more) ammonium sulfate, the pre-

cipitate collected by centrifugation and washed twice with 30 ml of 60% ammonium sulfate in 0.25 M sucrose. The 60% ammonium sulfate supernatant was concentrated to 100% (305 g/liter more) ammonium sulfate. The precipitate plus some undissolved ammonium sulfate was collected by centrifugation and washed twice with 30 ml of saturated ammonium sulfate in 0.25 M sucrose. The 100% ammonium sulfate supernatant was saved as such. A similar fractionation procedure was employed on nonradioactive rat liver for the preparation of tissue blanks for background determinations. All of the protein precipitates were each dissolved or suspended in about 10 ml of distilled H_2O. Protein was determined by the biuret method (25), with a bovine serum albumin standard. It was necessary in the 100% ammonium sulfate fraction to correct for color formation in the presence of ammonium sulfate with an appropriate blank.

Radioactivity was determined on duplicate aqueous samples containing approximately 10 mg of protein. The appropriate aliquots were evaporated to dryness in scintillation vials at 50 C until dry (1-5 hr). Three milliliters of hydroxide of Hyamine were added and the samples were heated at 50 C until solvation was achieved (12-48 hr). Fifteen milliliters of 0.5% PPO-0.01% POPOP in toluene were used as the scintillation solution. Methanol extracts of the aqueous protein solutions or suspensions (about 10 mg protein) were prepared after lyophylization, by extracting the dry protein sample three times with 3 ml of methanol using centrifugation at 1000 x g x 10 min to precipitate the protein each time. The supernatants were combined and aliquots evaporated by heating at 50 C and dissolved in 1 ml of hydroxide of Hyamine and 15 ml of 0.5% PPO-0.01% POPOP in toluene.

RESULTS AND DISCUSSION

The data in Table I show the results of the in vivo metabolism experiment. An analysis of the tissue data shows that retinoic acid is rapidly lost with 60% of the injected radioactivity appearing in the urine after 27 hr. There seems to be a general loss of materials from all tissues. The rates of loss appear to be different as indicated by a relative decrease in the radioactivity in liver and a relative increase in small intestine plus contents, possibly indicating the excretion of metabolites via the bile (20-22). On a relative basis, kidney levels remained constant. There was a generally uniform ability to extract the radioactivity from all the tissues into methanol with an average extraction efficiency of about 85%. Methanol was used to maximize the extraction of more polar metabolites.

Column chromatography of the methanol extracts is shown in Figures 1, 2 and 3. The procedure utilized allowed direct column application of the methanol extracts without prior fractionation. The elution patterns should then be representative of the total amount of material present in an individual tissue at a given time due to the efficiency of extraction, as indicated by the data in Table I and the ability to put the entire extract immediately on the column. Degradation and artifact formation should be minimal. In the columns (not shown) of the extracts of liver and kidney 1 hr after injection, the only radioactive peak was eluted coincident with the marker retinoic acid. This indicated the method was suitable and did not produce artifacts, as had been indicated to be the case with ion exchange chromatography (14).

Chromatography of the intestine plus contents methanol extract (not shown) obtained 1 hr after injection of retinoic acid at the low levels of radioactivity analyzed indicated that most of the radioactivity was eluted with the second gradient and the methanol strip. Only about 5% of the recovered counts from this column were eluted with the marker retinoic acid. Three hours after the injection of retinoic acid the majority of the radioactivity eluted from the columns (Fig 1-3) was coincident with marker retinoic acid in the liver (62%) and the kidney (73%) extracts. Part of the end of gradient 1 in the column of the metabolites of liver after 3 hr was lost, causing its shortened nature. Only 2% of the total radioactivity in the intestinal extract was eluted coincident with marker retinoic acid. Column chromatographic analysis of the extracts 9 hr after injection of retinoic acid (not shown) also indicated the major radioactive material in liver and kidney to be retinoic acid with again a predominance of polar metabolites in the intestine plus its contents. At 27 hr (Fig. 1-3) there were traces of retinoic acid in liver and kidney with the major amount of radioactivity in several other compounds. At all times in the intestine and its contents, almost all of the radioactivity was present in materials eluted at more polar solvent concentrations.

The data show retinoic acid to be present in small amounts in the liver and kidney at least 24 hr after injection of these small, physiological doses. There was, however, a considerable metabolism to other compounds with the excretion of most of the administered radioactivity. It is not possible to relate precisely the

TABLE I

Tissue Distribution of Radioactivity After Intraperitoneal Injection of 17 μg (0.39 μc) of 6,7-^{14}C-Retinoic Acid

Time after injection, hr		Rat	Liver	Small intestine *plus* contents	Kidneys	Adrenals	Blood plasma	Urine	Feces
1	Weight, g	231	9.3	6.2	2.3	0.0415		0.8[a]	
	Total dpm		21,600	7,860	1,860	26	341[b]	1,070	
	% dpm in methanol extract		83	82	93	89			
	Relative %[c]		69	25	6				
3	Weight, g	219	8.5	6.8	1.9	0.0404		2.7	
	Total dpm		204,000	162,000	42,800	150	6,550	33,200	
	% dpm in methanol extract		89	91	91	93			
	Relative %		50	40	10				
9	Weight, g	217	6.8	7.2	1.6	0.0433		2.0	
	Total dpm		9,590	15,200	1,900	<10	418	14,900	
	% dpm in methanol extract		88	90	86				
	Relative %		36	57	7				
27	Weight, g	187	6.9	4.6	1.7	0.0528		11.3	
	Total dpm		9,570	20,200	2,800	28	649	525,000	159,000
	% dpm in methanol extract		75	90	79	72			
	Relative %		29	62	9				

[a]Total volume in ml.

[b]Dpm per milliliter of plasma.

[c]Per cent of total liver, small intestine plus contents and kidneys dpm from each individual rat.

present observed metabolites to compounds indicated in the literature, however, some possible relationships can be suggested. It is possible that one or more experimental variables such as method of administration, dosage level, vitamin A nutritional status of an animal, time of analysis after dosing, the tissue analyzed, and the species of animal studied could have an influence on the metabolites present. The present data indicate that metabolites of similar chromatographic character were obtained from several of the individual rat tissues at the different times of analysis after injection. More specific studies with more animals will be necessary to define more thoroughly the flow and extent of accumulation of these materials in the various tissues. Definite pathways of metabolism can then be postulated.

A material eluted immediately following retinoic acid appeared in the extracts of all three tissues studied 27 hr after injection. A material eluted at a similar position was also seen in the extract of intestine 3 hr after injection and the extract of liver 9 hr after injection (not shown). This is the predominant material which increases in relative concentration with time in all the tissues studied and which is not chromatographically similar to the major urinary metabolites. This compound may be a *cis* isomer. The 13-*cis* isomer elutes somewhat faster from silicic acid than all-*trans* retinoic acid (13). Methylated *trans* and *cis* isomers have variant chromatographic properties (14).

Small amounts of compounds similar in chromatographic character to the urinary metabolites were seen in the kidney 3 hr after injection. A compound chromatographically similar to the urinary metabolites was found in the intestine plus contents at both 3 and 27 hr after injection of retinoic acid, in addition to the presence of more polar materials. The major metabolite in liver 3 hr after injection of retinoic acid was chromatographically similar to the urinary metabolites. A compound was seen in the liver at 3 and 27 hr which was more polar in chromatographic character than the urinary metabolites, but similar to the polar intestinal materials. These polar liver and intestinal materials may be similar to the various conjugated forms of retinoic acid found in rat bile (20-22). Zile and DeLuca (26) found metabolites in the liver more polar than retinoic acid and Yagishita et al. (15) found metabolites in the intestine more polar than retinoic acid.

The results of the column chromatography of the urinary metabolites are shown in Figure 4. An analysis of the excreted metabolites from retinoic acid labeled at each end of the system

TABLE II

Liver Subcellular Distribution of the Radioactivity Derived From Intraperitoneally Injected 6,7-^{14}C-Retinoic Acid

Fraction	dpm/mg Protein
Nuclei and cell debris	192 ± 2[a]
Mitochondria	239 ± 11
Lysosomes	324 ± 3
Microsomes	340 ± 3 (82%)[b]
Soluble Supernatant	
0-40% ammonium sulfate precipitable	153 ± 1
40-60% ammonium sulfate precipitable	211 ± 1
60-100% ammonium sulfate precipitable	773 ± 28 (93%)
100% ammonium sulfate soluble	422 ± 4
Lipid (fluffy) layer	128 ± 2

[a]Range of duplicate samples subjected to radioactivity analysis.

[b]Percentage of radioactivity extractable into methanol.

of conjugated double bonds should give an indication of the extent of cleavage of the molecule at any point between the radioactive atoms. Two cleavage products would probably be different in their elution patterns from a column. The radioactivity elution pattern of metabolites derived from a molecule should then be different from a compound labeled on one side of the point of cleavage versus a compound labeled on the other side of the point of cleavage.

Fifty-eight per cent of the radioactivity of the injected 6,7-^{14}C-retinoic acid and 32% of the radioactivity of the injected 15-^{14}C-retinoic acid were recovered in the urine after 24 hr with respectively 14 and 11 ml of urine voided. Roberts and DeLuca (16) obtained a lesser amount of radioactivity in urine from 14- or 15-^{14}C-retinoic acid compared to 6,7-^{14}C-retinoic acid with urinary excretion essentially complete after one to two days. These results suggested the occurrence in urine of metabolites with the side chain not cleaved as well as metabolites with at least the C-15 and C-14 carbon atoms cleaved and converted to CO_2. The lack of any chromatographically different radioactive urinary metabolites from the 6,7-^{14}C-retinoic acid and the 15-^{14}C-retinoic acid (Fig. 4) suggest that these materials represent the metabolites in which the conjugated double bond chain is not cleaved. The postulated cleaved metabolites may have been eluted with the intact metabolites or may have remained on the column. Column recoveries from the urinary 6,7-^{14}C-retinoic acid metabo-

lites and the 15-^{14}C-retinoic acid metabolites were 50% and 40%, respectively. Dunagin et al. (18) have shown that the biliary metabolites obtained from rats injected with 6,7-^{14}C-retinoic acid or 15-^{14}C-retinoic acid have a similarity in their column chromatographic properties and have suggested that the biliary metabolites are not cleaved. The urinary metabolites have column elution properties similar to the fraction M-1 obtained by Zile and DeLuca (26).

To test the possibility that the urinary metabolites may be ester conjugates, an aliquot of the urine containing metabolites from 6,7-^{14}C-retinoic acid was made 0.1 N with NaOH, diluted with an equal volume of methanol and warmed at 45 C for 3 hr. The column chromatographic pattern of the resultant materials indicated no retinoic acid or compounds of greatly altered chromatographic character.

The results of a subcellular distribution study of the radioactivity derived from 6,7-^{14}C-retinoic acid are shown in Table II. The study was performed in an attempt to further define the site of action of the vitamin. The association of a large amount of radioactivity with a particular fraction would suggest a physiological significance of the vitamin in that fraction. The results indicate radioactivity to be located throughout all fractions. There is a base level of about 150-250 dpm/mg protein which possibly represents nonspecific binding to protein. There are two areas of a higher specific activity, the lysosomal-microsomal fraction and the 60-100% ammonium sulfate precipitable and 100% ammonium sulfate soluble fractions of the soluble supernatant. Two of these fractions were extracted with methanol after lyophylization to determine if there was covalent bonding of the radioactivity to methanol insoluble protein. The data in Table II indicate as thorough an extraction of radioactivity into methanol as was the case with the whole tissue methanol extracts. The level of retinoic acid given these rats was larger than desirable but was necessitated by limits in methods of detection. It may be that the size of doses, the method of administration, the time of killing after dosing, the tissue studied, or the vitamin A status of an animal could affect the subcellular localization of the radioactivity and the nature of the compounds. A thorough analysis would require a study of the effect of these variables.

Future problems will be concerned with determining optimal conditions for obtaining sufficient quantities of these various metabolites so that chemical characterization and thorough biological analyses can be performed. A final elucidation of the pathways of metabolism and the nature of the active forms of the vitamin will then unquestionably greatly aid the efforts of investigators concerned with determining the biochemical function of the vitamin in its growth promoting role.

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Obligatory Role of Bile for the Intestinal Absorption of Vitamin E

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ABSTRACT

Normal, white female rats subjected to cannulation of the abdominal thoracic duct have been utilized for a study on the essentiality of biliary and pancreatic secretions for the intestinal absorption of vitamin E. In all animals the thoracic duct lymph was collected. Some rats had the enterohepatic circulation undisturbed and in others bile or pancreatic juice or both were drained to the exterior by appropriate catheters in the common bile duct. On the first postoperative day, the animals received intragastrically an emulsion containing protein, carbohydrate, monoolein, 2 mg of *d,l*-α-tocopheryl acetate plus 50 μC of *d,l*-α-tocopheryl-1′,2′-^{3}H-acetate. The appearance of radioactive α-tocopherol and its derivatives was determined in lymph, hourly, after emulsion administration. The obligatory role of bile in intestinal absorption of *d,l*-α-tocopheryl-1′,2′-^{3}H-acetate has been established. Pancreatic juice seems to be necessary for the hydrolysis of the vitamin E acetate ester. The simultaneous infusion of bile and pancreatic juice promotes absorption of about 10% of the administered dose into the lymph. A chromatographic separation of the radioactive vitamin E fractions revealed that most of the vitamin E, which is actively transferred from the intestinal lumen to the lymph, is nonesterified. An oxidation product of α-tocopherol, presumably its *p*-quinone, appears in small amounts in the lymph, but almost no labeled α-tocopheryl acetate could be detected under these experimental conditions.

INTRODUCTION

Although it has been recognized for many years that bile salts and pancreatic secretions affect the absorption of dietary vitamin E (1,2) evidence on this matter has been incomplete (3). Infants with congenital atresia of the bile ducts or with cystic fibrosis of the pancreas have diminished concentrations of plasma tocopherol and creatinuria and show an abnormal erythrocyte hemolysis test. These defects are presumably due to poor absorption of the vitamin in the absence of biliary and pancreatic secretions from the intestine. However, this interrelationship has not been clearly substantiated.

Most studies made in animals and humans have employed either metabolic balance techniques estimating tocopherol absorption from measurements of tocopherol intake and fecal or urinary excretion (4-8) or measurements of changes in the vitamin levels of plasma, liver and other organs (9-15). Although these studies have provided excellent information about the distribution of small amounts of vitamin E and its metabolites in the body, the use of such techniques makes interpretation of the absorption data difficult.

Direct evidence for the absorption of ^{14}C-α-tocopherol into the intestinal lymph of rats was reported by Johnson and Pover (16). Since in that study samples of lymph were pooled over variable periods of time from 27 to 96 hr after feeding of the labeled vitamin, observations on the kinetics of absorption during the postabsorptive period were precluded. Blomstrand and Forsgren (17) have recently reported on the intestinal absorption of several labeled tocopherols in man. However, no complete study on the effect of biliary or pancreatic secretions or both on vitamin E absorption is available in the literature.

It was, therefore, considered important to carry out a study on the influence of biliary or pancreatic secretions or both on the intestinal absorption of vitamin E. This investigation was facilitated by the recent development of a new technique for the cannulation of the abdominal thoracic duct in the rat (18) and the availability of *d,l*,-α-tocopheryl-1′,2′-^{3}H-acetate. In addition, the total radioactivity and the vitamin E radioactive fractions of lymph were determined hourly in an attempt to obtain a more detailed picture on the kinetics of absorption of vitamin E in the presence or absence of bile.

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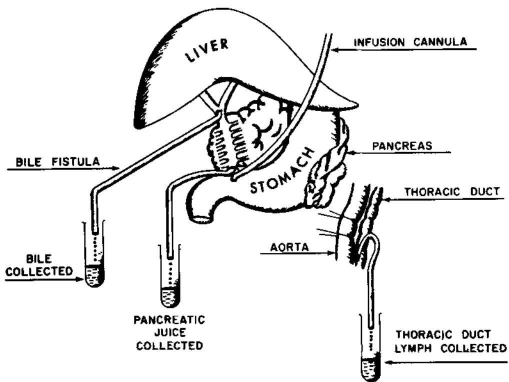

FIG. 1. Thoracic duct cannula and triple catheterization of the common bile duct in the rat. This preparation was used in some of the studies reported here.

MATERIALS AND METHODS

Female albino rats (300-350 g) of the CD Charles River Breeding Laboratories (North Wilmington, Mass.) were maintained on a standard Purina chow diet and water ad lib until the time of operation.

Surgery

The detailed procedure for the cannulation of the abdominal thoracic duct in the rat has appeared (18). In some instances, rats with thoracic duct fistula but with reconstituted enterohepatic circulation were used. In other instances, the animals had a lymph fistula plus two catheters in the upper part of the common bile duct; through the distal catheter, the infusion cannula, 3.6 ml of saline (0.85% NaCl plus 0.04% KCl) were infused. A third preparation used in the present studies consisted of a thoracic duct fistula plus three catheters in the common bile duct (Fig. 1). Use of this preparation permitted the infusion into the duodenum of either saline alone (duodenum devoid of bile and pancreatic secretions) or saline plus bile uncontaminated with pancreatic juice; in both instances the animal's own bile and pancreatic juice were diverted from the intestine. After operation, the animals were placed in a restraining cage (19) made by C. H. Stoelting Co., Chicago, Ill.

EXPERIMENTAL PROCEDURES

Absorption experiments began 18 to 24 hr after operation, according to the protocol reported for studies on cholesterol absorption (20). The detailed composition of the emulsion used has been described elsewhere (20). It contained basically 50 μC (140 μg) of an ethanolic solution of pure *d,l*-α-tocopheryl-1′,2′-^{3}H acetate (see below), protein, carbohydrate, monoolein, saline and 2 mg of *d,l*-α-tocopheryl acetate in 50 μl ethanol. Both the carrier and the labeled vitamin E ester were added to tubes containing 4 ml of the emulsion and mixed by means of a Vortex Jr. Mixer (Model K-J, Scientific Industries, Inc.). Animals were given the emulsion by stomach intubation without the aid of anesthesia. Lymph was collected at hourly intervals for 12 hr. Bile samples were also collected along with the lymph in the cases of animals with common bile duct catheterizations.

Purification of Radioactive Material

D,l-α-tocopheryl-1′,2′-^{3}H-acetate (F. Hoffmann-La Roche & Company, Ltd., Basle, Switzerland) had a specific activity of 356 μC/mg in 1962. By using glass fiber paper chromatography techniques described below, it was found that the original material contained breakdown products of α-tocopherol, and, therefore, it was purified by passing it through a 15 x 3.5 cm column of silicic acid (100 mesh, Mallinckrodt). After activation and packing, the column was first washed with 400 ml of *n*-hexane (Chromatoquality Reagent, 99 + mole %, Matheson Coleman & Bell, East Rutherford, N.J.); 90 mg of the tritiated α-tocopheryl acetate were dissolved in 5 ml *n*-hexane and poured into the column. Mixtures of *n*-hexane-benzene (100:5 up to 100:50) were used to successively wash the column. Benzene (Reagent, A.C.S., Lehigh Valley Chemical Co., Easton, Pa.) was distilled before use and kept dry by the addition of calcium chloride to the bottle. Eluents of each solvent mixture were collected in 25 ml fractions; 5 μl aliquots were spotted in small portions on glass fiber paper and the radioactivity counted in a Packard Scintillation Spectrometer by procedures similar to those described for the counting of cholesterol and its esters (20). Most of the radioactivity was found in the eluates consisting of a mixture of hexane-benzene, (100:35). Final purity of the *d,l*-α-tocopherol ester was checked by glass fiber chromatography immediately prior to use. Using suitable standards of free α-tocopherol and *d,l*-α-tocopheryl acetate (both from Hoffmann-La Roche Inc., Nutley, New Jersey), it was found that the fraction corresponding to the radioactive tocopheryl acetate was more than 99% pure.

Vitamin E Extraction Procedures

Lymph samples were extracted immediately after collection. The method used for the

extraction of lipids from lymph is reported in detail elsewhere (20). Briefly, this procedure involved extraction of the lymph with ethanol-isopropyl ether (2:1 v/v) and in the ratio of 1 ml of lymph per 20 ml of extracting mixture. After centrifugation, the supernatant was dried under N_2 and the samples were made up to a known volume (0.3 to 1.0 ml) with *n*-hexane, and aliquots of 100 μl used for chromatography. Absolute ethanol (U.S.P. 200 Proof, Distilled by Publicker Industries, Inc., Philadelphia, Pa.) was freed of aldehydes by redistillation over $KMnO_4$ (Baker Analyzed Reagent, J. T. Baker Chemical Co., Phillipsburg, N.J.) and KOH pellets (Fisher Scientific Co., Fair Lawn, N.J.) and kept in dark bottles until use. Isopropyl ether (Practical, Matheson Coleman & Bell, East Rutherford, N.J.) was freed of peroxides by procedures reported previously (20). The lymph extraction mixture was prepared immediately after purification of isopropyl ether.

Chromatography and Radioactivity Measurements

The separation of free tocopherol from tocopheryl acetate and from other lipid components was carried out by glass paper chromatography using glass fiber paper obtained from Gelman Instruments Co., Ann Arbor, Michigan (ITLC-SG) and employing two solvent systems. The first solvent system consisting of isooctane-benzene (100:15 v/v) is the same used for the separation of cholesterol and cholesterol esters in blood (21) and lymph (20). Using this system, free tocopherol traveled just below cholesteryl arachidonate and separated quite well from α-tocopheryl acetate, which traveled much lower than the unesterified vitamin. Oxidation products of α-tocopheryl remained at the origin of the chromatogram. These oxidation products were chromatographed by using an acidic solvent system consisting of isooctane-ethyl ether-formic acid (100:10:0.5). Anhydrous ethyl ether (Reagent A.C.S.) and formic acid (both purchased from Allied Chemical, Morristown, N.J.) were used without further purification. Using this solvent system, free tocopherol and its acetate ester traveled close to the solvent front. The oxidation product comparable to the one obtained after reaction of α-tocopherol with $FeCl_3$ in absolute ethanol, presumably the α-tocopheryl-*p*-quinone (22), remained distinctly isolated in the middle of the chromatogram.

Glass fiber paper chromatography of the lymph extracts was done in duplicate and excellent agreement was obtained. Suitable mixtures of standards were applied to one side of the chromatograms as 5 μl (0.4 μg) aliquots; after developing and drying of the chromatogram, the side (about 3 cm wide) containing the mixtures of standards was cut, sprayed with concentrated sulfuric acid and charred over a hot plate. The corresponding radioactive bands were systematically removed and prepared for counting as described (20). The counting was carried out in a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3380, with Model 544 Absolute Activity Analyzer attachment. The results are thus expressed in dpm per hour of lymph production.

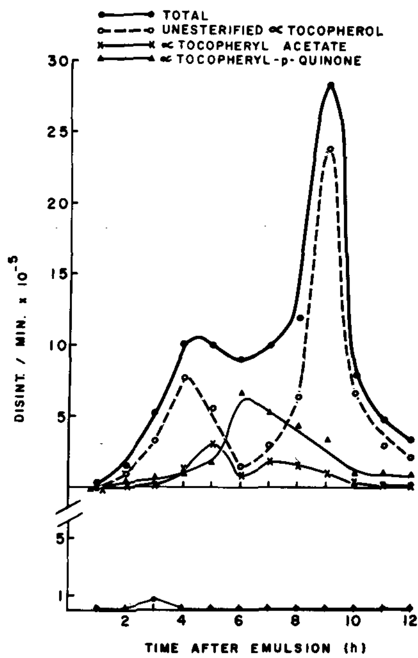

FIG. 2. The appearance of radioactive α-tocopherol and derivatives in the thoracic duct lymph of rats at hourly intervals in the presence of biliary and pancreatic secretions (upper graph) or in their absence (lower graph). In both instances, an emulsion was administered containing protein, carbohydrate, monoolein and tritiated α-tocopheryl acetate. Each graph is representative of three experiments.

RESULTS

Simultaneous Effect of Bile and Pancreatic Juice on Tocopherol Absorption

Three animals were prepared in which a thoracic duct fistula was performed but had the enterohepatic circulation reconstituted by an external shunt. Saline was given ad lib. The emulsion, containing α-tocopheryl-1′,2′-$^3H_2$-acetate was given 20 hr after operation. The appearance of radioactive α-tocopherol and its derivatives in lymph is shown in Figure 2. Two

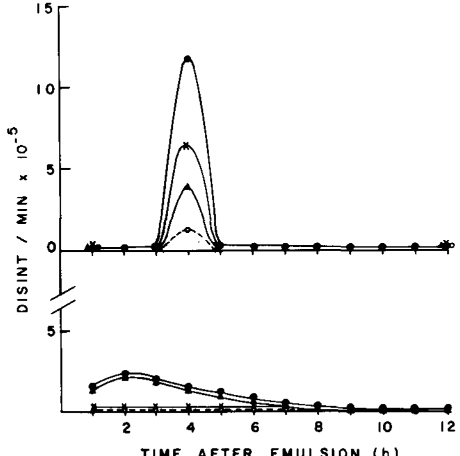

FIG. 3. The appearance of radioactive α-tocopherol and derivatives in the thoracic duct lymph of rats when either pancreatic juice (upper graph) or bile (lower graph) is present in the intestine. Other experimental conditions as described in Figure 2. Each graph is representative of three experiments.

distinctive radioactivity peaks are seen, an early peak, occurring at 3 or 4 hr after feeding and a second, more significant peak, occurring 6 to 9 hr after administration of the emulsion. Most of the radioactivity was found in the form of free tocopherol; however, there was some in the form of an oxidized product, presumably α-tocopheryl-*p*-quinone and a very small amount in the fraction corresponding to the tocopheryl acetate ester. About 10% of the administered dose of radioactivity was recovered in the 12 hr following the intragastric administration of the emulsion under these experimental conditions.

In a second group of experiments, three animals were prepared in which a thoracic duct was created and both biliary and pancreatic secretions drained to the exterior by means of a cannula in the lower part of the common bile duct; through the same opening on the bile duct, another catheter was inserted and introduced 2-3 mm into the intestine. This cannula was used to infuse saline (3.6 ml/hr) into the duodenum. Negligible amounts of radioactivity were detected in the thoracic duct lymph of the rats under these circumstances (Fig. 2). There was no detectable peak absorption in any of the three experiments and the amount of radioactivity appearing in the lymph was 0.1% of the administered dose (on the average) during the 12 hr after feeding. At this extremely poor level of absorption, there was a predominance of the oxidized form of the tocopherol and virtually no free tocopherol appeared in lymph during this period.

The Effect of the Infusion of Pancreatic Juice Or Bile Alone on Tocopherol Absorption

The previous experiments showed that both pancreatic and biliary secretions, when given simultaneously, promote a considerable enhancement of the absorption of vitamin E from the intestine of the rats. It was decided, therefore, to study the effect, separately, of each of these two secretions.

Three rats were used in which bile was drained to the exterior, pancreatic juice left undisturbed, and a thoracic duct fistula created. The animals were hydrated as described in the Materials and Methods section. Figure 3 shows that, after administration of the radioactive α-tocopheryl-acetate-containing emulsion, the absorption rate of tocopheryl is very much reduced owing to the lack of bile. An average of 1.5% of the total radioactivity administered was recovered in the 12 hr following feeding. There was an absorption peak by the third or fourth hour. Most of the radioactivity found in lymph was due to the labeled α-tocopheryl acetate and significant amounts were found in the oxidized form. Radioactive free tocopherol was either absent or very low during the 12 hr of lymph collection.

In a last group of experiments, the effect on tocopherol absorption of infusion of bile alone into the duodenum was studied. For this purpose, bile, uncontaminated with pancreatic juice (18), was collected for up to one week from several rats and the pooled collections kept at 4 C until use. Three animals were cannulated in the thoracic duct and thrice cannulated in the common bile duct (Fig. 1). The catheter in the upper part of the common bile duct drained pure bile; one of the two catheters in the lower part of the common duct was used to drain pure pancreatic juice from the animal's body. Through the infusion cannula, each rat received 1.5 ml bile (from experimental data on bile flow in these well hydrated fistulated animals, it has been found that from 0.8 to 1.5 ml/hr of bile is produced) plus 2.1 ml saline per hour into the intestine. Owing to the lack of pancreatic juice, a severely reduced rate of absorption was observed in lymph during the 12 hr following administration of the emulsion containing radioactive α-tocopheryl acetate (Fig. 3). It was seen that there is no real peak absorption; total radioactivity was somewhat higher during the first 5 hr after feeding, and about 1.3% of the administered radioactive dose was recovered in the 12 hr lymph collection. As in the previous instances of poor absorption, the fractions of vitamin E corresponding to the oxidized form and to tocopheryl acetate had most of the radioactivity

and very little, if any, free tocopherol was found.

DISCUSSION

The studies reported here are the first in which the appearance of radioactive tocopherol fractions has been followed as a function of time during conditions of presence and absence of bile in the intestine. This was accomplished after feeding identical amounts of the radioactive *d,l*-α-tocopheryl acetate in an emulsion. Since the same concentration of the vitamin E acetate ester was administered in each instance, it was possible to carry out appropriate comparisons under the several conditions studied. Infusion of saline at a constant rate has assured not only an adequate hydration, but a uniform lymph flow as well (18). The results of this study have provided definitive evidence that the presence of bile salts and pancreatic juice in the intestine is a requisite for the intestinal absorption and the lymphatic transport of vitamin E.

During the simultaneous infusion of bile and pancreatic juice into the duodenum, labeled vitamin E appears in lymph mostly as the unesterified tocopherol and very little as the tocopherol ester. Two peaks of radioactivity appear and this biphasic type of kinetics suggests the existence of a dual mechanism of absorption. Data obtained indicate that the infusion of pancreatic juice alone can support appearance of vitamin E in lymph, which, quantitatively, is very similar to that seen in the early peak of absorption under conditions of reconstituted enterohepatic circulation. However, qualitative differences were evident and more experiments would be necessary to further clarify this observation.

The recovery of 10% of the radioactivity from the administered dose agrees with that reported by Simon et al. (6) after feeding *d*-α-tocopheryl-5-methyl-^{14}C-succinate to rabbits. However, in the present study, unlike that of Simon et al. (6), no recirculation of the absorbed isotope to the intestine is possible because the absorbed labeled vitamin E is diverted outside the animal's body owing to the lymph fistula. Since data reported herein were obtained under ideal conditions for absorption, i.e., normal animals with undisturbed enterohepatic circulation to which a physiological dose of tocopheryl acetate (2.14 mg) was administered in a fat-containing emulsion, it can be concluded that α-tocopheryl acetate is a fat-soluble ester which is poorly absorbed from the intestinal tract, even though this compound is liquid at room temperature and presumably might be soluble in micellar bile salt solutions.

The poor absorption of the vitamin E acetate ester is in sharp contrast with that of other lipid materials, notably, glycerides, which are absorbed, after intestinal digestion, with an efficiency close to 100%. This observed dissimilarity is perhaps indicative of a lack of parallelism between vitamin E and glycerides during digestion, intestinal absorption and lymphatic transport. From the above considerations, it is also clear that absorption data, based on fecal excretion of a substance or its metabolites after feeding of the radioactive compound, should be interpreted with caution, especially if the compound, as in the case of vitamin E (23), undergoes an enterohepatic circulation. Recirculation of the absorbed radioactive material might possibly affect the fecal excretion data.

The appearance of radioactive vitamin E in lymph was at a minimum in the absence of either bile or pancreatic juice. Two main radioactive fractions are detected in lymph under these very poor absorption conditions: the α-tocopheryl-*p*-quinone and traces of the administered ester; unesterified vitamin E is almost completely absent under these conditions. These data suggest that the small quantity of radioactive vitamin E that appears in lymph in the presence of only pancreatic juice in the lumen is the result of an entirely different and inefficient mechanism than the one operating when both bile and pancreatic juice are present in the duodenum. These findings clearly emphasize the obligatory role of biliary secretions in the absorption of α-tocopheryl acetate. They also explain why vitamin E deficiency syndrome develops under conditions in which there is insufficient bile or pancreatic juice in the gastrointestinal tract (1,2).

The requirement for bile indicates that emulsification of this water-insoluble vitamin is required prior to its penetration into the intestinal mucosa. Pancreatic juice presumably might contain an enzyme (α-tocopherol ester hydrolase) the existence of which, as well as its site of action (intraluminal, surface or interior of the intestinal cell), remains to be demonstrated. Indeed, from the present studies, it is evident that in the rat the first step in the absorption of α-tocopherol ester is hydrolysis. In those cases of good absorption, there was extensive hydrolysis of the administered α-tocopheryl acetate, which presumably occurred in the intestinal lumen, and accounts for the virtual absence of tocopheryl acetate in the lymph. This absence of vitamin E ester in the lymph of the rat is in complete agreement with reported data on the absorption and distribution of α-tocopheryl acetate in chicks (8). Aside from its

emulsifying properties, the presence of bile salts could be a requisite for the hydrolysis of α-tocopherol acetate prior or during penetration of vitamin E into the mucosal cell. It was demonstrated by Greaves and Schmidt (1) that the oral administration of deoxycholic acid increases the absorption of vitamin E in bile-fistulated rats, thus, these authors provided indirect evidence that an individual bile acid is able to promote the absorption of vitamin E. It would, therefore, be of interest to demonstrate whether free or conjugated individual bile acids promote the absorption of unesterified or esterified vitamin E or both at different rates into the thoracic duct lymph.

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Correlation Between Post-Heparin Lipase and Phospholipase Activities in Human Plasma[1]

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ABSTRACT

The hypothesis that a single lipolytic enzyme in post-heparin plasma effects the hydrolysis of both triglyceride and phospholipid was tested. After intravenous heparin, activity in plasma with the two substrate classes appeared and disappeared in parallel. The activities were not separable by the fractionation methods of zone electrophoresis, gel filtration, anion-exchange, ultracentrifugation, or by combinations of these techniques. The degree of purification of the two activities with the use of *n*-butanol was similar. Lipolytic activity appeared to be associated with a large high density molecular aggregate. However, the concept of a single post-heparin enzyme does not explain all the observations since the ratio of activity with triglyceride substrate to activity with phospholipid substrate decreased markedly in some subjects after increased amounts of intravenous heparin.

INTRODUCTION

It has been shown that post-heparin plasma of man contains a phospholipase activity that degrades phosphatidyl ethanolamine (PE) and phosphatidyl choline (PC) to their lyso derivatives LyPE and LyPC (1,2). In contrast to the site of action of snake venom phospholipase or of heat-treated human pancreatic extract, post-heparin plasma phospholipase activity attacks the fatty acid (FA) at the α' (C-1) position of egg PC or PE (2,3). In post-heparin rat plasma the endogenous plasma lipoprotein PC can serve as a substrate for the phospholipase activity which is also α' specific (4,5). In addition, evidence has been obtained for an in vivo action of post-heparin phospholipase activity in conjunction with that of the well-known post-heparin lipase activity (mainly on plasma very low density lipoprotein) (6).

The similarities of optimal incubation systems with phospholipid (PL) or triglyceride (TG) substrate, of effects of inhibitory factors, and of in vivo action led to the suggestion that a single heparin-released lipolytic enzyme was involved (1,2,6). This concept was strengthened by the demonstration of (a) the positional specificity with PL substrate; (b) the presence in various animal tissues of α' specific phospholipase activity (7,8); and (c) the isolation from tissue of a single enzyme, active on both TG and PL (α' specific) substrates (9). In the present study, the possibility that the hydrolysis of both TG and PL results from the action of a single post-heparin lipolytic enzyme, was investigated.

MATERIALS AND METHODS

Post-heparin plasma was obtained from blood samples collected 10 min after a test dose (10) of 0.1 mg/kg body weight or 15 min after 5000 units (50 mg) IV heparin, in tubes containing the anticoagulant disodium ethylenediaminetetraacetate (1 mg/ml whole blood). Large collections of 400 ml blood were obtained 15-22 min after 10,000 units (100 mg) IV heparin (Invenex Pharmaceuticals, San Francisco, Calif.) in bottles containing sodium oxalate (1 mg/ml) anticoagulant. The separated plasma was frozen in aliquots of 5.5 ml after a processing interval of 1.5-2 hr at about 22 C. It was accepted that post-heparin lipolytic activity had effected some changes in plasma lipoproteins in vitro. Such changes proceed even at 4 C (11).

Egg PE and PC were obtained from a crude egg yolk preparation (2), PE after a chromatographic separation on a column of silicic acid (1) and PC after elution from a column of alumina (12). Other materials used included: Ediol, a 50% coconut oil emulsion (Calbiochem, Los Angeles, Calif.); olive oil (Matheson, Coleman and Bell, Los Angeles, Calif.); and bovine albumin, fraction V (Armour Pharmaceutical Co., Kankakee, Illinois).

Lipid substrates, prepared as previously described (1,2) contained: 90 μmoles of PC in 7.0 ml of 0.1 M glycine-NaOH (pH 9.6) containing 80 μmoles of deoxycholate; 52 μmoles of PE or olive oil in a 5.0 ml solution containing 400 mg albumin and 0.45 ml 1 M

[1] Presented in part at the AOCS Meeting, Washington, D.C., April 1968.

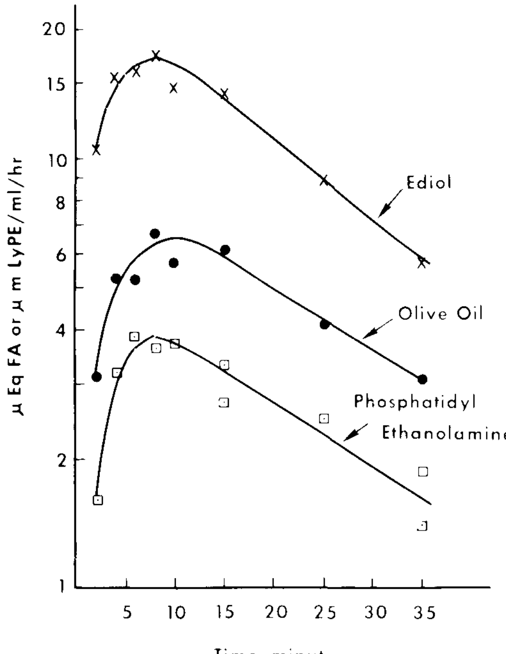

FIG. 1. The turnover of lipolytic activity in post-heparin plasma. Plasma was obtained at intervals of 2-35 min after a test dose of heparin (0.1 mg/kg body wt.). Lipolytic activity was measured with Ediol, olive oil and PE substrate.

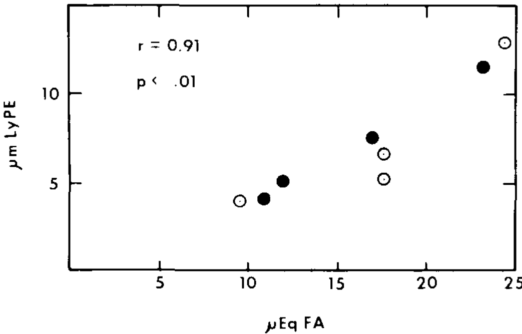

FIG. 2. The correlation of post-heparin lipolytic activity measured with phospholipid or triglyceride substrate. Four subjects were maintained on a moderate fat intake (40% fat calories) and later on a fat-free diet. On each diet plasmas were obtained 10 min after heparin (0.1 mg/kg body wt.). Each plasma activity (ml/hr) with PE substrate was plotted against the activity with olive oil substrate. Solid circles are of activities measured on the fat-free diet.

$(NH_4)_2SO_4$ (pH 9.1 with NaOH); 1.0 ml of diluted Ediol reagent (1 to 10 with 0.15 M NaCl) plus 5.0 ml of albumin-$(NH_4)_2SO_4$ solution above.

Assays of lipolytic activity with PE, olive oil and Ediol were carried out by incubation at 38 C of 1.0 ml samples with the substrates described above. The method of Dole and Meinertz (13) was used to measure FA release in incubations with olive oil or Ediol substrate. Quantification of LyPE formed from PE substrate was obtained by a measurement of the phosphorus of the LyPE fraction separated chromatographically on silicic acid impregnated paper (1,2). To monitor enzyme purification procedures, a qualitative demonstration of the location or distribution of lipolytic activity was often considered sufficient. For this purpose a 10 μl aliquot of each incubation mixture (with occasional variation in the volume ratios of substrate to tested samples) was applied directly to a silicic acid chromatoplate. The plates were developed for phospholipid or neutral lipid separation (1-3). For detection of hydrolysis of PE, a spray of 0.15% ninhydrin in lutidine-acetone (10:90 v/v) was used.

Zone electrophoresis of post-heparin plasma was accomplished on blocks of starch or on the synthetic polymer, Pevikon C-870 (Fosfatbolaget, Stockholm, Sweden).

Gel filtration of post-heparin plasma, and purification of lipolytic activity upon an anion-exchange medium, employed Sephadex G-200 and DEAE-Sephadex (both of particle size 40-120 μ). These media were prepared and used in Sephadex column assemblies in accordance with recommendations of the manufacturer (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.).

The absorption at 280 mμ of solutes in column effluents was continuously monitored with a UV absorption meter and fraction collector assembly (Gilson Medical Electronics, Middleton, Wis.). A simultaneous record was obtained of fraction collection and absorption at 280 mμ in per cent Transmission (Model RR Recorder, Texas Instruments, Inc., Houston, Texas). A faulty 280 mμ interference filter was used in the experiments of Figures 5 and 6. The distribution of peak components was correctly recorded, however the magnitude of decrease of per cent transmission was reduced.

RESULTS

Turnover of Plasma Lipolytic Activity

Plasma lipolytic activities with TG or PE substrates have a similar turnover in the circulation after a test dose (10) of IV heparin. One of two studies showing similar results is shown in Figure 1. With PE or 2 TG substrates, the same order of appearance, peak and decline of activity was obtained in plasma during intervals

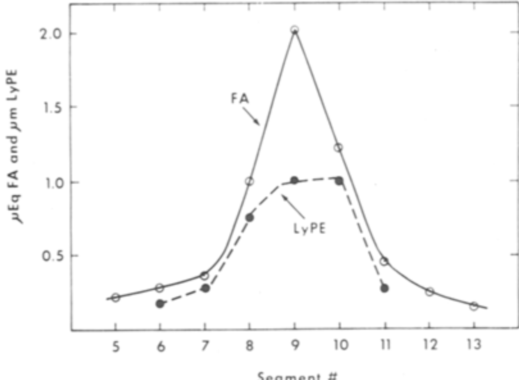

FIG. 3. Location of lipolytic activity after zone electrophoresis of post-heparin plasma. Fifteen minutes after 5,000 units of IV heparin, plasma was obtained without and with the inhibitor diethyl-*p*-nitrophenyl phosphate (11). The plasmas were kept chilled until applied to a block of Pevikon. Fifteen milliliters of uninhibited and 1.5 ml of inhibited plasma samples were applied in separate parallel slits. Electrophoretic conditions as previously described (14): barbital buffer, pH 8.6, 0.1 μ; 5 V/cm for 26 hr at 25 C block temperature, 18-20 C environmental temperature. Segments of the block (1/2 in.) were cut from the point of application towards the anodal end. Each segment (uninhibited plasma separation) was thoroughly mixed with 7.5 ml of 0.15 M NaCl and the Pevikon sedimented by centrifugation. Activity in supernatants was measured with olive oil and PE substrate in 4 hr incubations. The ratio of activity olive oil/PE per ml/hr in segment 9 of 2/1, equaled the activity ratio per ml/hr of the original plasma of 25/13.

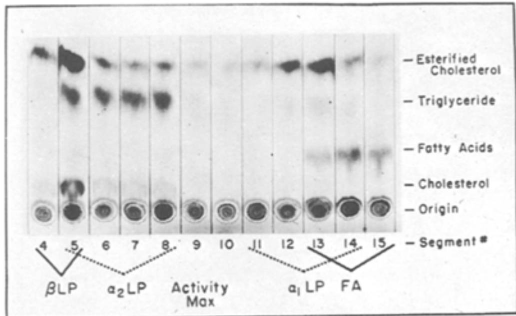

FIG. 4. Location of lipolytic activity relative to location of plasma lipoproteins after electrophoresis of post-heparin plasma. In the separation described for Figure 3, the 1/2 in. segments of the inhibited plasma separation were processed with 2 ml of 0.15 M NaCl. Equal amounts of the lower phase of lipid extracts, prepared by the procedure of Folch et al. (15), were applied to a chromatoplate and developed for neutral lipid separation. The chromatoplate was photographed immediately after exposure to iodine vapor.

of 2 to 35 min after IV heparin. A similar curve of activity was obtained with PC substrate.

Ratio of Activities With Triglyceride and Phospholipid Substrates

In a limited study (four subjects) of the effect of diet on the enzyme response to IV heparin (10), a significant correlation of lipolytic activity measured with PE or TG (olive oil) substrate was found (Fig. 2). The lipolytic response to heparin on a fat containing diet was higher with three of the four subjects than that on a fat-free diet. The ratio of activities olive oil/PE substrates was about 2 (Fig. 1 and 2) and Ediol/PE substrates about 4 (Fig. 1). However, in three subjects after 10,000 units of heparin (versus about 700 units in the studies of Fig. 1 and 2) the ratio of lipolytic activities Ediol/PE substrates was reduced to 1.3 to 1.5. Therefore, lipolytic activities were measured in the plasmas of four normal subjects after different amounts of IV heparin (Table I). With subjects 1 and 2 a greater increase in lipolytic activity was measured with PE than with Ediol substrate after the larger amount of IV heparin. This effect was moderate in subject 3 and not observed with subject 4.

Zone Electrophoresis of Post-Heparin Plasma

After zone electrophoresis of post-heparin plasma in blocks of starch or Pevikon, the peak activity with PE or TG substrate was recovered in the same area (Fig. 3). From the distribution of the plasma neutral lipids (of inhibited plasma) in the block segments as shown in the chromatogram of Figure 4, the peak enzymatic activity appeared to be located in an intermediate position between the triglyceride-rich very low density lipoprotein and the high density lipoprotein (provided that in vitro lipolytic activity upon lipoproteins did not affect electrophoretic migration in uninhibited samples). Larger aliquots of lipid extracts from segments 8 and 9 showed some neutral lipid and phospholipid. This lipid and all the enzymatic activity were recovered in the infranatant when aqueous eluates of segments 8 and 9 were subjected to ultracentrifugation at d=1.006. Thus the lipolytic activity was separated from very low density lipoprotein by electrophoresis and ultracentrifugation.

Gel Filtration of Post-Heparin Plasma

The hydrolysis of PE and PC with post-heparin lipolytic activity proceeds effectively in a glycine medium (1,2). Thus, molecular filtration of post-heparin plasma (10,000 units IV heparin) on Sephadex G-200 was carried out in 0.1 M glycine-NaOH pH 8.0 (Fig. 5). The chro-

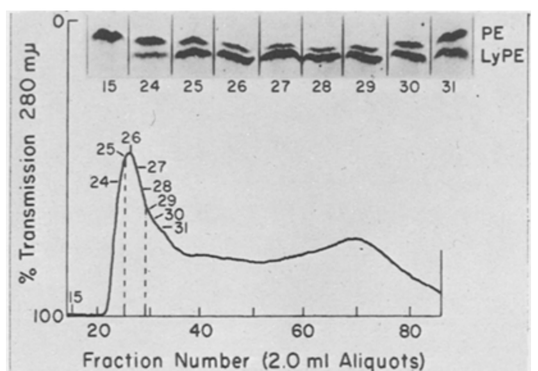

FIG. 5. Gel filtration of post-heparin plasma. The plasma (5.0 ml) was filtered on a 2.5 x 38.5 cm column of Sephadex G-200 prepared in 0.1 M glycine-NaOH (pH 8.0). Elution with the same buffer was at 22 C with a flow rate of 26 ml/hr. Eluates 15 and 24-31 (0.1 ml) were each incubated with 0.1 ml of PE substrate for 90 min. A segment of the chromatogram shows the location of maximum lipolytic activity.

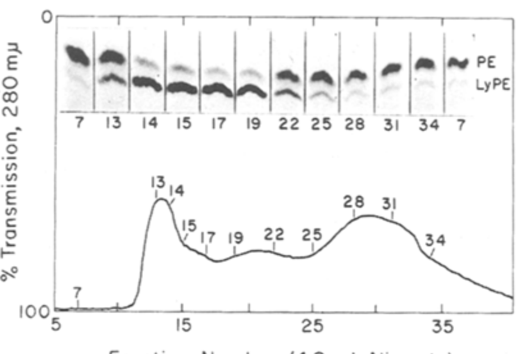

FIG. 6. The effect of salt upon the location of lipolytic activity in gel filtration of post-heparin plasma. The plasma (5.0 ml) was filtered on a 2.5 x 37.5 cm column of Sephadex G-200 prepared in 0.2 M NaCl-0.1 M glycine-NaOH (pH 8.0). Elution with the same medium was at 22 C and flow rate of 20 ml/hr. Chromatogram: 180 min incubations of 0.1 ml of eluates with 0.1 ml PE substrate.

matogram shows that the conversion of PE to LyPE was at a maximum in the 8 ml eluate (lipid rich) of aliquots 26-29. A virtually identical distribution of lipolytic activity with TG and PC substrate was also found. The elution of the lipolytic activity along with plasma lipoproteins of high molecular weight in the column void volume indicates that the enzyme has a high molecular weight or is present in aggregate form.

In a filtration comparable to that of Figure 5, 5.0 ml plasma (after 10,000 units IV heparin) had a lipase activity of 53.1 μeq FA released per ml/hr and activity with PE substrate of 36.6 μmoles LyPE formed per ml/hr. In three successive 4 ml aliquots of eluate the total recoveries of activity with Ediol and PE substrate were 90% and 69%, respectively. In the successive aliquots the ratios of activity Ediol/PE substrate were 2.2, 2.0, and 2.0, respectively, versus 1.45 in the original plasma. Thus, while recovered activities with each substrate were not equal, the ratio of activities in each eluate was constant.

TABLE I

The Effect of the Amount of Intravenous Heparin Upon Plasma Lipolytic Activities With Triglyceride or Phospholipid Substrates

Subject	Weight, kg	Intravenous heparin,[a] units	Lipolytic activity[b] Ediol	PE	Activity ratio Ediol/PE	
1	80	400	22.8	4.9	4.7	
		10,000	80.0	40.7		2.0
2	79	790	26.0	6.1	4.3	
		10,000	51.4	29.0		1.8
3	84	840	38.7	10.6	3.7	
		10,000	69.8	22.2		3.1
4	94	940	27.0	8.8	3.1	
		10,000	61.9	18.9		3.3

[a]The plasmas of normal adult male subjects were obtained from blood collected with oxalate anticoagulant and processed as described under Materials and Methods for collection of large amounts of blood. Blood (100 ml) was collected 8-10 min after the low dose of heparin and 400 ml blood was collected 15-25 min after 10,000 units of heparin.

[b]Lipolytic activity: μeq FA released or μmoles LyPE produced per hr/ml plasma with Ediol or PE substrate, respectively. Since plasma may contain more glycerol from in vitro hydrolysis of TG after the larger amounts of IV heparin, and since glycerol may activate hydrolysis of PE (3), all incubations with PE substrate were carried out in a medium of 0.5% glycerol.

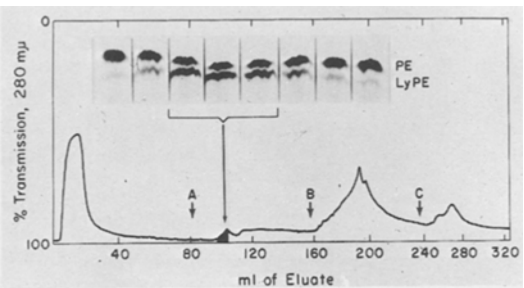

FIG. 7. Purification of post-heparin lipolytic activity utilizing *n*-butanol in the elution from an anion-exchange column. A column (2.5 x 4.0 cm) of DEAE-Sephadex was prepared at 4 C in 0.02 M $(NH_4)_2SO_4$-0.1 M glycine-NaOH (pH 8.0). Initially the exchanger was suspended several times in excess solvent at 22 C in order to remove fine particles in the supernatant, equilibrated in the column with solvent at 22 C and finally resuspended and packed at 4 C. Lipolytic activity applied: 11.8 ml of the eluates of peak activity of 5.0 ml plasma filtered on G-200 as in Figure 5, with 1 M $(NH_4)_2SO_4$ added to effect a 0.02 M concentration. Initial eluation flow rate was 60 ml/hr. Solvent changes: at A, 8:4:88 v/v/v butanol/methanol/0.075 M $(NH_4)_2SO_4$-0.1 M glycine-NaOH (pH 8.0); at B and C, 0.1 and 0.2 M $(NH_4)_2SO_4$, respectively, in 0.1 M glycine-NaOH (pH 8.0). Chromatogram: 135 min incubations of 0.1 ml each of eight consecutive 2 ml eluates with 0.4 ml PE substrate.

The nature of the protein elution curve of Figure 5, which is quite different from elution pattern obtained with normal plasma (16-18), apparently reflects the extensive in vitro (rather than in vivo) changes in lipoproteins that have occurred in post-heparin plasma. Blood also was obtained before and 15 min after 10,000 units IV heparin and in each case was immediately treated with diethyl-*p*-nitrophenyl phosphate to inhibit in vitro lipolysis (11). With this procedure gel filtration of each plasma yielded three distinct protein peaks, comparable to published reports. An observable difference in the elution patterns of pre- and post-heparin plasma was a moderate decrease in the height of the first peak (area of very low density lipoprotein) in the post-heparin sample.

An increased salt concentration has been recommended in gel filtration of normal plasma in order to limit protein-protein interactions (16,17). With post-heparin plasma, both the protein elution pattern and the distribution of lipolytic activity were indeed markedly affected. In Figure 6 an aliquot of the same plasma as used in Figure 5 was filtered in the presence of 0.2 M NaCl. The lipolytic activity was spread over more than 24 ml (aliquots 14-19); activity with PC and olive oil substrate corresponded to that shown with the chromatogram of activity with PE substrate. Recovery of

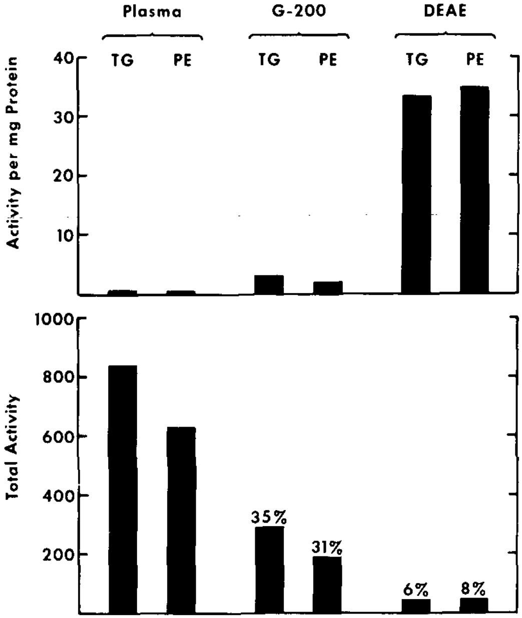

FIG. 8. Purification of post-heparin lipolytic activity by gel filtration and anion-exchange. Three 5 ml amounts of a plasma pool were separated on three separate G-200 columns as in Figure 5 and the eluates of peak activity were pooled (30.8 ml). Two 12 ml aliquots were fractionated on two separate DEAE-Sephadex columns by the technique illustrated in Figure 7; and the 8 ml of eluates of peak activity of each column were dialyzed against 0.1 M glycine-NaOH (pH 8.0) for 3 hr. Activity measurements with TG (Ediol) and PE substrates, and measures of protein, were calculated on the basis of the original 15 ml plasma volume. The original plasma (10,000 units IV heparin) had a lipase activity of 56.2 μeq FA released per hr/ml and activity with PE substrate of 42.2 μmoles LyPE formed per hr/ml, ratio of activity Ediol/PE substrate of 1.3. The inhibition of color development by glycine in the protein measurement (21) was corrected by addition of glycine to the blank and standards.

original activity in the 24 ml peak was reduced (40% for activity with PE substrate and 32% with Ediol substrate).

The elution of peak enzymatic activity was also moderately delayed when heparin (0.1 mg/ml) was added to the 0.1 M glycine-NaOH of pH 8.0. Furthermore, an adverse effect of increased pH was observed on refiltration of the recovered peak of activity as in the experiment shown in Figure 5. At pH 8.0 a symmetrical protein elution peak was obtained with a corresponding distribution of activity; at pH 9.2 (in the optimal pH range for assays of activity with PE and PC substrate) a considerable loss of activity occurred and the peak of activity (all substrates) was recovered in an eluate of decreasing protein content.

TABLE II

Characterization of Post-Heparin Lipolytic Activity by Ultracentrifugation and Gel Filtration

Sample	Ultracentrifugal data (Spinco 50 Rotor)	Slice of 10 ml tube		Filtration on G-200 Location of activity Nature of protein elution
		Supn	Infn	
A. Plasma	*d* 1.063 (D_2O) 1.2 X 10^8 *g* • min, 4°	6 ml	4 ml	Supn: no activity in a single symmetrical component at void volume. Infn: activity at void volume, protein elution curve very similar to that of whole plasma.
B. Peak activity of eluates of plasma filtered on G-200	*d* 1.210 (NaCl/KBr) 2.8 X 10^8 *g* • min, 4°	8 ml	2 ml	Supn: trace of activity at void volume in one symmetrical component. Infn: activity at void volume,[a] in an asymmetrical peak, and apparently a small albumin component.
C. Plasma	*d* 1.210 (NaCl/KBr) 2.8 X 10^8 *g* • min, 4°	8 ml	2 ml	Infn: activity at void volume, considerable trailing protein eluted to the albumin component.

[a]The pool of peak activity contained 24% of the activity of the fraction subjected to ultracentrifugation, and 7% of original plasma activity.

Ultracentrifugation and Gel Filtration

After ultracentrifugation of samples, as described in Table II, the fractions were filtered on Sephadex G-200. (The filtration technique effectively separated the activity from the high concentration of salt.) The peak of activity with PE, PC or TG substrate appeared in the same eluate (void volume) in these filtrations. Quantitative measurements of lipids were not done; thin layer chromatography for lipids showed the aliquot of peak lipolytic activity contained phospholipid and neutral lipid. Thus, the lipolytic activity, sedimenting at d=1.210, has a density greater than that of most plasma lipoproteins and, by its appearance in the void volume after gel filtration, is still characterized as a large molecular aggregate.

Purification With Anion-Exchange and n-Butanol

The activity eluted from a G-200 filtration (Fig. 5) was further purified on a short (2.5 x 4.5 cm) column of DEAE-Sephadex (Fig. 7) utilizing the lipoprotein solubilizing effect of *n*-butanol (19,20). Enzymatic activity could be eluted after the rapid elution of a large inactive component. The adsorption of the enzymatic activity was critically dependent on the pH of 8.0 and a 0.02 M $(NH_4)_2SO_4$. The sharp elution of activity, following elution change A, required an increased $(NH_4)_2SO_4$ concentration as well as the use of butanol-methanol (or butanol). The changes in elution medium at B and C show the further elution of inactive protein. Without butanol, the lipolytic activity was eluted at the start of the component eluted following change B. Pigmented material at the top of the column was not eluted with further increases of $(NH_4)_2SO_4$ concentration or at a higher pH. The incubations of aliquots of eight consecutive 2 ml eluates with PE, PC or TG substrate showed the same peak of enzymatic activity as the chromatogram of activity with PE substrate in Figure 7. The arrow indicates the area of the elution of the 6 ml of peak activity. This active fraction was filtered on G-200; a single component, eluted at the void volume, contained activity with the three lipid substrates.

The recovery and purification of post-heparin lipolytic activity with TG and PE substrate proceeded in parallel (Fig. 8). Of the total activity, 35% and 31% was recovered after gel filtration and 6% and 8% was recovered after fractionation on DEAE-Sephadex. In activity per milligram of protein (specific activity), the purification, also in parallel, was about 56-fold (47 and 65 with TG and PE substrate, respectively). Of the total plasma protein, 0.1% was recovered in the last fractionation.

In the separation illustrated in Figure 7 definitive measurements of lipids were not made in the eluates containing lipolytic activity. While thin layer chromatography for lipids apparently showed components with the R_f of phospholipid and neutral lipid, lipid-like components were extracted from the medium and accessories of the column assembly by butanol. Such lipid-like material was not completely eliminated through the use of an all glass and

teflon column assembly and anion-exchanger pre-extracted with the butanol reagent. The possible interference of this material in tests of lipolytic activity was not explored.

DISCUSSION

The post-heparin plasma lipolytic activity with TG and PL substrate appears to be a large molecular aggregate of density greater than most plasma lipoproteins. The activity eluted with the void volume of gel filtration (G-200) during initial filtration of whole post-heparin plasma, after prior sedimentation at d=1.210, and after separation on a short column of DEAE-Sephadex. The latter method demonstrated the potential value of *n*-butanol in the purification of the enzyme. Conceivably, other anion-exchangers used with butanol could further improve the purification procedure.

The major in vivo substrates of the post-heparin lipolytic activity are PC and TG of very low density lipoprotein (6). The activity with TG substrate has been separated from its natural substrate in vitro by ultracentrifugation at high density (22) and by electrophoresis (23). The activity with PE substrate was also recovered with the activity for TG substrate in the present comparable studies.

Some results of this study seem to support the hypothesis that the hydrolysis of PL and TG is effected by a single lipolytic enzyme in post-heparin plasma. The lipolytic activity with PL and TG varied in parallel: (a) in turnover of activities in plasma; (b) in zone electrophoresis alone or combined with ultracentrifugation of the separated activity at low density; (c) in gel filtration; (d) in sedimentation at high density alone or combined with gel filtration; (e) in elution from an anion-exchange medium with or without added *n*-butanol; (f) and in the specific activity and recovery after purification.

However, some observations are at divergence with the single enzyme hypothesis: (a) there was some variation of the ratios of recovered activities TG/PL substrates in block segments after electrophoresis (Fig. 3); (b) a decreased ratio of plasma activities TG/PL substrates was found in three of four subjects after increased amounts of intravenous heparin (Table I); (c) while plasma may initially have a greater activity with TG than PL substrate the purification procedure of Figure 8 yielded a preparation essentially equal in activity with TG and PL substrates.

These observations may be explained (if the purification procedures have not removed plasma factors which activate TG hydrolysis or inhibit hydrolysis of PL) as follows. Intravenous heparin releases a number of closely related enzymes (24-26), some of which may be specific for TG or the products of its partial hydrolysis, but presumably one enzyme could hydrolyze both TG and PL (9); the possible presence of that enzyme and the closely similar properties of all activities are responsible for those tests which support the single enzyme hypothesis. A purification procedure (Fig. 8) may be relatively selective for specific enzymes. The differential release of these lipolytic enzymes may be related to the amount of intravenous heparin.

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Specificity of Flaxseed Lipoxidase

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ABSTRACT

Mass spectrometric data indicate that products of linoleate oxidation by flaxseed lipoxidase consist of 80% 13-hydroperoxyoctadecadienoate and 20% 9-hydroperoxyoctadecodienoate; the products of linolenate oxidation by flaxseed lipoxidase consist of 88% 13-hydroperoxyoctadecatrienoate and 12% 9-hydroperoxyoctadecatrienoate. Flaxseed lipoxidase possesses a high degree of specificity for attachment of oxygen to linoleic and linolenic acid molecules.

INTRODUCTION

Much work has been done on the oxidation of polyunsaturated fatty acids by the enzyme lipoxidase (E.C. 1.13.1.13) (1). Most of this work was accomplished by using the enzyme prepared from soybeans. Many other plant tissues have been tested for lipoxidase activity, but the products of lipoxidase activity and the mechanism of reaction are based solely on studies conducted with soybean lipoxidase (2,3). Because of renewed interest in pathways of fatty acid oxidation in flaxseed which involved lipoxidase (4), it was necessary to know the specificity of this enzyme toward linoleic and linolenic acids.

MATERIALS AND METHODS

Enzyme Reaction

Linoleic and linolenic acids were purchased from the Hormel Institute and emulsified with Tween 20 according to the method of Surrey (5). Flaxseed acetone powder, 11.0 g, was extracted with 110 ml of 0.1 M phosphate buffer, pH 6.5. After 45 min, the mixture was centrifuged at 12,000 x g for 20 min. The supernatant was decanted, diluted one to five with buffer and then heat treated for 10 min at 55 C to destroy hydroperoxide isomerase activity (4).

Linoleic or linolenic acid substrate, 72.0 ml, was added to 1800 ml of 0.1 M phosphate buffer, pH 6.5, along with 300 ml of the diluted, heat-treated enzyme extract. After 1 hr incubation at 24 C the mixture was placed under nitrogen and extracted with diethyl ether, once with 600 ml and twice with 400 ml. The combined ether extract was dried over anhydrous sodium sulfate and then concentrated to 20 ml under reduced pressure.

Sodium Borohydride and Catalytic Reductions

Reduction of the hydroperoxide group to a hydroxyl group was accomplished with sodium borohydride. Ethanol, 50 ml, was added to the ether concentrate and the ether removed under reduced pressure. Sodium borohydride, 90 mg, was added and the solution stirred for 1 hr under nitrogen. Water, 50 ml, was added, the solution acidified to pH 3.0 and extracted twice with 50 ml diethyl ether. Ethanol, 10 ml, was added and the ether removed under vacuum.

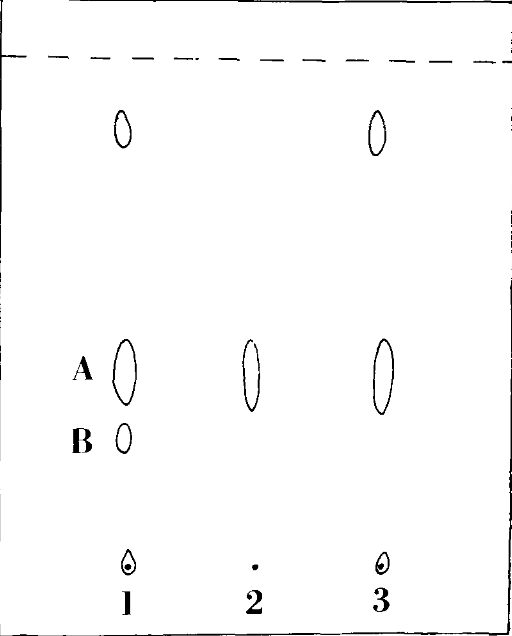

FIG. 1. Thin layer chromatogram of reaction products from flaxseed lipoxidase, after reduction, hydrogenation and esterification. 1. Linolenic acid substrate. 2. Methyl-9-hydroxystearate. 3. Linoleic acid substrate. Silica Gel N-HR/UV_{254} with petroleum ether-diethyl ether-acetic acid (60:40:1 v/v) solvent.

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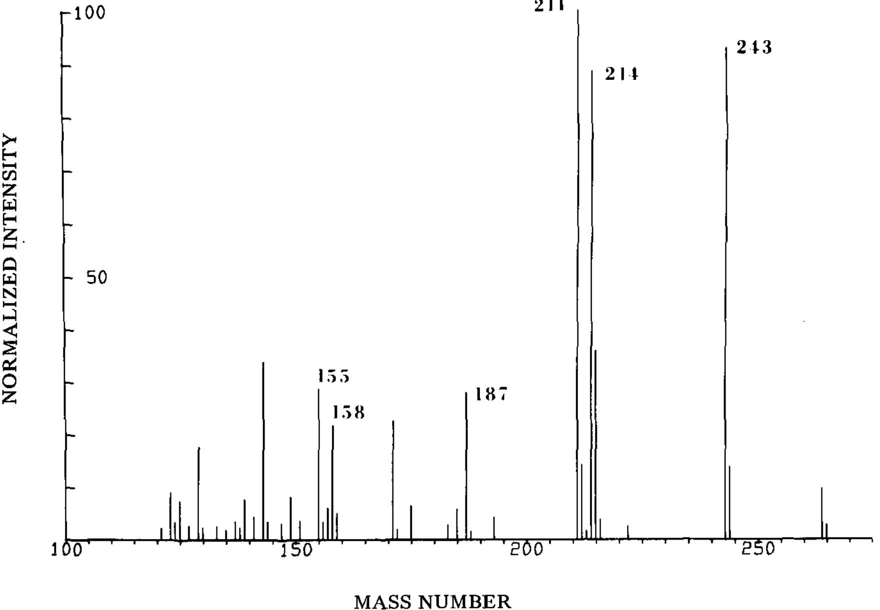

FIG. 2. Partial mass spectrum of methyl hydroxystearates resulting from the oxidation of linoleic acid by flaxseed lipoxidase. Sodium borohydride reduction in ethanol.

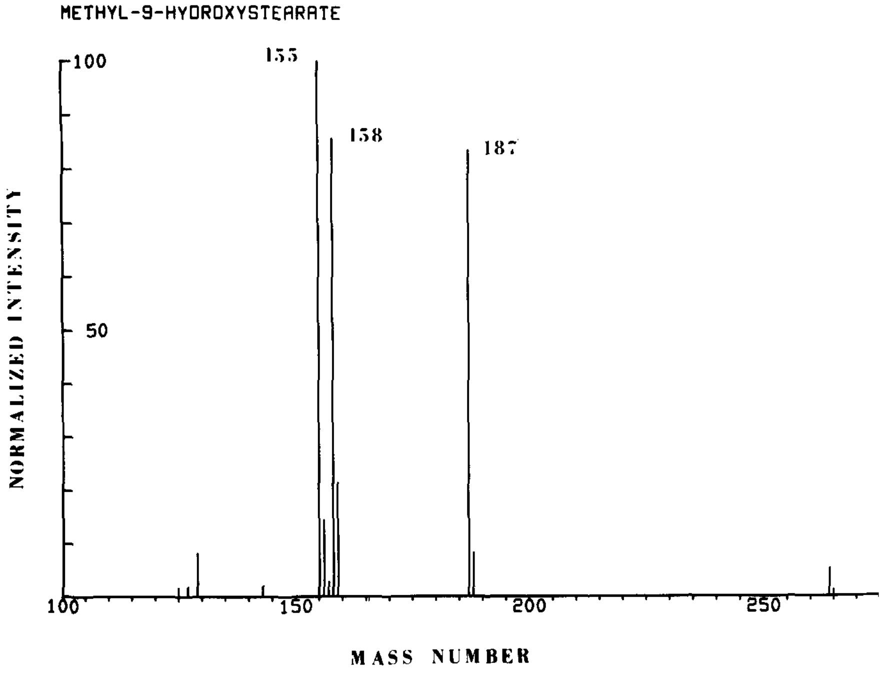

FIG. 3. Partial mass spectrum of methyl-9-hydroxystearate.

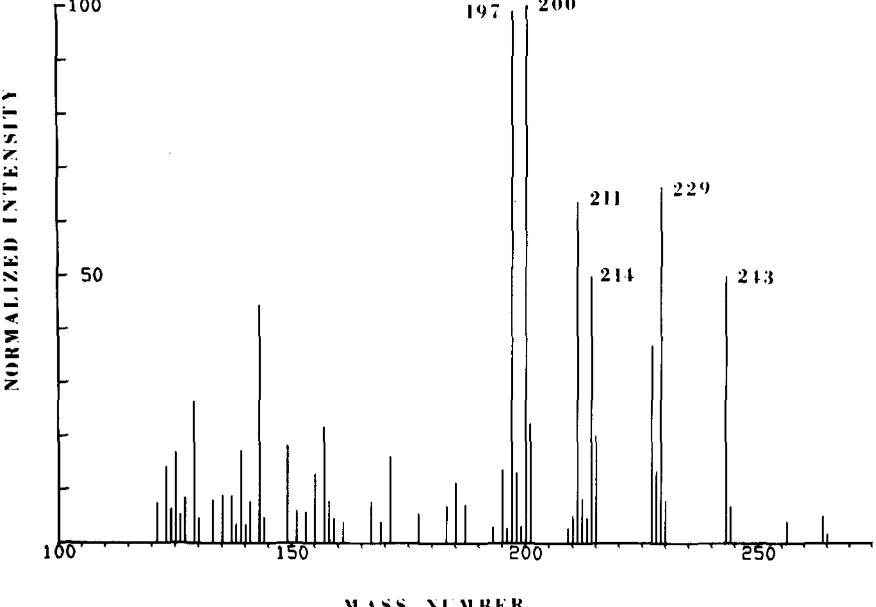

FIG. 4. Partial mass spectrum of methyl hydroxystearates resulting from the oxidation of linoleic acid by flaxseed lipoxidase. Sodium borohydride reduction in 0.1 M phosphate buffer, pH 6.5.

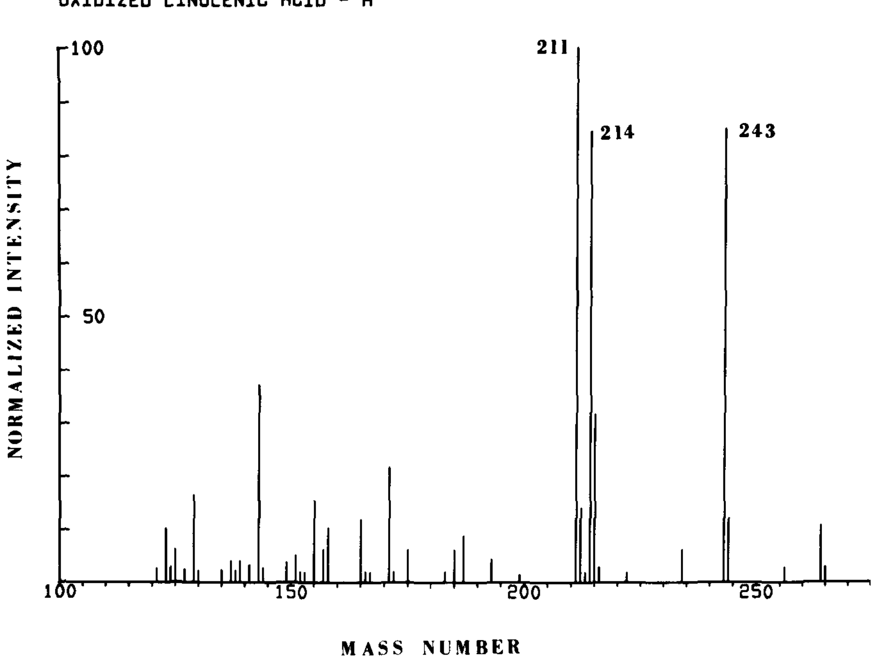

FIG. 5. Partial mass spectrum of methyl hydroxystearates resulting from oxidation of linolenic acid by flaxseed lipoxidase.

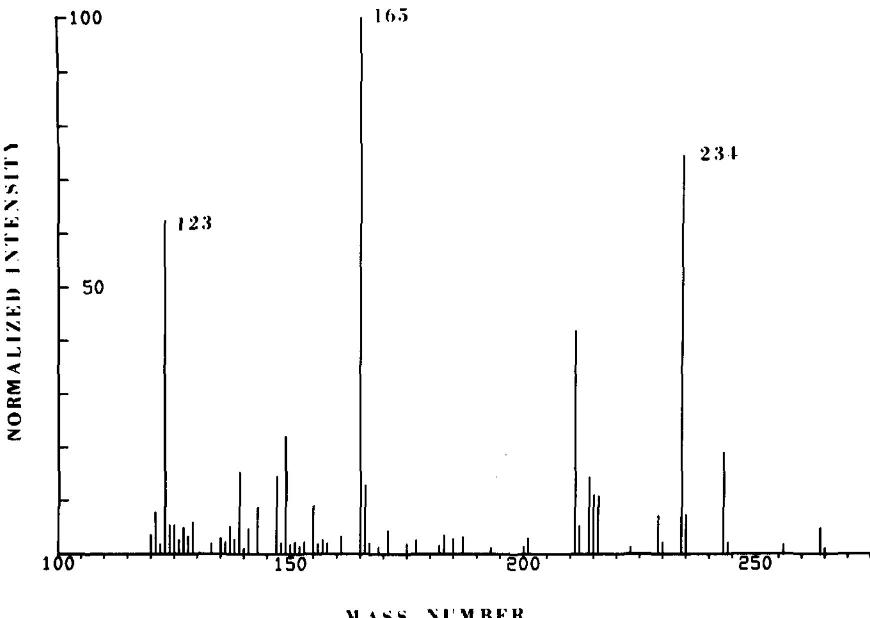

FIG. 6. Partial mass spectrum of Spot B, Figure 1.

The ethanolic solution was hydrogenated for 3 hr at 30 lb. pressure with 10 mg of 10% palladium on Norite as the catalyst. The solution was filtered, evaporated to dryness and esterified with boron trifluoride in methanol. The methyl esters of the reaction products were purified on thin layers of silica gel (MN Silica Gel N-HR/UV_{254}) using petroleum ether-diethyl ether-acetic acid (60:40:1 v/v) as the developing solvent. The major (>80% of material on plate) spot was eluted from the gel. The R_f of this material corresponded with methyl hydroxystearates spotted on the same plate. Mass spectra were obtained with a Hitachi-Perkin Elmer RMU-6E mass spectrometer equipped with a glass inlet. Inlet temperature was 60 C, source temperature 100 C, and electron potential 70 v.

RESULTS AND DISCUSSION

The oxidation of linoleic acid by flaxseed lipoxidase yielded primarily, if not exclusively, 13-hydroperoxyoctadecadienoic acid. The material which migrated on the thin layer plates as a mono-hydroxy fatty acid (Fig. 1) produced the spectrum shown in Figure 2. Table I shows the various ion species responsible for the different mass peaks. The mass peaks at 155, 158 and 187 arise from the scission of a molecule with the hydroxyl in the 9 position. Figure 3 shows the mass spectrum of methyl dimorphecolate. Mass peaks 211, 214 and 243 arise from methyl-13-hydroxystearate. Analysis of the spectrum shows that the material from the thin layer plate contains 80% of the 13-hydroxy isomer and 20% of the 9-hydroxy isomer. There was no evidence for the presence of an 11-hydroxy isomer.

Early attempts to reduce the linoleic hydroperoxides directly in the reaction mixture produced unexpected results. Figure 4 shows the mass spectrum of the product reduced in the phosphate buffer reaction mixture. The mass peaks at 197, 200 and 229 indicate attachment of a hydroxyl group in the 12 position (Table I). Analysis of the spectrum indicates the following composition: 58% of the 12-isomer, 36% of the 13-isomer, and 6% of the 9-isomer. The presence of phosphate ions in the reaction mixture or a pH of 6.5 apparently alters the mechanism of the borohydride reduction. Neither of the previous publications (3,6) observed this phenomenon when the reduction was performed in borate buffer at pH 9.0. Because of this hydroxyl shift, the hydroperoxide was isolated from the reaction mixture and placed in ethanol prior to reduction with sodium borohydride in all subsequent experiments.

TABLE I

Ion Species and Their Origin

Mass no.	Ion species	Parent compound
155	$CH(OH)-(CH_2)_6-CH=C=O$	methyl-9-hydroxystearate
158	$(CH_2)_7-C(=O)-OCH_3+H^+$	methyl-9-hydroxystearate
187	$CH(OH)-(CH_2)_7-C(=O)-OCH_3$	methyl-9-hydroxystearate
197	$CH(OH)-(CH_2)_9-CH=C=O$	methyl-12-hydroxystearate
200	$(CH_2)_{10}-C(=O)-OCH_3+H^+$	methyl-12-hydroxystearate
211	$CH(OH)-(CH_2)_{10}-CH=C=O$	methyl-13-hydroxystearate
214	$(CH_2)_{11}-C(=O)-OCH_3+H^+$	methyl-13-hydroxystearate
229	$CH(OH)-(CH_2)_{10}-C(=O)-OCH_3$	methyl-12-hydroxystearate
243	$CH(OH)-(CH_2)_{11}-C(=O)-OCH_3$	methyl-13-hydroxystearate

The mass spectrum of the monohydroxystearic acids (Spot A, Fig. 1) which resulted from the incubation of linolenic acid with flaxseed lipoxidase is shown in Figure 5. Analysis of the mass peak intensitities indicates that the product consists of 88% methyl-13-hydroxystearate and 12% methyl-9-hydroxystearate. There were no mass peaks indicating the presence of a hydroxyl group at the 11, 15 or 16 position.

The results with both linoleic and linolenic acid as substrates for flaxseed lipoxidase indicate a high degree of specificity for attack of oxygen at carbon 13. It is the author's belief that the formation of the 9-isomer is the result of autoxidation. The amount of autoxidation occurring during preparation of the substrate, the enzymatic reaction, extraction and analysis is difficult to control, even when working under nitrogen. Different experiments with the linoleic acid substrate showed varying amounts of the 9-isomer, ranging from 6% to 30%. This problem was also encountered by Hamberg and Samuelson. In their first publication (7) with soybean lipoxidase they reported 70% and 30% of the 13-isomer and 9-isomer, respectively, for linoleic acid and 80% and 20%, respectively, for linolenic acid. In a later publication (2) they reported 92% and 8% for the 13- and 9-isomer with linoleic acid and 100% of the 13-isomer with linolenic acid. When we analyzed the products of soybean lipoxidase and linoleic acid, we obtained 80% of the 13-isomer and 20% of the 9-isomer. Differences in the ratios of the two isomers could be explained by the varying amounts of two lipoxidase isozymes, each responsible for the formation of a single isomer. However, this is unlikely in our case since the enzyme extracts were prepared from a single flaxseed acetone powder. We believe that the varying amounts of the 9-isomer which were observed were due to different degrees of autoxidation in the various experiments. Although the formation of an 11-hydroperoxide isomer has been theorized for the autoxidation of methyl linoleate (8), no evidence for its presence has been found (3,9-11). Only the 9- and 13-hydroperoxy isomers have been observed. The formation of the 9-hydroperoxy isomer by autoxidation or enzymatic activity could be determined by the optical activity of the compound. We have not made this determination.

The incubation of linolenic acid with flaxseed lipoxidase resulted in the formation of a second product (Spot B, Fig. 1). This material ran below the monohydroxystearates on the thin layer plate and its mass spectrum showed that it was definitely not a methyl hydroxystearate (Fig. 6). The structure of this material is unknown, but it may be a cyclic compound similar to that found in the synthesis of prostaglandins.

Our results with flaxseed lipoxidase indicate that its specificity for attachment of molecular oxygen to linoleic and linolenic acid molecules is very similar to that of soybean lipoxidase. The function of the hydroperoxides formed by lipoxidase is not known. However, they do serve as substrates for an isomerase enzyme found in flaxseed, wheat and barley (4,12). 13-Hydroperoxyoctadecadienoic acid formed from linoleic acid can be isomerized to 13-hydroxy-12-keto-octadec-9-enoic acid by the flaxseed hydroperoxide isomerase enzyme. In a similar fashion, linolenic acid will give rise to 13-hydroxy-12-keto-octadec-9, 15-dienoic acid. The amount of 13-hydroperoxyoctadecadienoic acid formed by flaxseed lipoxidase from linoleic acid is in close agreement with the amount of 13-hydroxy-12-keto-octadec-9-enoic

acid formed by incubation of a crude flax extract with linoleic acid. The metabolism and metabolic significance of these compounds is under continuing investigation.

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Composition of the Surface Lipids of Pea Leaves (*Pisum sativum*)

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ABSTRACT

Surface lipid of pea leaves (*Pisum sativum* var. Frosty) was analyzed with column, thin layer and gas liquid chromatography in conjunction with mass spectrometry and infrared spectroscopy. It contained 42% *n*-hentriacontane and 7.3% *n*-hentriacontan-16-ol. About 5% was wax esters, C_{40}-C_{50} consisting of primarily C_{26} and C_{28} alcohols and C_{16}-C_{22} acids. Almost 5% was aldehydes, mainly C_{26} and C_{28}. Primary alcohols, chiefly C_{26} and C_{28}, made up 20% of the surface lipid.

INTRODUCTION

Several components of the surface lipids of plants were identified in the 1920's and 30's (1,2). However until chromatographic techniques became available, a complete analysis of the complex mixture of the lipids found on plant surfaces was almost impossible, and only a few plant surface lipids have yet been subjected to fairly complete analysis (3,4).

For biosynthetic studies on surface lipids, pea leaves were found to provide an especially suitable experimental material (5). In this earlier study only the hydrocarbon fraction was examined. Often ketones and corresponding secondary alcohols that might be biosynthetically related to the hydrocarbons were found in the surface lipids (1,6) and their biosynthetic relationships has been examined experimentally in a few cases (7,8).

In the course of further in vivo and in vitro studies on the biosynthesis of the surface lipids of pea leaves it became necessary to identify all such possible intermediates as well as other

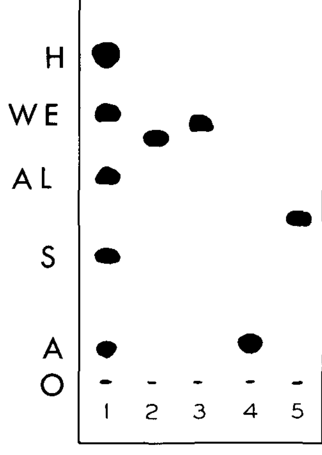

FIG. 1. TLC of the surface lipids of pea leaves. 1. Total surface lipids. 2. Ketone produced from the chromic acid oxidation of the secondary alcohol. 3. Acetate of secondary alcohol. 4. Alcohol produced by sodium borohydride reduction of the aldehyde fraction. 5. Acetate of the primary alcohols derived from the aldehyde. H, hydrocarbons; WE, wax esters; AL, aldehydes; S, secondary alcohols; A, alcohols; O, origin. The standards used to identify the various spots on the TLC were *n*-C_{30} alkane, hexacosyl docosanoate, hexacosanal, *n*-nonacosan-15-ol, hexacosanol and cabbage surface lipids. Total surface lipid, various column chromatographic fractions and isolated individual fractions were chromatographed several times always with standard mixture alongside. The relative migrations of the various components are summarized in this Figure.

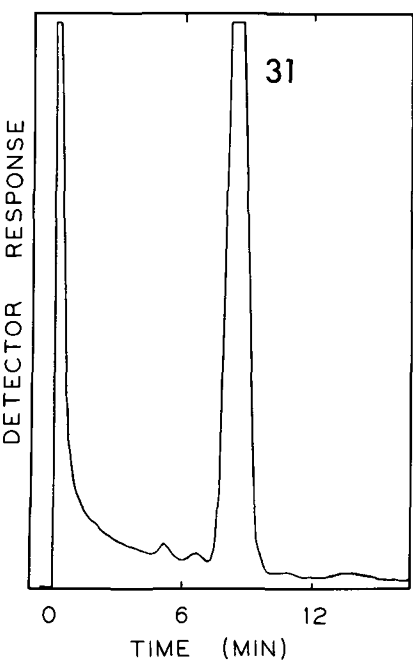

FIG. 2. GLC of the paraffin from pea leaves. 6 ft x 0.25 in. o.d. Coiled column packed with 3% SE-30 on Anakrom Q 80-90 mesh with the carrier gas nitrogen at 100 ml/min, and column temp. 280 C. Hydrocarbons isolated from cabbage leaf and *Senecio ordoris* leaf (16) and synthetic *n*-C_{30} alkane were used as standards.

major components in order to gain insight into the biosynthetic mechanisms involved. This paper describes the identification of the major components of the surface lipids of pea leaves.

EXPERIMENTAL PROCEDURES

Plants and Extraction of Wax

Pea plants (*Pisum sativum* var. Frosty) were raised in sand on a subirrigated bench in the greenhouse. Leaves of all ages were collected from mature plants (flowering) and they were immersed in chloroform for 20-30 sec. On evaporation under reduced pressure the crude surface lipid was obtained.

Fractionation

The crude surface lipid (1 g) was dissolved in ethyl ether and dried onto SilicAR-CC-4 100 mesh (Mallinckrodt Chemical Works, St. Louis, Mo.). This silica gel was placed on a column of SilicAR (2 x 40 cm) and eluted in turn with *n*-hexane (160 ml), benzene (300 ml), chloroform (360 ml) and methanol (400 ml).

Samples of 20 ml were collected and all fractions were evaporated to dryness under a stream of nitrogen. Thin layer chromatography (TLC) was used to monitor the fractionation. Hexane elution gave one thin layer chromatographically homogeneous component (Fraction 1) and benzene gave two fractions. The early one (Fraction 2) consisted of three components on thin layer, and the latter one (Fraction 3) showed only one spot on thin layer. Chloroform apparently completed the elution of Fraction 3 and gave a little more waxy material (Fraction 4). Methanol yielded a small amount of more polar materials (Fraction 5) which was not examined further.

Fraction 2 was further separated into three components by preparative TLC on Silica Gel G with benzene as the developing solvent.

When small quantities of the surface lipids were to be fractionated complete fractionation could be done by preparative TLC on Silica Gel G with benzene as the developing solvent.

Chromatography

TLC was carried out with 20 x 20 cm plates coated with either a 0.25 or 0.5 mm thick layer of Silica Gel G and activated overnight at 110 C (9). A lined tank was used with benzene as the developing solvent and a spray of 2',7'-dichlorofluorescein was used to detect the lipids. For isolation of the individual components silica gel, scraped off from the appropriate areas, was rinsed with ethyl ether.

Gas Chromatography

Either a Perkin-Elmer 810 or a Barber-Colman gas chromatograph equipped with flame ionization detector was used. Fatty acids were analyzed on 15% diethylene glycol succinate on 80-100 mesh Anakrom AB or on 4% silicone gum rubber (SE 30) on Anakrom SD (Analabs, North Haven, Conn.). The alcohol acetates, paraffins, ketones and secondary alcohol acetates were analyzed on a 4% SE-52

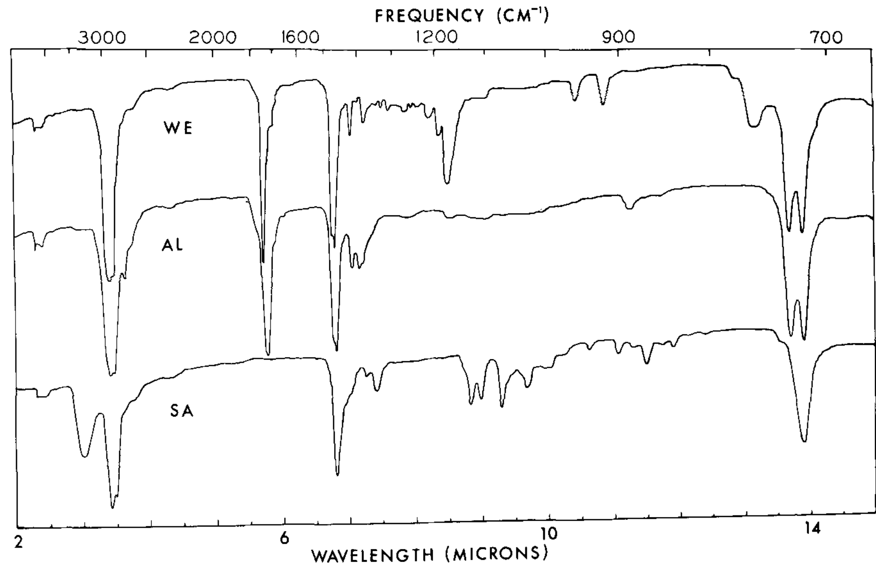

FIG. 3. Infrared spectra of the wax ester (WE), aldehyde (AL) and secondary alcohols (SA) isolated by TLC from Fraction 2.

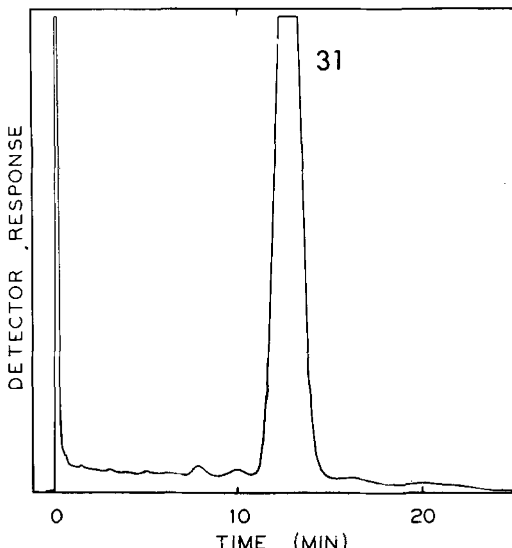

FIG. 4. GLC of the ketones produced from the secondary alcohol by chromic acid oxidation. Column and conditions same as in Figure 2.

column and a 3% SE-30 column, and unhydrolyzed wax esters were analyzed on 3% SE-30 column. Details of the chromatographic conditions are shown under the appropriate figures and tables.

Preparation of Derivatives

Aldehydes were reduced at room temperature (15-30 min) with a methanolic solution of sodium borohydride. Acetates of the primary and secondary alcohols were prepared by refluxing with an excess of acetic anhydride for 2 hr. Methyl esters of fatty acids were prepared either by refluxing the wax ester fraction with an excess of BF_3-methanol reagent for 2 hr or by heating the free acids with BF_3-methanol reagent for 5 min. In the former case the isolated products were subjected to preparative TLC with hexane-ethyl ether-formic acid (40:10:1) as the developing solvent to separate the alcohols and the methyl esters of the acids.

The secondary alcohols were oxidized to the ketones with CrO_3 in glacial acetic acid at 60 C for 15 min. After dilution of the reaction mixture with water the product was extracted with chloroform and purified by TLC with benzene as the solvent. Wax esters used as standards were prepared from C_{26} alcohol and appropriate acids to make a total length of C_{44}-C_{50}. The acids were refluxed with an excess of trifluoroacetic anhydride for 1 hr with enough benzene to give a single phase. After removing the excess trifluoroacetic anhydride with a stream of nitrogen, equimolecular amounts of C_{26} alcohol were added and refluxed for an additional 30-40 min. The reaction mixture was washed with water and the esters recovered by passing the benzene solution through SilicAR-CC-4, giving almost quantitative recovery of pure wax ester.

TABLE I

Composition of Pea Surface Lipids[a]

Fraction	R_f	Per cent of total
Hydrocarbon	0.85	42.2
Wax ester	0.65	4.8
Aldehyde	0.49	4.6
Secondary alcohol	0.32	7.3
Primary alcohol	0.1	20.0

[a]A combination of column and thin layer chromatography described under the experimental section was used to fractionate the total surface lipid and the per cent composition by weight is shown in this table.

Infrared Spectra

The IR spectra of thin films of various components were taken with a Perkin-Elmer Model 21 spectrophotometer.

Mass Spectra

Mass spectra were made with an MS mass spectrometer at 70 eV.

RESULTS

The composition of the surface lipids of pea leaves as obtained by a combination of column and thin layer chromatography is shown in Table I.

Fraction 1

TLC showed this fraction to contain hydrocarbons (Fig. 1) and the infrared spectrum showed them to be saturated long chain hydrocarbons. Gas liquid chromatography (GLC) revealed essentially one component, n-C_{31} alkane, with very small amounts (less than 1% of C_{29}, C_{30}, C_{32} and C_{33} and traces of C_{27} and C_{28} (Fig. 2). Mass spectrum confirmed these conclusions and showed the absence of branching that might not have been detected by the other techniques.

Fraction 2

TLC showed three components with their R_f suggesting that they were wax esters, aldehydes and secondary alcohols (Fig. 1). The wax ester fraction, separated by preparative TLC, gave an infrared spectrum consistent with the tentative

TABLE II

Fatty Acid Composition of the Eax Esters From the Surface Lipids of Pea Leaves[a]

Chain length	Per cent
C_{14}	1.8
C_{16}	28.8
C_{18}	30.3
C_{20}	17.1
C_{22}	22.1

[a]Methyl esters were analyzed on a coiled column (6 ft X 0.25 in. o.d.) packed with 15% stabilized diethylene glycol succinate on 60-70 mesh Anakrom AB.

TABLE III

Composition of Fatty Aldehydes and Free and Esterified Primary Alcohols of the Surface Lipids of Pea Leaves[a]

Chain length	Aldehyde	Free alcohol, %	Wax alcohols
C_{22}	Trace	Trace	1.8
C_{24}	1.4	2.1	2.1
C_{26}	55.7	57.7	56.2
C_{27}	1.0	1.4	1.9
C_{28}	40.9	38.8	38.0
C_{30}	1.0	Trace	Trace

[a]Aldehydes were analyzed as acetates of alcohols produced by sodium borohydride reduction. Free and wax alcohols were analyzed as acetates on coiled column (6 ft 0.25 in. o.d.) packed with 4% SE-52 or 3% SE-30 on Anakrom Q. Column temperature 285 C; flow rate 90 ml/min of Argon. Traces of C_{32} were found especially in the aldehyde fraction.

identification (Fig. 2). Saponification gave acids and alcohols from which derivatives were prepared (methyl esters and acetates respectively). These were further analyzed by GLC. The major acids were C_{16}, C_{18}, C_{20} and C_{22} (Table II) while the major alcohols were C_{26} and C_{28} (Table III). The mass spectrum of the unhydrolyzed ester fraction revealed molecular ions at 538 (C_{42}), 566 (C_{44}), 594 (C_{46}), 622 (C_{48}) and 650 (C_{50}). Fragments corresponding to $RCOOH_2$ were found for C_{16}, C_{18}, C_{20} and C_{22} acids. GLC of the unhydrolyzed ester fraction also showed C_{42}, C_{44}, C_{46}, C_{48} and C_{50} esters and their quantitative distribution is shown in Table IV.

Also included in Table IV is the distribution of wax esters expected from a random combination among the major acids (Table II) and the major alcohols (Table III). There is good agreement in most cases between the calculated and measured values.

The aldehyde fraction isolated by TLC gave an infrared spectrum consistent with the tentative identification (Fig. 3). On reduction with sodium borohydride at room temperature it gave primary alcohols (Fig. 1), acetates of which were subjected to GLC. The major aldehydes were C_{26} and C_{28}. The mass spectrum of the aldehyde fraction confirmed the identification, giving molecular ions at 380 (C_{26}) and 408 (C_{28}). The major ions corresponded to M-18 which is common among long chain aldehydes (10). The other series of ions expected of the long aldehydes were also present. M-29 ions were only minor and M-28 coincided with the molecular ion of the next lower homolog. Reference spectra were run with synthetic aldehydes to confirm the fragmentation pattern by direct comparison.

The secondary alcohol fraction gave an IR spectrum indicative of secondary alcohol (Fig. 3). Mass spectrum of this fraction gave a molecular ion at 452 consistent with n-C_{31} secondary alcohol and gave a major fragment at 434 (M-18) diagnostic of the alcohol. A very strong ion at 241, representing $C_{15}H_{31}$-CHOH showed the molecule to be a symmetrical n-C_{31} secondary alcohol. The acetate derivative of the secondary alcohol gave one peak on GLC the retention time of which was consistent with the assigned structure, with very small amount of C_{29}, C_{30}, C_{32} and C_{33}. Chromic acid oxidation of the alcohol gave a ketone (Fig. 1) which on GLC showed essentially one peak with the retention time corresponding to palmitone with a trace of nonacosan-15-one (Fig. 4). The mass spectrum of the ketone confirmed the assigned structure with the molecular ion at 450, the $C_{15}H_{31}$-CO ion at 239 and the three ions $C_{15}H_{31}CO{=}CH_2$, $C_{15}H_{31}COHCH_2$ and $C_{15}H_{31}COH_2CH_2$ at 253, 254 and 255 respectively. In the mass spectra of the secondary alcohols M-18 ions 406 (C_{29}), 420 (C_{30}), 462 (C_{33}) and 448 (C_{32}) could be detected in decreasing order of intensity. RCHOH ions where R = $C_{14}H_{29}$ and $C_{16}H_{33}$ were also detected. In the mass spectra of the ketone RCO ions where R = $C_{14}H_{29}$ and $C_{16}H_{33}$ were found. Thus the minor secondary alcohols are nonacosan-15-ol, triacontan-15-ol, dotriacontan-16-ol and tritriacontan-17-ol and possibly hentriacontan-15-ol.

Fraction 3

TLC indicated primary alcohols and the infrared spectrum supported this conclusion. Formation of acetate derivatives which had an R_f corresponding to synthetic hexadecanol acetate also agreed with the assigned structure. GLC showed that the major alcohols were C_{26} and C_{28}. The more polar materials, repre-

TABLE IV

Composition of Unhydrolyzed Wax Esters of the Surface Lipids of Pea Leaves

Chain length[a]	Calculated,[b] %	Experimental,[c] %
C_{40}	2.3	3.4
C_{42}	17.8	28.5
C_{44}	28.7	30.4
C_{46}	21.6	23.8
C_{48}	18.9	10.8
C_{50}	8.4	3.2

[a]Chain length refers to the total length of the acid and alcohol moiety.

[b]Calculated assuming random combination of the acid and alcohol moieties listed in Tables II and III.

[c]Determined by subjecting the unhydrolyzed wax ester to GLC on 3% SE 30 at 335 C at a flow rate of 400 ml/min.

senting only a small portion of the total surface lipids, were not studied further.

DISCUSSION

It appears that surface lipids of pea leaves represent one of the simplest mixtures found in plants. The alkane fraction is essentially pure n-C_{31} alkane. Unlike other tissues, such as *Brassica oleracea*, where the corresponding ketones and secondary alcohols are found, pea leaf lipids contain only the secondary alcohol. Trace amounts of ketones might have been present but they were not detected in the present studies. Alkanes have been detected in many plants but the analyses were not complete enough to detect the ketones and secondary alcohols with similar carbon skeletons. However there are instances where the alkanes are accompanied by the secondary alcohols of the same chain length in plants, and where mixtures of alkanes are found corresponding mixtures of secondary alcohols are also present (6). The results reported here clearly show that the chain length distribution of secondary alcohols correspond with the distribution of alkanes. How exactly these two classes of compounds are biosynthetically related is still not known. C_{29} secondary alcohols are the dominant ones found in most cases thus far studied (6) and even when C_{31} secondary alcohol was recently reported to be the major component, the hydroxyl group was found in the C_9 position (6). Pea leaf surface lipid described here represents the first report of the occurrence of hentriacontan-16-ol as the dominant secondary alcohol in plant surface lipids.

Besides in the present report aldehydes have been identified in sugar cane (11), grapes (4), and recently an unknown component encountered earlier (9,12) in the surface lipids of *Brassica oleracea* was shown to be aldehydes (13). Recently enzymatic reduction of acyl CoA to the aldehyde and subsequent reduction of the aldehyde to the alcohol by an aldehyde reductase has been demonstrated in broccoli and pea leaf acetone powders (14). Fatty acids are reduced to the alcohol in the presence of ATP, CoA and DPNH (but not TPNH) by a soluble enzyme partially purified from etiolated *Euglena gracilis*. (P. E. Kolattukudy, manuscript in preparation). If the aldehydes found in plant surface lipids are intermediates in the conversion of acids to alcohols the aldehydes would be expected to resemble the alcohols, and in fact in pea leaves the aldehydes were identical to the free alcohols in their chain length distribution. Furthermore, the free alcohols were identical to the esterified alcohols in chain length, suggesting that this free alcohol pool participated in the esterification process. Analysis of unhydrolyzed wax esters from marine organisms indicated that the acids and alcohols combined randomly (15) and the results reported here are generally in agreement with this conclusion. However there may be some preference for the longer alcohols to esterify with shorter acids giving rise to the slightly higher values than expected in the case of shorter esters and lower values for longer esters.

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Binding of Long Chain Fatty Acids to β-Lactoglobulin[1]

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ABSTRACT

β-lactoglobulin (BLG), a bovine milk protein that is available commercially in crystalline form, binds long chain free fatty acids (FFA). The binding data were analyzed with a model containing one primary FFA binding site and a large number of weak secondary binding sites. At 37 C and pH 7.4, the apparent association constant for binding of FFA to the primary site was of the order of 10^5 M^{-1} and that for binding to the secondary sites was approximately 10^3 M^{-1}. The strength of binding was: palmitate>stearate>oleate>laurate. The affinity of BLG for palmitate increased as the pH of the incubation medium was raised from 6.5 to 8.7 and decreased as the ionic strength of the medium was raised. Palmitate binding was decreased in the presence of 6 M urea and when the protein either was exposed to elevated temperature or was acetylated prior to incubation. BLG took up methyl palmitate, cetyl alcohol, hexadecane and cholesterol to a lesser extent than FFA. Binding of FFA to BLG was associated with a small increase in the intensity of the fluorescent emission of the protein at 333 mμ. BLG can serve as an FFA acceptor or carrier in biological experiments. FFA released from adipose tissue during in vitro incubation was taken up by BLG. Net transfer of fatty acid to the incubation medium ceased when the molar ratio of FFA to BLG exceeded 1.1. ^{14}C-1-Palmitate bound to BLG was taken up by Ehrlich ascites tumor cells in vitro. At a given palmitate-protein molar ratio, much more labeled fatty acid was taken up by these cells from media containing BLG than from those containing bovine albumin, apparently because FFA is bound less firmly to BLG than to albumin.

INTRODUCTION

β-lactoglobulin (BLG), a protein present in bovine milk, can be obtained commercially in crystalline form. We have noted that this protein will bind long chain free fatty acids (FFA) in an aqueous solution (1). Therefore, it was reasonable to assume that BLG might serve as a substitute for serum albumin in incubations that require the presence of an FFA carrier or acceptor.

There are two properties of long chain fatty acids that make them difficult to work with in biological systems. First, they are poorly soluble in aqueous solutions. Second, unbound FFA are injurious to mammalian tissues even in relatively low concentrations, e.g., 10^{-4} M. Thus, for most biological work with FFA, it is necessary to employ a fatty acid carrier in order to introduce the required amounts of FFA into the incubation medium while, at the same time, preventing exposure of the tissue to an excessive amount of unbound fatty acid. Serum albumin, the physiological FFA transport protein, is used almost exclusively for this purpose. The availability of BLG as an alternative to albumin may provide a useful tool for the elucidation of several points concerning the mechanisms of FFA metabolism. For example, comparative studies of media containing either BLG or serum albumin may indicate whether albumin possesses some special property for transferring FFA to or into cells or for removing fatty acid from adipose cells.

The interaction of FFA with proteins also has been studied almost exclusively with serum albumin. Hence, it is of interest to determine whether the mechanism of FFA association with albumin is unique for that protein or is applicable in general to other proteins that can bind FFA. Study of FFA binding to BLG should help to clarify this point. In addition, our initial observations indicated that BLG possessed a much smaller capacity to take up FFA than serum albumin (1). Preliminary analyses of these data suggest that BLG contained only one binding site with high affinity for FFA. In contrast, serum albumin contains approximately six high energy FFA binding sites (1). For studies of the detailed mechanism of FFA binding to proteins and FFA transfer from a carrier to a cell, the use of a protein having only a single strong binding site is

[1]Special abbreviations used in this text: $\bar{\nu}$, average molar ratio of bound FFA to total protein; c, molar concentration of FFA in free solution and in equilibrium with that bound to protein; n, number of binding sites in a given class; k′, apparent association constant for binding to a given class of sites.

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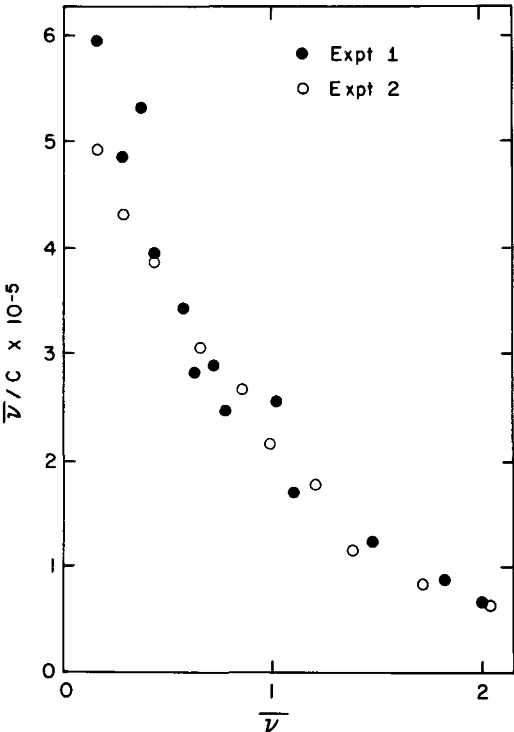

FIG. 1. Scatchard plot of data for binding of 1-^{14}C-palmitic acid to β-lactoglobulin at 37 C in phosphate-buffered salt solution, pH 7.4.

advantageous because it greatly simplifies the theoretical considerations and the mathematical analysis.

Because of the potential usefulness of BLG for these purposes we have investigated the binding of long chain FFA to this protein and the uptake and release of FFA by mammalian tissues in media containing BLG.

EXPERIMENTAL PROCEDURES

Materials

Crystalline BLG (lots 36 and 38) were purchased from Pentex, Inc. (Kankakee, Ill). These preparations contained less than 0.2 μeq FFA per μmole protein as measured by titration (2). BLG was dissolved in buffered salt solution and dialyzed against 4 liters of this solution for 24 to 48 hr at 4 C with mechanical stirring. The dialyzing solution was changed at least once during this time. Unless noted otherwise, the buffer solution contained 0.116 M NaCl, 0.004 M KCl, 0.0012 M $MgSO_4$ and 0.016 M sodium phosphate and was adjusted to pH 7.4 with 1.0 N HCl. This is referred to in the text as phosphate-buffered salt solution. Following dialysis, the BLG solution was adjusted to pH 7.4, and the protein concentration was determined by the biuret method (3). Dried samples of BLG served as the standard for this procedure. The molecular weight of BLG was taken as 42,000. Freshly prepared solutions of the protein migrated as a single band when subjected to electrophoresis at pH 8.2 on glass microscope slides coated with Agarose (1). Additional tests to detect heterogeneity in these BLG preparations were not done (4).

BLG was acetylated at pH 8 according to the method of Fraenkel-Conrat et al. (5). Free amino groups were measured by the ninhydrin method (6).

The commercial sources and methods for purification of the radioactive lipids used in this study have been described (1). ^{14}C-4-Cholesterol that had been purified by thin layer chromatography (>99% radiopure) was a gift from Dr. W. E. Connor.

TABLE I

Reversibility of Fatty Acid Binding to β-Lactoglobulin[a]

Time of incubation, hr	β-Lactoglobulin solution[b] Radioactivity, cpm ^{14}C	^{3}H	Fatty acid, mμeq
0	3160	0	25
0.5	2250	565	55
1	1810	425	43
4	595	1340	94
7	440	1980	135
16	260	2280	154

[a]Incubation was done at 37 C. 1-^{14}C-palmitate was added to BLG dissolved in phosphate-buffered salt solution by exposure to palmitate-coated Celite particles. This solution contained 0.2 μmole/ml BLG and 0.025 μeq of 1-^{14}C-palmitate. A heptane solution containing palmitate-9,10-^{3}H (20 μeq/ml) was prepared, and 1 ml of it was incubated with 1 ml of the BLG solution containing 1-^{14}C-palmitate in a series of flasks. At each of the time intervals shown, two flasks were removed and the content of ^{14}C and ^{3}H in the heptane and aqueous phases was determined.

[b]Each value is the mean of two determinations.

Measurement of Binding

Association of labeled FFA with BLG was measured by a modification of the equilibrium partition analysis method devised by Goodman (1,7). The reaction was carried out in specially constructed glass flasks (1). Incubation was done with shaking in a temperature-controlled water bath. Increasing amounts of labeled FFA of known specific radioactivity were dissolved in 1 ml of *n*-heptane and incubated with 1 ml of an aqueous solution containing BLG (2 x 10^{-4} M). The partition ratio of FFA between

heptane and the protein-free buffered salt solution was determined in a separate set of flasks (1,8). Equilibrium was reached in this system after 6-10 hr at 37 C and, for convenience, an overnight incubation usually lasting 16 hr was used. Both heptane and aqueous phases remained optically clear during incubation, and precipitates did not form at the interface.

Radioactivity was measured with a Packard Tri-Carb 3375 liquid scintillation spectrometer. The scintillator solution contained 0.3%, 2,5-diphenyloxazole and 0.01% 1,4-bis [2-(4-methyl-5-phenyloxazolyl)]-benzene in toluene and methanol (7:3 v/v) (1). Quenching was monitored initially both with the external standard and by addition of ^{14}C-1-palmitate as an internal standard. Both methods indicated that the amount of quenching varied between 1% and 8%, and only the external standard was employed in subsequent experiments.

From the binding data, the unbound FFA concentration, c, at each molar ratio of bound FFA to protein, ν, was calculated (1,7). A computerized curve-fitting procedure (9) was employed to analyze these results in terms of number of classes of binding sites, number of sites within a given class, n, and the apparent association constant for binding to each class of sites, k'. Corrections for electrostatic interactions were not made, and the intrinsic association constants were not determined.

Incubation with Celite

Celite 545 was coated with labeled lipids of known specific radioactivity (10). Weighed amounts of these Celite preparations were incubated with BLG solutions or protein-free buffer solutions in a temperature-controlled water bath with shaking (1). The Celite was sedimented by centrifugation, and the uptake of radioactivity by the protein solution was determined (1).

Fluorescence Measurements

Fluorescence emission spectra of BLG and bovine albumin excited at 280 mμ were recorded between 280 and 450 mμ (11). All fluorescence measurements were made with the Aminco-Bowman spectrophotofluorometer calibrated by Chen (12).

Incubations with Mammalian Tissues

Epididymal fat pads obtained from fasted Osborne-Mendel rats were incubated at 37 C under air with shaking in 10 ml of phosphate-buffered salt solution containing protein and adrenocorticotropic hormone (11). One fat pad from each rat was incubated in medium containing BLG, the other in medium containing albumin. The FFA content of 1 ml aliquots of the incubation medium were measured by titration (2).

Isolated, washed Ehrlich ascites tumor cells were incubated with shaking under air at 37 C (13) with ^{14}C-1-palmitate bound to either BLG or bovine albumin. Incorporation of the labeled palmitate into the cell lipids or into CO_2 was measured (14).

RESULTS

FFA was taken up by BLG when an aqueous solution of the protein was incubated with a soap solution, fatty acid-coated Celite particles, a rat epididymal fat pad or fatty acid dissolved in heptane. If the BLG solution was exposed subsequently to heptane, fatty acid was released into the organic phase. As indicated by the data contained in Table I, this was due to the reversibility of FFA binding to BLG and not damage to the protein from exposure to heptane. ^{14}C-1-Palmitate was added to BLG, and the resulting solution was incubated with a large excess of palmitate-9,10-^{3}H in heptane. As the incubation proceeded, the ^{14}C content of the BLG solution decreased to less than 10% of the original amount while increasing amounts of ^{3}H appeared in the aqueous phase. This was accompanied by the appearance of increasing quantities of ^{14}C in heptane and a decrease in the ^{3}H content of the heptane. The total palmitate content of the BLG solution increased sixfold during the incubation. Thus, most of the ^{14}C-1-palmitate content of the BLG solution was exchangeable even under conditions that produced a large net increase in the total palmitate content of the protein solution.

Data obtained for palmitic acid binding to BLG at 37 C in phosphate buffered salt solution are shown in Figure 1. In this graph, ν is plotted against ν/c according to the method of Scatchard (15). These data were obtained in two separate experiments, each with a different commercial preparation of BLG. Note the excellent agreement of the two sets of data points. The data show a nonlinear correlation between ν/c and ν. This suggests that BLG, like the serum albumins (1,7), contains more than one class of palmitate binding sites. However, the possibility that the nonlinear correlation results at least in part from electrostatic effects that occur in the binding process cannot be excluded. Results similar to these were obtained with lauric, stearic and oleic acids.

Analyses of these data were made with a model consisting of two independent classes of

TABLE II

Constants for the Binding of FFA to β-Lactoglobulin as Derived From a Non-integer Model[a]

Fatty acid	Number of data points	Primary sites, M^{-1}			Secondary sites, M^{-1}			Root mean square error[c]
		$n_1k'_1 \times 10^{-5}$	$k'_1 \times 10^{-5}$	n_1[b]	$n_2k'_2 \times 10^{-5}$	$k'_2 \times 10^{-5}$	n_2	
Lauric	23	0.55±0.02[d]	0.41±0.01	1.34	0.06±0.08	0.58±3.8	0.11	0.042
Palmitic	23	5.17±0.26	3.91±0.38	1.32	3.12±0.29	0.76±0.17	4.11	0.033
Stearic	22	2.00±0.78	3.86±7.3	0.52	8.54±6.50	0.33±0.06	26.1	0.110
Oleic	23	0.39±0.09	0.41±0.01	0.95	32.35±0.49	1.34±0.35	24.1	0.018

[a]Incubation at 37 C in phosphate-buffered salt solution.

[b]Estimates for n. These were derived by dividing nk' by k'.

[c]This parameter is a measure of the average deviation of the data from the computed curve (1).

[d]Parameter uncertainty, i.e., asymptotic estimates of the standard error (9).

BLG binding sites. This treatment assumes that BLG contains a fixed number of total FFA binding sites and that the sites are not altered by the binding process. Starting estimates for fitting the experimental points to a binding curve were obtained with a system that did not constrain the number of binding sites, n, to integer values (1,9). These estimates are shown in Table II. The range of estimates for n, (0.52 to 1.34) suggested that the primary class of BLG sites consisted of only a single site. In marked contrast, the computed estimates for n_2 (0.1 to 26.1) suggested that a single integer value for the secondary class of sites may not be applicable to each of the fatty acids. Moreover, the large uncertainties in $n_2k'_2$ and k'_2 for lauric and stearic acids further suggested that different integer values of n_2 should be considered for each acid (9).

With the values listed in Table II as initial estimates, these data were fitted to models constrained so that n_1 and n_2 must be integers. Table III contains the most satisfactory integer models for binding of each fatty acid. The best fit for oleate was obtained when $n_1 = 1$ and $n_2 = 24$. A best fit for stearate also was obtained with this model, but other integer values produced equally good fits. This is consistent with the large uncertainty in $n_2k'_2$ with stearate (Table II). The best fits for lauric and palmitic acids were obtained with a 1,2 and 1,6 integer model, respectively. In order to compare more readily the binding of each of the four acids and to satisfy our initial assumption that BLG has a fixed number of pre-existing binding sites, we attempted to fit all of the data to a single model. The value of 24 was selected as most representative for the actual number of secondary binding sites because it was best for oleate, the ligand with which we obtained the highest experimental values of ν. The fits obtained with the 1,24 model for laurate and palmitate, although adequate, were not as good as the respective best case for these acids. With the 1,24 model, k'_1 was of the order of $10^5 M^{-1}$ and k'_2 was of the order of 10^3 to $10^2 M^{-1}$ for each of the four acids. Note that even when n_2 was varied over a wide range, there was very little change in the value obtained for k'_1. This indicates that the character of the single high energy BLG binding site is relatively insensitive to the assumptions made concerning the class of weaker secondary binding sites.

Figure 2 shows plots of the data for each acid and the binding curves derived from the values of n and k' for the 1,24 model listed in Table III. These are graphs of ν (the molar ratio of bound FFA to BLG) against the negative logarithm of c (the unbound FFA concen-

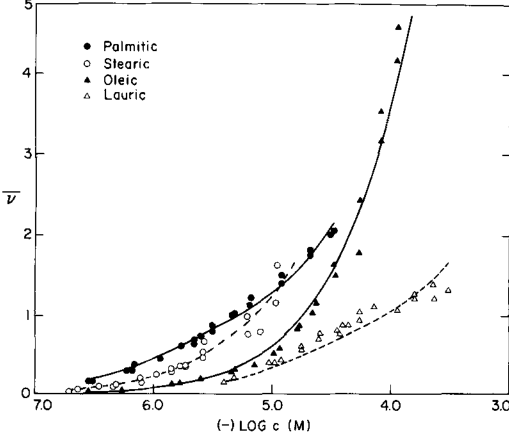

FIG. 2. Graphs for the binding of radioactive free fatty acids to β-lactoglobulin at 37 C in phosphate-buffered salt solution, pH 7.4, for the 1,24 model.

tration). The strength of association was palmitate>stearate>oleate>laurate. The maximum values of ν that were obtained in these experiments were: oleate, 4.6; palmitate, 2.0; stearate, 1.6; laurate, 1.3. Similar maximum ν values were obtained when FFA adsorbed on Celite were incubated with BLG. With each acid, the concentrations of c that occurred at the maximum ν values were equal to the maximum solubility of that particular fatty acid in the protein-free aqueous phase (1). A large excess of FFA was present in the heptane phase or on the Celite under conditions where the maximum ν values were obtained.

Effect on Binding of Changes in Lipid and Protein Structure

The maximum quantity of ligand that was taken up by BLG from Celite decreased when the palmitate carboxyl group was modified or removed. The highest ν values that were obtained were: palmitic acid, 1.9; cetyl alcohol, 0.67; methyl palmitate, 0.28; hexadecane, 0.92. Cholesterol also was taken up to a limited extent, the maximum ν being 0.40. In these systems, large excesses of ligand in the Celite phase also were present when the maximum ν values were reached.

Alterations in the structure of BLG by either physical or chemical means affected the binding of palmitate (Fig. 3). Both the maximum uptake and the strength of association were reduced when the incubation medium contained 6 M urea and when the protein was acetylated or exposed to elevated temperatures prior to incubation.

Effect on Binding of Changes in the Incubation Medium

Palmitate was bound more firmly by BLG at pH 7.4 in 0.05 M Tris-HCl than in phosphate-buffered salt solution (Table IV). In Tris buffer, palmitate was bound more tightly at pH 8.7 than at pH 7.4. In contrast, we did not observe these differences when palmitate binding to bovine albumin was studied (1). In phosphate buffer, palmitate was bound more tightly at pH 7.4 than at pH 6.9. Similar results were noted for FFA binding to both bovine and human serum albumin (1). This observation also is consistent with the finding that palmitate uptake by tumor cells from media containing BLG increased as the pH was decreased below 7.4 (16).

The strength of binding of palmitate to BLG in 0.02 M sodium phosphate, pH 7.4, decreased as the NaCl concentration was raised from 0-1.0 M. Similar results were noted for palmitate binding to bovine albumin (1).

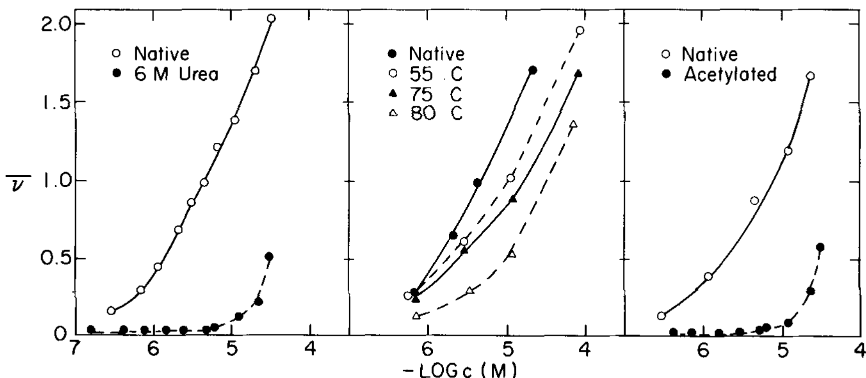

FIG. 3. Binding of 1-^{14}C-palmitate to physically or chemically altered β-lactoglobulin. The incubations were done in phosphate-buffered salt solution, pH 7.4, at 37 C. Approximately 90% of the free amino groups were modified in the acetylated BLG preparation.

TABLE III

Constants for the Binding of FFA to β-Lactoglobulin as Derived From Integer Models[a]

Fatty acid	Primary sites, M^{-1} n_1	$k'_1 \times 10^{-5}$	Secondary sites, M^{-1} n_2	$k'_2 \times 10^{-3}$	Root-mean-square root
Lauric	1	0.52±0.15	2	1.12±0.14	0.085
	1	0.40±0.06	24	0.10±0.02	0.130
Palmitic	1	6.61±0.39	6	7.09±0.14	0.052
	1	7.00±0.51	24	1.58±0.06	0.063
Stearic	1	1.61±0.39	2	4.22±5.50	0.110
	1	1.79±0.28	4	16.98±0.59	0.110
	1	1.56±0.24	6	11.87±0.21	0.110
	1	1.51±0.19	12	5.87±0.01	0.110
	1	1.87±0.24	24	2.48±0.18	0.110
Oleic	1	0.40±0.04	24	1.34±0.01	0.067

[a]The data are the same as those described in Table II.

The temperature dependence of binding of palmitate to BLG between 23 and 37 C was too small to be measured accurately by the present incubation techniques.

Uptake of FFA from Adipose Tissue

Like serum albumin, BLG took up FFA released from adipose tissue (Table V). However, when equimolar amounts of BLG and bovine albumin were present, the net release of fatty acids was greater in the media containing albumin (Experiments 1-3). In these incubations, the rate of FFA release was constant for 90 min when albumin was present but diminished gradually in the media containing BLG. When the BLG concentration was 3.7 times greater than that of albumin (Experiments 4-6), the rates of FFA release in media containing either BLG or albumin were approximately equal, and FFA release was linear during 90 min of incubation in both media. Six additional fat pad incubations were done with media containing low concentrations of protein in order to determine the maximum molar ratios of total FFA to BLG that could be reached in this system. A 4 hr incubation period was employed. The maximum molar ratio that was observed with BLG was 1.1. Under these conditions, there was very little increase in the FFA content of the BLG medium after 2 hr of incubation. In contrast, a molar ratio of 4.7 was reached after 4 hr of incubation when albumin was substituted for BLG, and FFA release continued at a linear rate over the entire 4 hr period. Taken together, these results indicate that BLG is less effective than bovine albumin as an acceptor for FFA released from adipose tissue, apparently because its binding sites have a weaker affinity for FFA than do those of albumin.

TABLE IV

Effect of pH on the Binding of Palmitate to β-Lactoglobulin[a]

$\bar{\nu}$	(–) log C[b], M Phosphate buffer[c] pH 6.9	pH 7.4	Tris buffer[d] pH 7.4	pH 8.7
0.4	5.6	6.0	6.5	6.8
0.7	5.1	5.7	6.2	6.5
1.2	4.6	5.2	5.6	6.1
2.0	e	4.5	4.9	5.3

[a]Incubation was done at 37 C.

[b]Negative logarithm of the unbound palmitate concentration.

[c]Phosphate-buffered salt solution.

[d]0.05 M Tris adjusted to the required pH with I N HCl.

[e]The maximum molar ratio of palmitate to β-lactoglobulin that could be achieved at pH 6.9 was 1.2.

TABLE V

FFA Release From Adipose Tissue[a]

Expt No.	Net FFA release, μeq/g x hr	
	β-Lactoglobulin	Bovine albumin
1	1.4	3.6
2	1.0	1.9
3	1.7	2.9
4	2.6	2.3
5	2.6	2.6
6	2.1	2.6

[a]Epididymal fat pads were taken from Osborne-Mendel rats. One fat pad was incubated under air at 37 C in 10 ml of phosphate-buffered salt solution containing BLG, the other in 10 ml of this solution containing Fraction V bovine albumin. Porcine adrenocorticotrophic hormone (5 units) was present in each of the media. In experiments 1-3, both the BLG and bovine albumin media contained 0.5 μmole/ml protein. In experiments 4-6, the BLG media contained 1.1 μmole/ml protein, but the albumin media contained only 0.3 μmole/ml protein. The FFA content before incubation and after 20, 40, 60 and 90 min of incubation was measured in duplicate by titration of an extract of 1 ml of the medium (2).

Uptake of FFA by Tumor Cells from Media Containing BLG or Albumin

Ehrlich ascites tumor cells took up ^{14}C-1-palmitate when BLG served as the FFA carrier (Table VI). This confirmed our previous preliminary observations (16). At a given FFA-protein molar ratio, much more ^{14}C-1-palmitate was bound, esterified and oxidized to CO_2 when the cells were incubated in media containing BLG than when they were incubated in media containing bovine albumin. We have shown that the magnitude of FFA uptake (13), esterification (14) and oxidation to CO_2 (14) increase as the unbound FFA concentration is raised. At a given ν, palmitate is bound less firmly by BLG than by bovine albumin. Hence, at a given ν, the unbound palmitate concentration is greater in a BLG medium than in an albumin medium. Therefore, one would expect palmitate uptake and utilization to be greater in the BLG medium, and this was observed experimentally. At each ν value, the ratio of FFA to lipid ester radioactivity in the cells was much higher when the medium contained BLG. This observation is compatible with our previous observations concerning FFA utilization (13,14). As the unbound concentration to which a cell is exposed is raised, FFA incorporation in unesterified form increases exponentially whereas fatty acid esterification and oxidation approach limiting values (14). Therefore, one would expect the ratio of FFA to lipid ester radioactivity in the cells to be much higher in the experiments shown in Table VI that were done in the BLG media, for the unbound palmitate concentration with BLG at ν 0.8 is 12 times greater than that present with bovine serum albumin at $\nu = 1.6$ (1).

Similar distributions of radioactivity in the cell lipid esters occurred when incubation was done in either a BLG or an albumin medium. Phospholipids contained from 1.5-2.0 times more radioactivity than glycerides and from 10-40 times more radioactivity than cholesterol esters.

Ultraviolet Fluorescence Spectra

The fluorescence emission spectrum of BLG excited at 280 mμ was altered slightly when FFA were added (Fig. 4). The wavelength of maximum fluorescence occurred at 333 mμ whether or not FFA were added to BLG. However, the presence of 1 μeq of palmitate per μmole BLG produced an 8% increase in fluorescence intensity. Addition of more palmitate resulted in very little further increase in fluorescence intensity. In contrast, the addi-

TABLE VI

Utilization of 1-^{14}C-Palmitate by Ehrlich Ascites Tumor Cells[a]

Fraction	1-^{14}C-Palmitate incorporated, mμeq/10^8 cells			
	$\bar{\nu} = 0.8$		$\bar{\nu} = 1.6$	
	β-Lactoglobulin	Albumin	β-Lactoglobulin	Albumin
FFA	200	10	600	19
Lipid esters	250	100	560	140
CO_2	32	23	40	28

[a]Cells were incubated for 1 hr at 37 C under air with 1-^{14}C-palmitate bound to either β-lactoglobulin or Fraction V bovine albumin. The medium contained 3.0 μeq 1-^{14}C-palmitate (670,000 cpm/μeq) and sufficient protein so that the molar ratio ($\bar{\nu}$) of total palmitate to total protein was that listed in the Table. In addition, the medium contained 0.058 M NaCl, 0.0025 M KCl, 0.0006 M $MgSO_4$ and 0.085 M sodium phosphate, pH 7.4.

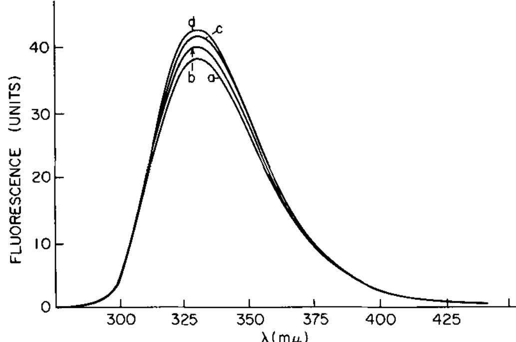

FIG. 4. Effect of palmitic acid on the ultraviolet fluorescence spectrum of β-lactoglobulin. The wavelength of excitation was 280 mμ. Spectra were recorded at room temperature in phosphate-buffered salt solution, pH 7.4. The BLG concentration was 10^{-4} M. The curves are labeled as follows: (a), no added FFA; (b), $\nu = 0.49$; (c), $\nu = 1.4$; (d), $\nu = 1.7$.

tion of FFA to bovine albumin produces a progressive decrease in fluorescence intensity and a blue shift in the wavelength of maximum emission of from 4-7 mμ (11).

Similar effects on ultraviolet fluorescence occurred when oleate, stearate or laurate were added to BLG. An increase in fluorescence intensity occurred independently of the method used for adding FFA to the protein, mixing with a soap solution, incubation with FFA-coated Celite, incubation with FFA dissolved in heptane, or incubation with rat epididymal fat pads in the presence of adrenocorticotrophin.

DISCUSSION

These results demonstrate that BLG can function as a long chain FFA carrier or acceptor in in vitro incubation systems. The affinity of BLG for FFA is considerably less than that of albumin (1,7). BLG contains only one high energy binding site for FFA. The association constant for FFA binding to this site ($k'_1 = 10^5 M^{-1}$) is one tenth that for FFA binding to the primary class of albumin sites (1); it is similar in magnitude to the association constant of the secondary class of albumin binding sites (1,7). Therefore, one can predict that at low values of ν, much more FFA should be available for uptake by tissues from media containing BLG than from those containing bovine albumin. This was confirmed experimentally by incubation with Ehrlich ascites tumor cells (Table VI). The weak secondary binding sites of BLG have an affinity for FFA (k'_2 of the order of $10^3 M^{-1}$) that is similar to the third class sites of the serum albumins (1,7). Hence, these sites are not effective in solubilizing much FFA in aqueous media. According to this interpretation, the factor that limits the quantity of fatty acid that can be taken up by BLG in these incubation systems is not saturation of available FFA binding sites. In fact, many unfilled FFA binding sites remain when the maximum ν values that can be attained experimentally are reached. We suggest that FFA uptake is limited by the solubility of the particular fatty acid in the aqueous incubation medium i.e., the maximum activity of unbound FFA anion that can exist in the aqueous phase.

Goodman has shown that the constant for association of palmitate with the binding sites of the human erythrocyte is in the range of $10^5 M^{-1}$ (17). Hence, one might predict that only the single primary binding site would be able to compete effectively with a mammalian cell membrane for FFA. This prediction was confirmed by the adipose tissue experiments (Table V) which demonstrated that BLG will remove net amounts of FFA only until the ν is in the range of 1.0. Under identical conditions of incubation, much more FFA was released from the fat pads when the medium contained an equimolar amount of bovine albumin. We conclude that the larger FFA release in the media containing albumin results from the greater ability of albumin to bind FFA; not from any special property that enables albumin to remove or enhance the release of FFA from the adipose cell.

FFA binding to serum albumin involves simultaneous electrostatic and nonpolar interactions (1,7,18,19). The present data concerning FFA binding to BLG are compatible with a similar interpretation. Palmitate analogues in which the carboxyl group is modified or removed were taken up to a lesser extent than the acid, and acetylated BLG bound palmitate very poorly. These observations are consistent with the presence of an electrostatic component in the binding process-attraction between the ionized FFA carboxyl group and a cationic protein site. In addition, the association constants varied with the structure of the FFA hydrocarbon chain, and binding was reduced when BLG conformation was altered by exposure to urea or heat. These findings indicate that nonpolar interactions also are involved in the binding process. In fact, binding of large organic ligands can occur in the absence of ionic interactions, for BLG took up small amounts of cholesterol, methyl palmitate, cetyl alcohol and hexadecane.

The binding constants listed in Tables II and III were obtained by making the following

assumptions: (a) that FFA anions interact with BLG; (b) that the measured total unbound FFA concentration is an accurate approximation of the unbound FFA anion activity (1,8); (c) FFA bind to pre-existing BLG sites, and binding sites are neither formed nor altered in the binding process; and (d) each class of binding sites competes independently for available FFA. Recent evidence indicates that some dimerization of FFA occurs in sodium phosphate solutions even when the FFA concentration is low (20). If subsequent work should confirm these results, then our assumption concerning total unbound FFA concentration would not be valid and small corrections will have to be made in these binding constants. In addition, studies on the interaction of organic ligands with serum albumin led Karush to suggest that proteins display conformational adaptability, that is, binding is associated with conformational changes in which binding sites are either formed or altered (21). Recent work by Lovrien also demonstrated that the association of dodecyl sulfate with alkaline bovine serum albumin produces considerable conformational change in the protein (22). Our inability to obtain a single value for n_2 that was suitable for each of the four acids may be considered as further support for the conformational adaptability hypothesis. If the secondary BLG sites are formed or altered when the first FFA molecule is bound, then each acid may induce a somewhat different change. Therefore, n_2 may be different for each acid, and our attempt to fit all of the data to a single model may be an oversimplification.

Another possible source of error stems from the fact that small quantities of heptane were present in the aqueous phase in the equilibrium partition incubations (8). If heptane binds to BLG, then the binding of FFA might be altered. Since small amounts of hexadecane were taken up by BLG, it is likely that some heptane also was bound to the protein. In spite of this potential difficulty, we employed the partition method for the following reasons. Other procedures that are used to study protein-ligand association also present serious difficulties when long chain FFA serve as the ligand. Long chain FFA are very poorly soluble in aqueous solutions of neutral pH, they do not pass through ordinary dialysis tubing, and they adsorb onto many types of solid phases. Furthermore, our data suggest that incubation in the presence of heptane did not grossly alter the capacity of BLG to bind FFA. The maximum ν value that was obtained with palmitate was almost identical in heptane partition and Celite incubations. In addition, others have shown that the apparent association constant for binding of testosterone to bovine albumin is of similar magnitude when measured by either equilibrium partition or dialysis (23).

FFA binding was associated with a small change in the ultraviolet fluorescence intensity of BLG. Hence, it should be possible to monitor reactions involving FFA uptake or release from this protein by spectrophotofluorometric techniques. However, this can be done more readily with bovine albumin, too, because the FFA-induced fluorescence changes are larger (11). These effects upon fluorescence result from alterations in the environment of one or more protein tryptophan residues. This could occur because FFA interacts directly with a segment of the peptide chain that contains tryptophan. Alternatively, this could result from a small conformational change in the region of one or more tryptophan residues that is secondary to binding of FFA at a distant region of the protein.

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Incorporation of 1,2-^{14}C-Ethanolamine Into Subfractions of Rat Liver Phosphatidylethanolamines and Phosphatidylcholines

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ABSTRACT

The incorporation of 1,2-^{14}C-ethanolamine into the liver phosphatidylethanolamines (PE) and phosphatidylcholines (PC) in female rats was studied. These phosphatides were fractionated according to their degrees of unsaturation and the specific activities of monoenoic, dienoic, tetraenoic and hexaenoic fractions were measured at intervals of 1, 20, 60 and 300 min after injection of the labeled precursor. Hexaenoic and dienoic PE incorporated and lost the label rapidly. Although the labeled precursor was incorporated into tetraene PE at a similar rate, this fraction attained a relatively low specific activity that remained essentially constant between 10 and 300 min after injection of the label. Hexaenoic PC had the highest specific activity among the PC fractions at all time periods. Estimations of the rate of loss of radioactivity in the hexaenoic PE fraction and its appearance in hexaenoic PC indicate that the docosahexaenoic acid is conserved, possibly by being reincorporated into PE after becoming a part of the hexaenoic PC species. The high rate of turnover of the hexaenoic PE also suggests that this fraction might have some special role in endogenous choline synthesis.

INTRODUCTION

Methylation of phosphatidylethanolamines (PE) to form phosphatidylcholines (PC) is the only known pathway for de novo synthesis of choline in higher animals. Estimates by Wise and Elwyn (1) indicate that this pathway may provide choline in amounts equivalent to the dietary intake, or about 13 μmoles/day/gram liver.

In the rat the conversion of PE to PC takes place mainly in the liver. When $^{14}CH_3$-methionine is injected, PC containing fatty acids with two to six double bonds incorporate most of the activity. A PC fraction containing docosahexaenoic acid (22:6) quickly attains the highest specific activity, but then loses this activity while the other fractions continue to increase in specific activity (2,3). During the incorporation of methionine, the amounts of PE and PC in the liver do not change and the amounts of the fractions within these classes remain the same (3). Therefore, the labeled PC molecules containing 22:6 are being replaced by unlabeled molecules. The experiments, however, did not indicate whether this replacement was through formation of new molecules by methylation of PE, or if the label was being lost through exchange with different fractions of PC.

If the entire PC molecule were being replaced, a probable source of unlabeled hexaenoic PC would be the methylation of hexaenoic PE. Therefore, we injected rats with radioactive ethanolamine to label their liver PE. The rate of appearance of this label in hexaenoic PC should indicate whether sufficient PE could be methylated to replace the PC which was apparently rapidly removed; and the rate of loss of label from hexaenoic PE should be consistent with its rate of appearance in hexaenoic PC. Arvidson has also studied the incorporation of radioactive ethanolamine under somewhat different experimental conditions (4); our results agree closely in comparable cases.

EXPERIMENTAL PROCEDURES

Female Long-Evans rats were fed a semipurified diet (Table I) ad lib. for two weeks and were allowed access to food until they were killed. Each rat (average weight 215 g) was injected intraperitoneally with a 0.9% saline solution containing 1.5 μc of 1,2-^{14}C-ethanolamine (International Chemical and Nuclear Co., Industrial City, Calif.) per 100 g body weight. At intervals of 10, 20, 60 and 300 min after injection, the animals were exsanguinated by decapitation, and their livers were removed, frozen on solid CO_2, and lyophilized. The dehydrated livers were pulverized and extracted twice for 1 hr each with 100 ml chloroform-methanol (2:1 v/v) containing 0.1 mg hydroquinone. Solvents were removed with a rotary vacuum evaporator, and the lipids were extracted into redistilled petroleum ether (boiling range 30-55 C) for storage at -20 C until analysis. All solvents and chemicals were reagent grade. Three animals were used for each time period.

TABLE I

Diet Composition

Component	Per cent by weight	Comment[a]
Casein	20.0	Provided about 500 μmoles of methionine per day.
Cerelose	64.95	
Fortified oil[b]	1.0	
USP XIV salts	4.0	
Cottonseed oil[c]	9.0	Linolenate intake was about 15 μmoles/day.
B vitamin mix[d]	1.0	
Choline chloride	0.05	Intake was about 83 μmoles of choline per day.

[a]Food intake averaged 20 g/day/rat.

[b]Provided, per 100 g diet, 1700 IU of Vitamin A; 100 U of D_3; and 6.6 mg of α-tocopherol.

[c]This cottonseed oil contained 0.21% linolenic acid.

[d]Provided, as mg/100 g diet, the following: Thiamine HCl, 0.5; riboflavin, 0.5; pyridoxine HCl, 0.25; Ca pantothenate, 2.0; inositol, 10.0; biotin, 0.010; folic acid, 0.02; nicotinic acid, 2.5; B_{12} (0.1%), 0.002; menadione, 0.05.

Liver phospholipids were separated on 0.37 mm layers of purified Silica Gel H (Merck, Darmstadt) with the solvent system of Skipski et al. (5). The plates were sprayed with 0.4% 2′,7′-dichlorofluorescein (Eastman Organic Chemicals) in absolute methanol, and phospholipid bands were identified under ultraviolet light. The bands were scraped into vials and counted (see below). Phosphatidylethanolamines and phosphatidylcholines accounted for about 88% of the radioactivity recovered from the plates, and the remainder appeared near the origin at the R_f of ethanolamine, or between PC and PE. The same sample was chromatographed on another plate; phospholipid bands were eluted and their phosphorus content was determined (6). Phosphatidylcholines represented about 48% of total liver phospholipid and PE about 22%.

Sufficient amounts of PE and PC were isolated by thin layer chromatography (TLC), for separation into subfractions according to the degree of unsaturation by argentation TLC, as previously described (7,8).

Fatty Acid Analyses

Fatty acids were analyzed on an F and M gas liquid chromatograph (Model 810) equipped with hydrogen flame detectors. The instrument was calibrated daily with standards containing known amounts of methyl esters of lauric, myristic, palmitic, stearic, oleic, linoleic and arachidonic acids (Cal Biochem. Corp., Los Angeles, Calif.; Hormel Institute, Austin, Minn.). Pentaene and hexaene fatty acids were identified by relative retention times and by comparison with an authentic sample of methyl docosahexaenoate (Hormel Institute). Methyl esters of the sample fatty acids were prepared by transesterification (8).

Radioactivity Measurements

Radioactivity of the lipid samples was determined in a Beckman liquid scintillation counter (Model LS-100). Extracted lipids were dried in counting vials and counted in 0.5% 2,5-diphenyloxazole in toluene. Lipid and silicic acid scraped from TLC plates were added directly to the counting vials and counted in a solution of 0.4% 2,5-diphenyloxazole in dioxane containing 10% naphthalene and diluted with 0.2 vol of water. Counting

TABLE II

Fatty Acids of Phosphatidylethanolamine and Phosphatidylcholine Fractions in Normal Female Rats

Fraction	μMoles PL in liver	Fatty acids, mole %									
		<16:0	16:0	16:1	18:0	18:1	18:2	>18:2	20:4	>20:4[a]	22:6
Unfractionated PE	72.6[b]	1.0	13.3	0	32.1	2.7	7.6	0.2	29.4	3.2	10.5
PE 18:1 + 18:2	11.0	0.1	20.9	0.1	29.1	5.8	43.6	0.4	0	0	0
PE 20:4	47.4	0.7	9.1	0	37.0	1.9	0.5	0.1	44.9	4.0	1.6
PE 22:6	14.2	0.5	20.6	0.1	23.7	1.9	0.9	0.1	9.6	3.8	38.8
Unfractionated PC	158.7	1.1	17.8	0	29.0	3.0	15.6	0.8	28.0	0.8	3.8
PC 18:1	9.5	1.4	43.7	0	17.0	31.5	6.3	0	0	0	0
PC 18:2	49.5	0.8	24.0	0.2	23.9	2.4	45.6	0.1	0	0	0
PC 20:4	88.9	0.5	12.1	0	32.9	1.2	0.7	0.2	50.9	0.9	0.6
PC 22:6	14.6	0.6	14.7	0.1	24.2	1.2	2.9	0.2	14.1	5.4	36.6

[a]Principally 22:5 fatty acids.

[b]Neither the distribution nor the fatty acids of the fractions changed during the course of the experiment, so values obtained from three individual rats at each time period have been averaged.

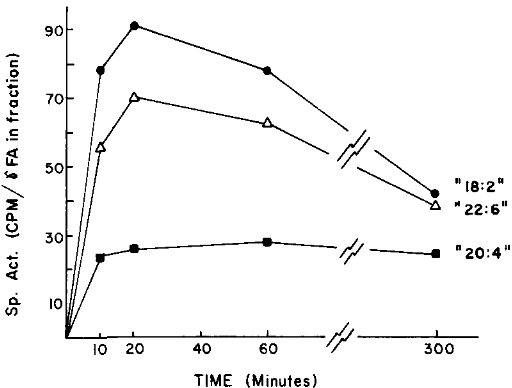

FIG. 1. Incorporation of 1,2-^{14}C-ethanolamine into subfractions of liver phosphatidylethanolamines from unfasted female rats.

efficiency of ^{14}C in the dioxane mixture was 76%; in the toluene mixture it was 82%.

RESULTS

The fatty acids of liver PE and PC, and of the PE and PC fractions obtained by argentation TLC are shown in Table II. A diene fraction containing a small amount of monoene represented about 15% of the total liver PE, whereas the tetraene fraction was about 65% and the hexaene fraction nearly 20% of the PE. The diene and tetraene fractions appeared to be quite well resolved, but we were unable to obtain a hexaene fraction free from arachidonic or pentaenoic acids. The proportion of 22:6 in this fraction was about the same as that observed by Arvidson in fed female rats (2).

Four main PC fractions were separated. The monoene and hexaene fractions constituted about 6% and 9% respectively of the total liver PC. The diene (30%) and tetraene (55%) represented the major portions of this phospholipid. The proportions of these fractions in PC are similar to those we had obtained previously from animals fed the same diet (3). The higher proportions of monoene and hexaene fractions reported by Arvidson (2) probably result from the different animal diets used in the two laboratories.

Table III shows the proportion of administered radioactivity found in PE and PC during a 5 hr period. Within 10 min after administration, most of the radioactivity appeared in the PE and maximum incorporation had already been attained. As time after injection increased, the proportion of radioactivity in PE slowly declined while that in the PC progressively increased. Figure 1 shows the specific activities of the isolated PE fractions. Initially, the diene and hexaene fractions had very high specific activities relative to that of the tetraene fraction. By 5 hr, however, specific activities of the diene and hexaene fractions had declined to about half the levels attained in the first 20 min, while the tetraene specific activity remained low and did not change appreciably during the course of the experiment.

TABLE III

Incorporation of 1,2-^{14}C-Ethanolamine Into Phosphatidylethanolamine and Phosphatidylcholine in Female Rat Liver[a]

Time, min	Phosphatidyl-ethanolamines, % of dose	Phosphatidyl-cholines, % of dose
10	27.1±1.7	0.7±0
20	26.0±1.7	1.9±0.7
60	26.7±1.0	4.7±0.1
300	22.1±0.6	9.8±1.3

[a]Three rats were analyzed separately for each time period.

In Figure 2 the incorporation of ethanolamine into the fractions of PE is expressed as the percentage of total radioactivity in PE. At 10 and 20 min, hexaenoic PE had the largest proportion of radioactivity, which gradually decreased by 300 min. Dienoic PE incorporated the lowest percentage of the label and its proportion remained low during the entire period. Tetraenes incorporated an intermediate proportion of activity at 10 and 20 min and the percentage in this fraction increased as that in the diene and hexaene fractions declined. By the end of the experimental period, the distribution of the label in the fractions resembled the proportions of the fractions in the PE; that is, their specific activities had converged (Fig. 1).

Figure 3 shows the change in specific activities of the PC with time after administration of the ^{14}C-ethanolamine. Specific activities of the monoene, diene and tetraene fractions increased slowly for the entire period, but the specific activity of the hexaene fraction increased more rapidly and remained considerably higher than the other fractions.

DISCUSSION

The hexaene and diene fractions of PE lost their radioactivity much faster than did the tetraene (Fig. 1). Previously, a similar loss of activity had been shown for hexaenoic PC but not dienoic PC after labeling with $^{14}CH_3$-methionine (3). During the experiments, there were no detectable changes in amounts of the fractions in either PE or PC, which indicates

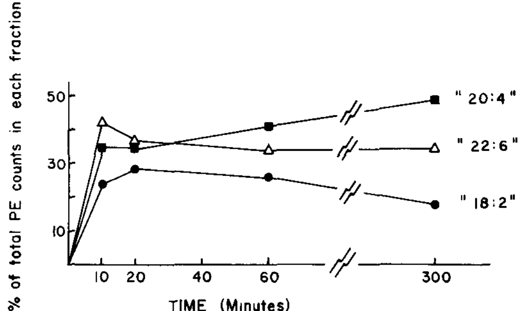

FIG. 2. Percentage distribution of radioactivity in subfractions of liver phosphatidylethanolamines.

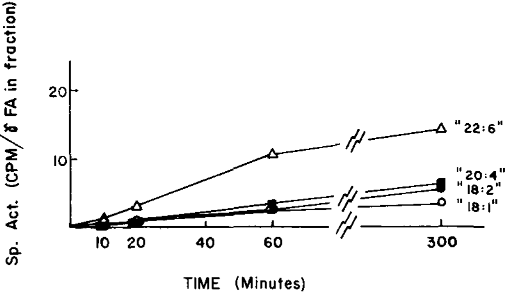

FIG. 3. Incorporation of 1,2-^{14}C-ethanolamine into subfractions of liver phosphatidylcholines from unfasted female rats.

that the radioactive molecules were being replaced with unlabeled ones. The number of counts lost from the PE hexaene fraction was at least equalled by the number gained in the PC hexaene fraction, thus, the loss of activity from 22:6 PE appears to result from conversion of this PE species to the hexaene fraction of PC. If so, radioactive hexaenoic PE is also being replaced by unlabeled molecules.

The labeling pattern in the PE and PC fractions must be a result of the concentrations of precursors available, or of a specificity of the enzyme that selects certain structures from the precursor pool, or both. The labeling pattern of $^{14}CH_3$-methionine incorporated into PC strongly reflects the composition of the PE precursors (2,3) though there is also evidence of some selectivity for certain PE structures (2). The immediate precursor of PE-fatty acids is diglyceride, when ethanolamine is incorporated through the CDP-ethanolamine pathway. In liver diglyceride from male Sprague-Dawley rats (180-200 g) Akesson found 25.2% oleate, 43.2% linoleate, 2.1% arachidonate and only 0.8% docosahexaenoate (9). Despite the small proportion of docosahexaenoate in the presumed precursor, our rats incorporated a large proportion of the label into docosahexaenoate PE. This preferential incorporation therefore must be attributed to enzyme specificity, unless there is another *important* precursor in addition to liver diglyceride. It is interesting that the microsomal enzyme responsible for PC formation from diglyceride precursors apparently has no such specificity and incorporates various diglycerides into PC regardless of their fatty acids (10). Also, in vivo, the incorporation of radioactive choline into PC fractions (11) is roughly proportional to the unsaturated fatty acids present in rat liver diglycerides (9). These results suggest that the formation of PE species from ethanolamine is distinctly different from the analogous synthesis of PC from choline.

Docosahexaenoic acid is biosynthesized from dietary linolenic acid (18:3 ω-3,6,9), its only known source. Linolenic acid is usually present in small quantities in the diet; the present diet supplied about 15 μmoles/rat/day. The animal converts it to docosahexaenoic acid and concentrates this fatty acid in the PE particularly, and also in the PC of many tissues. In livers of EFA-deficient rats, in which no dietary source of linolenic acid is provided, docosahexaenoic acid is retained proportionally much more than members of the linoleate family (ω-6) (e.g., 3,12). Arvidson has suggested that the rapid turnover of ethanolamine label in the 22:6 PE is consistent with a more rapid turnover of linolenate metabolites in comparison with linoleate metabolites (4). Such a fast turnover seems inconsistent with the retention of docosahexaenoic acid in the PE and PC of EFA-deficient rats. Thus it would appear that the PE molecule containing 22:6 is turned over rapidly in its conversion to PC, but in some way the polyunsaturated fatty acid is conserved.

With the available data and certain assumptions, the rate of 22:6 PC formation can be estimated. The rat livers in the present experiment contained about 14 μmoles of 22:6 PE per liver [This fraction contained a small amount of pentaenoic fatty acids (Table II) which conceivably could be of metabolic importance in these reactions. Because of the small amount of pentaene present, and for convenience, this fraction is described simply as "hexaenoic PE."]. The radioactivity in this PE fraction declined to about half in a period of 280 min, which corresponds to a removal or transmethylation of approximately 35 μmoles of 22:6 PE in a day. Secondly, if the radioactivity found in 22:6 PC is assumed to be derived from 22:6 PE labeled with ethanolamine, one can estimate roughly the amount of PC formed by using the specific activity of the PE precursor. This calculation leads to a value

of about 50 μmoles of 22:6 PE converted to 22:6 PC, per day. Finally, with data from an earlier experiment on the incorporation of $^{14}CH_3$-methionine conducted under nearly identical experimental conditions (3), calculations show that the replacement of labeled 22:6 PC amounted to about 40 μmoles/day. The dietary intake of linolenic acid, the precursor of 22:6, was only 15 μmoles/day, yet 35 to 50 μmoles of 22:6 appear to be moving from the PE to the PC, in the liver alone.

These results have at least two possible interpretations. First, it is possible that the conversion of PE to PC is restricted to some metabolic compartment of the liver (microsomes, for example) which does not mix with liver total PE and PC. Thus the replacement of radioactive molecules with unlabeled ones might represent a much smaller amount of 22:6 PE or PC than is present in the entire liver. If these conversions occur within a small metabolic pool, the rates of 22:6 PC formation, and therefore also choline formation, would be much lower than those calculated. It should be noted, however, that rats given adequate methionine but no choline are quite capable of meeting their choline requirements, presumably by methylating PE (13). Also, Bjornstad and Bremer found considerable labeled choline in both the supernatant and the mitochondrial fractions, as well as in the microsomes, of rat liver 10 to 30 min after the injection of female rats with C^3H_3-methionine (14). It is unlikely, therefore, that this reaction is restricted to only a small fraction of the liver PE and PC.

These calculated rates can also be interpreted to indicate that a part of the molecule containing the 22:6 moiety is being recycled. If the labeled 22:6 PC were split (presumably the labeled choline-containing portion would be reutilized), the glyceride moiety containing 22:6 might be reincorporated into unlabeled 22:6 PE. Such a process could account for the apparent transfer of a large amount of hexaenoic PE into PC, and would explain why the content of 22:6 PE does not decrease under these conditions. It would also provide an explanation for the conservation of 22:6 in EFA-deficient rats.

In the foregoing discussion, the possible contributions of other PE fractions to the biosynthesis of choline have been neglected. The specific activity of tetraenoic PE hardly changed after the initial incorporation of about 35% of the activity into PE at 10 min (Fig. 1,2). This kinetic behavior is difficult to interpret since several different mechanisms could be responsible. The present experiments do not show whether tetraenoic PE is being converted to tetraenoic PC, because the activity in tetraenoic PC could also have come from hexaenoic PC by transacylation or by equilibration through CDP choline.

Incorporation of activity into dienoic PE was rapid, and loss of activity was also fast, much like the incorporation curve of hexaenoic PE (Fig. 1). However, the counts lost from the dienoic PE were greater than the counts appearing in dienoic PC, so that the loss of activity cannot be attributed solely to PC formation. The several known mechanisms for removal of the label from dienoic PE seem indistinguishable under these experimental conditions.

The apparent conversion of the hexaenoic PE fraction to PC suggests that it may be of importance in the endogenous synthesis of choline. Rough calculations made using the data of Arvidson (2) lead to a rate of 22:6 PC formation in female rats similar to ours, and both calculations are consistent with the rate of choline synthesis given by Wise and Elwyn (1). If 13 μmoles of choline can be synthesized in a day by 1 g liver, then the rats in this experiment would have synthesized about 13 x 7.3 g/liver or about 95 μmoles of choline per day. Thus about half the total endogenous synthesis of choline might be attributable to the conversion of 22:6 PE to 22:6 PC.

ACKNOWLEDGMENTS

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Analyses of Fatty Acids From Newfoundland Copepods and Sea Water With Remarks on the Occurrence of Arachidic Acid

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ABSTRACT

Comparisons of the total fatty acids from some North Atlantic (Newfoundland) spring copepods (mostly *Temora longicornis*) and from associated sea water have been made. The animal fatty acids were similar in composition to published data for other copepods and this suggests a basic differentiation among planktonic crustacea by which species and genus fatty acid compositions may be compared. The fatty acids recovered from sea water unexpectedly showed a high proportion (~9%) of arachidic acid, which is normally a very minor component in marine lipids of either plant or animal origin. The evidence bearing on widespread occurrence of this acid in sea water and sediments is reviewed.

INTRODUCTION

In the course of other studies a zooplankter tow was made in the surface water of Long Harbour, Placentia Bay, Newfoundland. Live zooplankters, subsequently identified as copepods, were transported to Halifax by air, and further maintained in their native sea water for 48 hr prior to analysis for fatty acid composition.

It was suspected that GLC analysis might be complicated by contaminants originating in the plastic container. A control analysis, including all chemical treatments, was therefore carried out on the sea water and revealed unexpected and interesting detail in the fatty acid pattern.

This report does not constitute a detailed analysis by type of lipid for the copepods, or the sea water, nor have all the precautions sometimes observed (1,2) in analyses of sea water for lipids and fatty acids been taken. However, the use of open tubular (capillary) GLC reveals details in fatty acid composition of considerable interest, and also demonstrates that precautions ordinarily observed in lipid laboratories in handling solvents, etc., to prevent contamination, can provide satisfactory fatty acid analyses from minimal raw marine lipid samples.

EXPERIMENTAL PROCEDURES

The plastic (polyvinylchloride) container used to transport the zooplankton was of the type and size (5 gal) popularly used for transporting gasoline, water, etc., but had been used to deliver reagent grade hydrochloric acid to Newfoundland. It was thoroughly rinsed prior to filling with the collected zooplankters and additional water. Aeration was continuous except during a few hours of air transport. After an elapsed time from capture of 72 hr the water was transferred to glass containers and allowed to stand without aeration for 1/2 hr. At the end of this period a small amount (estimated at 2-3 ml by volume) of loose, green-brown, solid material had settled out but the water was essentially clear to the naked eye. The small crustacea were attracted to the surface by a bright light and removed individually with a minimum volume of sea water by aspiration. Approximately 200 individuals were recovered, and 14 of these were removed at random for later identification. One hundred milliliters of the same top layer of water were removed as the water control and the balance was decanted (discarded) to leave the solids. Microscopic examination suggested that this mass consisted mostly of intact strands of filamentous algae, 2-3 mm long, and this was also discarded. No dead copepods were observed.

The final concentrate of animals in sea water, some 100 ml, was filtered through a filter paper which had been prerinsed with ethanol. The animals and filter paper were transferred to a round bottom flask and refluxed in water-MeOH-KOH for 1/2 hr. The fluid was transferred to a separatory funnel and nonsaponifiables were extracted with petroleum ether. The soap solution was then acidified and the fatty acids extracted with petroleum ether. Methyl esters were prepared with MeOH-BF_3 reagent. Gas liquid chromatography was carried out on a butanediolsuccinate (BDS) polyester open-tubular (capillary) column with tentative identifications of minor components as described elsewhere (3,4). The results are given in Table I. Major components should be accurate to ± 5%, and components >1% to ± 10%, but accuracy is less with components occurring at a level <1%. The minor C_{16} fatty

TABLE I

Weight Per Cent of Fatty Acids and Other GLC Components Derived From Total Lipids of Some Newfoundland Spring Copepods (Mostly *Temora longicornis*) and Sea Water

Fatty acid[a]	Copepod	Sea water	Fatty acid (cont.)	Copepod	Sea water
12:0	2.2	1.3	18:0	2.6	19.1
Unknown (r, 0.129; ECL, 12.46)	1.5	1.3	18:1ω11	0.3	---
13:0	0.2	0.4	18:1ω9	7.3	13.2
Unknown (r, 0.187; ECL, 13.46)	0.7	0.8	18:1ω7	1.7	0.4
Iso 14:0	0.4	0.4	18:1ω5	0.5	0.1
Unknown	0.2	---	18:2ω9?	~0.1	---
14:0	9.1	3.5	18:2ω6	1.3	2.3
Unknown (r, 0.243; ECL, 14.18)	0.5	0.4	19:0	0.1	0.7
14:1ω7?	2.3	1.3	18:3ω6	0.1	---
14:1ω5? (ECL, 14.46)	0.8	0.6	19:1ω?	<0.1	---
Iso 15:0	1.6	0.4	18:3ω3	0.3	---
Anteiso 15:0	0.2	0.5	18:4ω3	1.2	---
15:0	1.0	1.1	20:0	0.2	8.8
15:1ω8?	2.4	1.8	20:1ω11	~0.1	---
15:1ω6? (r, 0.387; ECL, 15.44)	0.4	0.3	20:1ω9	0.2	---
Iso 16:0	0.1	0.2	20:1ω7	~0.1	---
Unknown (r, 0.44; ECL, 15.78)	1.3	0.4	20:2ω6	~0.1	---
16:0	14.1	11.0	Unknown (r, 2.82; ECL, 20.85)	10.6	16.9
16:1ω9	0.7	Trace	20:4ω6	0.1	---
16:1ω7	11.8	4.6	20:3ω3	~0.1	---
16:1ω5	0.2	0.4	20:4ω3	0.2	---
Iso 17:0	0.2	0.1	20:5ω3	12.4	6.2
Anteiso 17:0	0.2	0.2	21:5ω2?	<0.1	---
17:0	0.7	0.4	22:4ω6	~0.1	---
ΣC_{16} polyenes (misc.)[b]	0.2	}	22:5ω6	<0.1	---
16:4ω3	0.3	} 0.8	22:5ω3	~0.1	---
16:4ω1	0.3	}	22:6ω3	5.9	---

[a]Shorthand notation for chain length, number of double bonds, position relative to terminal methyl.
[b]Mostly at the 0.01-0.10% level.

polyene acids were too low in concentration and too poorly defined to permit even tentative identifications. The control sea water was treated by the same procedures, including alkali digestion in the presence of ETOH-washed filter paper. The zooplankters set aside were identified by R. J. Conover, Bedford Institute, as copepods belonging to the Calanidae. Eight were adult females and five were adult males of the species *Temora longicornis*. One was a member of the Tortanidae, *Tortanus discaudatus* [There are various systems of descriptive nomenclature and terminology used in discussion of crustacea. Most of these are based on morphological considerations. For the purposes of this paper the classifications have been simplified as follows: Phylum: arthropoda; Class: crustacea; Orders: Copepoda, Decapoda, Euphausiacea, etc. Within the Copepod order, Families: Calanidae, Temoridae, etc. Genera: *Calanus*, *Temora*, etc. Species: *C. finnmarchicus*, *T. longicornis*, etc.].

RESULTS AND DISCUSSION

The Temoridae, including *Temora longicornis*, are often dominant members of the copepod order of zooplankters found in or near the surface water layers in the North Atlantic and elsewhere in the spring (April-May) zooplankter bloom. It is probable that adults in the natural state exist on a mixed diet of phytoplankters and particles of organic detritus (5). Tortanidae on the other hand are reportedly carnivorous, but it is difficult to be specific in these matters without an examination of actual gut contents (5).

The present experimental data benefits from the fact that the animals had been held long enough to clear most of the original gut contents, eliminating the effect of recent heavy feeding as an influence on lipid analysis. The animals were also freshly killed and promptly analyzed, so post mortem oxidation of unsaturated fatty acids did not take place.

Conversely, the type of lipid has not been defined due to the limited amount of sample, and as has been shown for other marine invertebrates, the fatty acids of triglycerides and phospholipids are quantitatively different in detail although both are subject to some modification by diet (3,4,6). Nevertheless certain features distinguish the fatty acid composition, which may be taken as essentially that of *T. longicornis*, from those recently detailed for lipids of the larger euphausiids *Meganyctiphanes norvegica* (predominantly a carnivore) and *Thysanoessa inermis* (predominantly a herbivore) (4,7). The high proportion (for total fatty acids) in Table I of 14:0 relative to 16:0, 16:1 and 18:1 is similar to that reported for fatty acids of Pacific samples of the Calanidae: *Calanus cristatus* (8), *Calanus finmarchicus* (9), *Calanus plumchrus* (9), and of North Atlantic *Calanus finmarchicus* (10), and relatively very much higher than reported for a number of other types of marine crustacea (7,9,11). A high absolute percentage (10.7%) is also reported for Pacific *Calanus helgolandicus* (12). Probably this high level of 14:0 is a characteristic of the copepods. The absence of 22:1 acids, and the low level of 20:1 acids, may not be strictly a copepod characteristic, as total fatty acids from several euphausiids and other zooplanktonic crustacea show low levels of these acids (4,9,11). These monoethylenic C_{20} and C_{22} acids occur prominently in depot fat and to a lesser extent in other lipids of the larger marine invertebrates and vertebrates (3,13,14), but in zooplankters they may be associated with, rather than replaced by, high levels of 18:1 (4,11,15).

Detailed analysis of lipid from *T. inermis* showed that crustacean triglycerides need not include substantial proportions of 20:1 and 22:1 (4), and their absence from the analysis of Table I does not preclude triglyceride as a substantial part of the lipid of the copepods. However, our present scanty knowledge of the life history, feeding habits and lipid metabolism of these animals requires much elaboration. For example, a high proportion of unsaponifiable material is characteristic of copepods (9), and may affect total fatty acid composition.

Another interesting feature of the monoethylenic fatty acids is found in the isomer distribution patterns within the 16:1, 18:1 and 20:1 acids. These are similar to those observed in the larger euphausiids (4) and also in herring (*Clupea harengus*) (13) which feed heavily on euphausiids and more especially on copepods (5). This common isomer distribution in marine organisms with a diversity of food sources, including those dependent on phytoplankter fatty acids, suggests comparable basic metabolic processes based on desaturation and chain elongation (13).

Caution should be observed in drawing detailed conclusions from even a number of analyses from any one laboratory. For example the selection or collection by different laboratories of species for lipid and detailed fatty acid analysis sometimes tends to group experimental species in categories with $20{:}5\omega3 > 22{:}6\omega3$ (9), which is suggestive of herbivores (4), or the converse (11).

Filter feeders, mostly sessile molluscs and zooplanktonic crustacea, have in many instances been maintained on pure phytoplankter cultures (5), and the possibility of a simple food chain based on fatty acids from a single species of algae: plant→invertebrate filter feeder→carnivore (invertebrate or vertebrate), has thus been demonstrated (6,16). This is, however, a most unlikely occurrence in nature. Organic detritus, fecal pellets, moulted integuments, etc., are as important to many deeper-dwelling species as are algae with original fatty acids (17,18) intact. The surface living zooplankters, which may include those migrating upwards periodically from greater depths to feed in the upper 10-30 meters of the sea, are afforded a great diversity of foods since even spectacular blooms of single species of phytoplankters (and also some of their attendant grazing predators) have a shorter life span than the major types of crustacean zooplankters.

The latter types of animals likely have order, family, genus or species-peculiar fatty acid compositions in all types of lipids representing basically integration of fatty acids from a long-term average food intake modified as necessary for the particular metabolism developed by the animal to cope with overwintering or other periods of food scarcity. Within an order, as evidenced by the copepod data, many families, genera and species would have similar fatty acid compositions if found in a similar habitat and exposed to similar food sources. Deeper-dwelling animals are likely to be less influenced by transient effects, and their fatty acids of exogenous origin should be ingested from a diet already averaged by many precursors closer to the surface. Accordingly their fatty acids should show a more strict genus or species-specific composition pattern. As an example of specificity in total fatty acids, samples of the euphausiid *Systellaspis delibis* collected as far apart as 1,000 miles show a remarkable similarity in fatty acids (11), although this is not a particularly deep-dwelling species.

Sea Water Fatty Acids

Most of the solids suspended in the sea water had settled prior to concentration of the copepods. However, the filtration step for the animals would collect fine particulate matter as well as possibly result in absorption of some dissolved lipids. The sample of sea water, free of any obvious large particles or animals, which was treated as a control in a fashion similar to that of the drained filter paper and animals, thus corresponded roughly to normal sea-water in terms of organic nutrient materials, both particulate and dissolved. The fatty acids recovered, as methyl esters, were estimated from GLC response as about 1/50 to 1 1/100 of the esters recovered from the animals, and this, together with substantial differences in fatty acids such as 20:0 (Table I), makes it unlikely that contamination by similar materials would materially affect the copepod GLC fatty acid analysis.

The fatty acids in this sea-water analysis could be derived from a variety of plant, animal, or microbial sources. Several minor components coincided with fatty acids suspected as probable minor components of most marine lipids (e.g., 15:1ω6, 15:1ω8), or observed as unidentified peaks in the copepod fatty acids. There were some noticeable exogenous contaminants probably associated with the container, and many trace peaks perhaps derived from laboratory solvents. Some may have been artifacts from treatment of lipids with strong reagents such as the MeOH-BF_3 (probably including that with ECL 20.85). These suspect components are identified solely by retention data, or tentatively identified with query if in a position likely for a fatty acid, in Table I. A homologous series with FCL values of 0.44-0.46 appears to be included.

The overall definite fatty acids are primarily the saturated acids from 12:0 to 20:0, the C_{16} and C_{18} monounsaturated acids usually observed in marine plants and lower animals, and a modest amount of 18:2ω6 (linoleic) acid and rather more 20:5ω3 (eicosapentaenoic) acid. With the exception of the polyunsaturates, these are all oxidation resistant fatty acids, and have in most cases been reported by others in sea water, either dissolved or in particulate matter (1,2,19-21). The reports of polyunsaturated acids are scanty and depend mostly on the state of the GLC technology employed. In some cases other materials are differentiated from methyl esters of fatty acids (20). The homogeneity possible in sea waters of diverse origins, except where local effects such as plankton blooms supervene, is demonstrated by the similarity of our fatty acid results to those determined by Ushakov (21) for Black Sea water.

A few deductions may be made for the origin of these acids, and the low proportion of 18:1ω7 and 18:1ω5 to 18:1ω9 suggests that a substantial part of the acids are of plant origin, as the ratio of 18:1ω7 to 18:1ω9 is usually 1:10 to 1:2 in marine animals (3,4,13,14), whereas plants produce predominantly the 18:1ω9 isomer (17). Similarly 18:2ω6, and 20:5ω3 which is the polyunsaturated fatty acid most characteristic of marine algae (17), are probably derived from very recent breakdown of plant cells. The *iso* and *anteiso* C_{15} and C_{17} acids are originally of animal (or bacterial) origin (22,23).

One curious feature of this composition pattern deserves discussion. This is the high proportion of 20:0, relative to 18:0 and of 18:0 relative to 16:0. In most marine life forms 20:0 to 18:0 to 16:0 as 0.1 to 1 to 10 would be a likely proportion. The ratio of 16:0 to 14:0 is, however, not abnormal for marine lipids of either animal or vegetable types (17,22). Broadly the same composition of saturated fatty acids, but with less 20:0, was found in filtered Halifax harbor water. The ratios for the higher fatty acids in sea water are similar to those of certain other analyses (19-21), although not all analyses include 20:0.

This factor affects other data. For example 20:0 may not have been prevalent in samples from the Gulf of Mexico due to a recent plant origin for the fatty acids (1,19), or a tentative identification of 20:1 (2) may be taken as 20:0 in Pacific Ocean samples. The latter study would then put 20:0 in the dissolved category of lipids. Possibly this is why it is reported as a significant component only for animals where seawater circulates freely internally such as in a jellyfish *Cyanea capillata* (24) or in a deepwater holothurian *Scotoplanes theeli* (15).

The relative enrichments in higher longer-chain saturated acids observed in progressively deeper mud samples (23,25), although 20:0 was notably absent from these results [compare (2) as noted above], suggests that C_{14} and C_{16} acids of surface origin are more successfully catabolized by microorganisms or mud feeding animals than, for example, 18:0. Possibly a similar selective microbial action accounts for the accumulation of 20:0 and 18:0 in seawater also. Further studies of seawater and sediments are obviously needed to clarify the status of arachidic acid.

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Fatty Acid Biosynthesis and Incorporation Into Lipid Classes in Seeds and Seed Tissues of Flax[1]

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ABSTRACT

Fatty acid metabolism in developing flaxseeds was studied by incubating whole seeds or isolated seed tissues in buffered solutions of 1-^{14}C-acetate, 2-^{14}C-malonate and $^{14}CO_2$. Lipid classes were separated by thin layer chromatography, and fatty acid labeling in phospholipids, diglycerides and triglycerides was determined by combined thin layer and gas liquid chromatographic techniques. Incorporation of ^{14}C from acetate into embryo lipids was very rapid with phospholipids and 1,2-diglycerides becoming highly labeled in treatment times as short as 5 min. Triglycerides were labeled more slowly. Phospholipid radioactivity was largely associated with the phosphatidyl choline fraction. Oleic acid had the highest specific activity of all major fatty acids in short treatment periods. This was followed in decreasing order of activity by palmitic, linoleic, stearic and linolenic acids. As the treatment period was lengthened to 90 min or longer, linoleic and linolenic activities were markedly increased. Use of malonate or CO_2 rather than acetate as the substrate increased the labeling of the saturated acids. Incorporation of ^{14}C from acetate into lipids of endosperm tissues and whole flax seeds was slower than incorporation into embryo lipids. Stearate had the highest specific activity of the fatty acids in endosperm and whole seeds.

INTRODUCTION

Radioactive metabolites have frequently been used to elucidate fatty acid biosynthesis in developing flaxseeds. These metabolites have been introduced as products of photosynthesis (1), injected into attached or cut shoots bearing developing fruits (2,3), or incorporated by incubation of seed tissue slices or isolated seed parts (4-8). The findings of such studies have demonstrated active lipid synthesis in flaxseeds at all ages from 10 to 40 days after fertilization (5,6,8) and have revealed effects of biotin (4) and certain environmental variables (4,6). In addition, the apparent interconversion of unsaturated acids by sequential desaturation of oleic to linoleic and linolenic acids has received considerable support (1,3,7). The technique has failed to provide evidence of the conversion of stearate to oleate, as claimed for other plants (9,10), and it has not been used thus far to analyze changes in lipid classes during periods of active lipid deposition.

The studies reported here sought to further evaluate fatty acid interconversions in developing flaxseeds, incorporation of newly synthesized fatty acids into various lipid classes, effects of several substrates on fatty acid labeling patterns, and differences in labeling in the two lipid-accumulating tissues of the seed. Emphasis was placed on stearate metabolism by use of a flax line known to be high in this acid (8), since part of the difficulty of detecting rates of stearate labeling in previous studies was due to the low levels in the tissues (1).

EXPERIMENTAL PROCEDURES

Materials and Apparatus

Chemicals used were reagent grade; solvents were distilled prior to use. 1-^{14}C-sodium ace-

TABLE I

Lipid Class Distribution in Embryo and Endosperm of C.I. 2138 Flax as Influenced by Age

Seed age, days	mg/1000 Seeds		
	PL	DG	TG
Embryo			
14	8.6	2.9	109.9
20	27.0	20.8	809.2
21	29.0	21.0	814.8
24	25.9	35.7	1091.3
26	25.9	46.9	1555.6
28	25.9	44.9	1569.8
Endosperm			
26	12.5	25.7	524.0

[1]Presented in part at the AOCS Meeting in New York, October 1968.

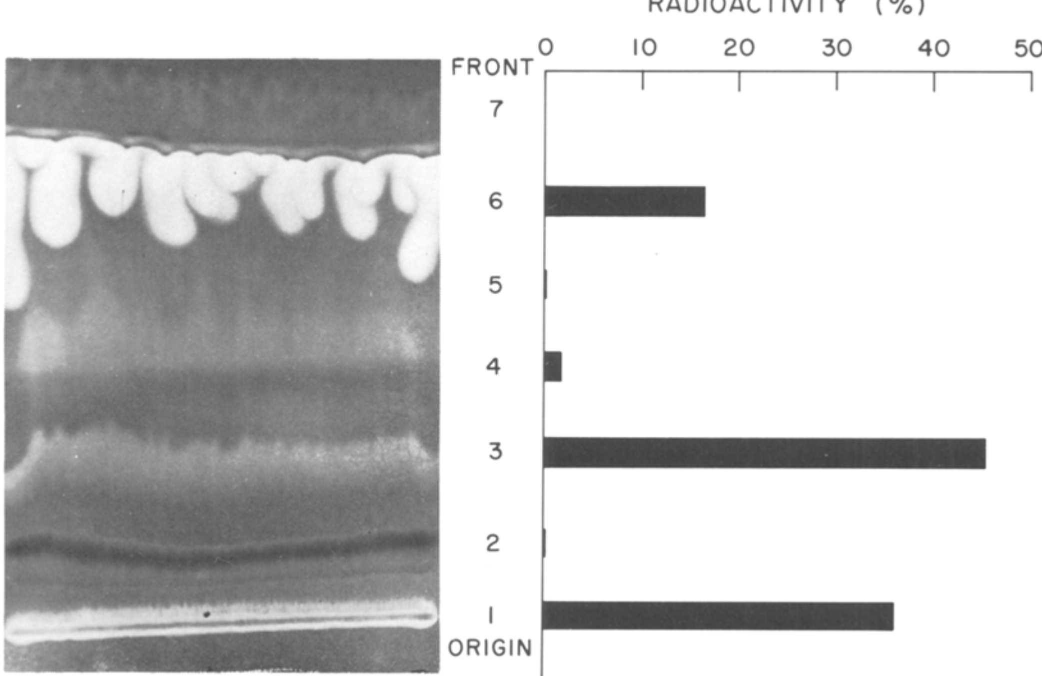

FIG. 1. Separation of radioactive lipid classes from flax embryos incubated in 1-^{14}C-acetate. Sandwich development of 20 x 24 cm plate (0.6 mm layer thickness) in petroleum ether-diethylether (70:30 v/v). Layer contained 1 ml H_3PO_4 (85%) per 30 g Silica Gel G. Visualization by 2,7-dichlorofluorescein, 0.2% in ethanol. Fraction identification: 1, PL and MG; 2, pigments; 3, DG; 4, unknown; 5, FFA; 6, TG; 7, hydrocarbons and pigments. Total load, approximately 175 mg.

tate, sp. act. 44 mc/mM, and 2-^{14}C-sodium malonate, sp. act. 18.3 mc/mM, were obtained from Radiochemical Center, Amersham, England. Both were found to have radiochemical purity greater than 99% when analyzed by paper chromatography. $Ba^{14}CO_3$, sp. act. 1.9 mc/g, was obtained from the same source. Silica Gel G for thin layer chromatography (TLC) was obtained from Brinkman Instruments, Inc. Preparative thin layer plates were developed in methanol to remove impurities prior to separation of lipid classes. Pure fatty acid methyl esters, monostearin, dipalmitin, tripalmitin were produced in the laboratory. Egg phospholipids, and alfalfa glycolipids were prepared by methods previously described (11). Columns and conditions for gas liquid chromatography (GLC) included: (a) 3 ft x 3/16 in. o.d. copper column containing SE-30 on Chromosorb W, 60-80 mesh (1:6 w/w), operated at 190 C and 60 ml/min flow of He in a unit of conventional design using a thermal conductivity cell for detection; and (b) 8 ft x 1/8 in. o.d. stainless steel column containing *o*-phthalic ethylene glycol polyester on 60-80 mesh acid washed C_{22} firebrick (1:4.5 w/w), operated at 205 C and 60 ml/min flow of He in an F & M 5750 Research Chromatograph with flame ionization detector and Infotronics 470 digital integrator. Radioactivity was analyzed by liquid scintillation analysis using the Nuclear Chicago Mark I Spectrometer with 2-(4-*tert*-butylphenyl)-5-(4-biphenylyl)-1,3,4-oxadiazole (CIBA, Ltd.) as the scintillator at a concentration of 5 g/liter in toluene. Counting efficiencies were determined by the channels ratio method.

Tissue Preparation and Treatment

Flax plants, line C.I. 2138 of the U. S. Department of Agriculture flax collection, were grown in nutrient solution in a temperature-controlled greenhouse at 18 C. Bolls of known age were obtained, seeds removed, and tissues dissected by methods previously published (8). Isolated seed tissues were placed in 60 mm petri dishes, 110 to 300 per dish, on filter paper moistened with 1 ml of a medium consisting of pH 6.0 phosphate buffer and 25 μg/liter biotin. Steps in the experimentation, listed in order, were: (a) application of labeled substrates, (b) incubation at 25 C and 200 ft-c illumination,

TABLE II

Incorporation of ^{14}C Into Components of Embryo Phospholipids and Glycolipids[a]

Fraction		Staining reaction			Radioactivity,
No.	Identification	Molybdate	Dragendorff	Ninhydrin	%
1	3 bands, unidentified	+		+	6.7
2	Phosphatidyl choline	+	+		65.4
3	3 bands, unidentified	+			15.0
4	Phosphatidyl ethanolamine and digalactosyl diglyceride	+	+	+	6.2
5	Phosphatidic acid and 1 band, unidentified	+			0.8
6	Monogalactosyl diglyceride		+		2.2
7	Neutral lipid				3.8

[a]Embryos, 24 days old, incubated 10 min in 20 μc 1-^{14}C-acetate. Identification made on basis of Rf's of standard compounds (phosphatidyl choline, phosphatidyl ethanolamine, phosphatidic acid, digalactosyl diglyceride and monogalactosyl diglyceride) and on basis of staining reactions (15,16). Solvent system for TLC: chloroform-methanol-water (65:25:4 v/v).

(c) washing with water to remove unmetabolized substrate, (d) freezing over dry-ice, (e) lyophilization, and (f) storage at -10 C. Exposure of embryos to $^{14}CO_2$ was accomplished by similar methods except the culture dishes were held in a glass desiccating jar and the $^{14}CO_2$ was introduced into the atmosphere. Preliminary studies showed that lipid class and fatty acid labeling were directly proportional to substrate concentration and time of application as long as embryo age, counting from anthesis, was not less than 20 days. Actual treatment conditions in the experiments varied from 5 to 40 μc labeled substrate per culture dish with 5 to 1310 min incubation periods and 20 to 28 days embryo age at initiation of the experiments.

Lipid Class Analysis

Lipids were extracted with chloroform-methanol (2:1 v/v) in an extraction tube containing stainless steel ball bearings (12). The slurry was then filtered, and the residue was washed with solvent. Solvent was removed from the combined extracts under a stream of nitrogen; lipids were dissolved in chloroform, washed with water, and applied to 20 x 24 cm glass plates for separation of the lipid classes. The layer was 0.6 mm thick and contained 1 ml H_3PO_4 (85%) per 30 g of silica gel (13). Development was accomplished by the sandwich method in a solvent system of petroleum ether (Skellysolve B; bp 40-60 C) -diethyl ether (70:30 v/v). Lipid components were located by 2,7-dichlorofluorescein straining (0.2% in ethanol). Minor components were scraped into counting vials containing 5 ml of methanol and assayed directly for radioactivity. Fractions containing phospholipids, diglycerides, and triglycerides (PL, DG and TG, respectively) were transferred separately from the plate to 1 x 30 cm columns for recovery of lipids by elution with 3 ml methanol followed in sequence by 10 ml diethyl ether and 10 ml methanol. These fractions were saponified, acidified with acetyl chloride, esterified with BF_3, recovered in petroleum ether, and made to final volume of 10 ml (14). Aliquots were then assayed for radioactivity and analyzed for fatty acid composition by GLC using the polyester column. Methyl eicosenoate was used as an internal standard for GLC to determine weights of the counting aliquot, the lipid fraction, and each fatty acid in the fraction.

Distribution of radioactivity among the various components of the PL fraction was determined in a separate experiment by conventional methods. The PL fraction was obtained from radioactive embryo lipids by preparative TLC as described above. It was further divided into seven fractions by chromatography on 5 x 24 cm plates (0.25 mm layer thickness) in a solvent system of chloroform-methanol-water (65:25:4 v/v/v). The seven zones were located both by fluorescein staining and charring with 50% H_2SO_4. Staining reactions (15,16), along with Rf's of standard preparations of phosphatidyl ethanolamine, phosphatidyl choline, phosphatidic acid, monogalactosyl diglyceride, and digalactosyl diglyceride, provided identification of some of the embryo PL components.

Fatty Acid Analysis

Aliquots of samples containing fatty acid methyl esters prepared from each of the three major lipid classes were fractionated into component esters in two steps: (a) argentation TLC

TABLE III

Lipid Class Specific Activities in Embryos Incubated for Varied Periods in 1-^{14}C-Acetate

Treatment period, min	dpm per mg per 15 μc substrate[a]		
	Phospholipids	Diglycerides	Triglycerides
5	134,500	189,600	898
10	401,000	438,400	4,575
15	444,900	741,500	6,066
25	659,800	1,023,600	10,960
30	673,600	1,120,000	11,160
60	846,500	1,106,000	20,210
90	1,043,400	1,596,100	35,570
240	1,559,700	1,518,500	72,920

[a]Culture medium contained 5 to 45 μc substrate per 170 embryos. Specific activities corrected to standard 15 μc treatment. Average standard error, 17% of the mean. Total number of trials in experiment, 19. Embryo age, 20 to 28 days.

using 5 x 24 cm plates (0.25 mm thickness) developed in chloroform-acetic acid (99.5:0.5 v/v); and (b) final separation of the saturated and monoene fractions into their component fatty acids by GLC on a SE-30 column with the esters trapped from the effluent gases in glass tubes. Diene and triene fractions were used directly for linoleic and linolenic acids, respectively. The monoene fraction was purified by GLC in most cases, but less than 0.5% of the monoene radioactivity was associated with acids other than oleic. Specific activities of esters recovered from TLC plates and GLC traps were calculated from ester weights determined by GLC with the internal standard. Total and percentage activities of acids in each of the three lipid classes were calculated from fatty acid specific activities and lipid class fatty acid composition data. Summation of values for the three major lipid classes provided an estimate of overall fatty acid labeling in total lipids.

RESULTS

Embryo Lipid Class Labeling From Acetate

Preparative TLC of embryo lipids, 100 to 250 mg per analysis, permitted isolation of seven fractions: 1, phospholipids and monoglycerides; 2, pigments; 3, 1,2-diglycerides; 4, unknown; 5, free fatty acids; 6, triglycerides; and 7, hydrocarbons and pigments (Fig. 1). On a weight basis, TG were the major lipid components at all ages (Table I). TG and DG increased in weight up to 26 days of age, while PL reached a maximum level at 21 days. Average TG fatty acid composition was 5.6% palmitic, 5.9% stearic, 36.6% oleic, 10.7% linoleic, and 41.2% linolenic with little variation in embryos older than 20 days of age. Compositions of DG and PL were similar to TG except PL was slightly higher in palmitic and lower in oleic.

Radioactivity in the embryo lipids was largely associated with the PL, 1,2-DG, and TG fractions (Fig. 1). Fractions 2, 4, 5 and 7 contained only 3% of the activity, and this was mostly located in the unidentified components of Fraction 4. Monoglycerides (MG) did not appear as a discreet class on the preparative plates and were assumed to be mixed with PL. Free fatty acids (FFA) and 1,3-DG also were not visible on the preparative plates. The fact that FFA were not mixed in the TG fraction was experimentally verified by adding 8 mg of myristic acid to a sample containing 200 mg of embryo lipids and searching by GLC for myristate in the methyl esters from each of the seven fractions. PL radioactivity was largely associated with phosphatidyl choline with only minor labeling occurring in other phospholipids, in glycolipids, or in neutral lipids associated with the crude PL fraction (Table II).

Time course experiments showed that incorporation of ^{14}C from acetate proceeded rapidly in embryos with high activity being incorporated into PL, 1,2-DG and TG in periods as short as 5 min (Table III). DG and PL were labeled very rapidly while TG became labeled more slowly. With continuous exposure to acetate up to 240 min, PL and TG increased progressively in specific activity. DG, in contrast, appeared to reach maximum specific activity at 90 min. When the experimental procedure was modified so that a 10 min exposure to 1-^{14}C-acetate was followed by prolonged incubation in unlabeled acetate after a water wash to remove unmetabolized substrate, PL and DG declined sharply in specific activity, while TG increased (Fig. 2, part A). However, there was a slight increase in total lipid radio-

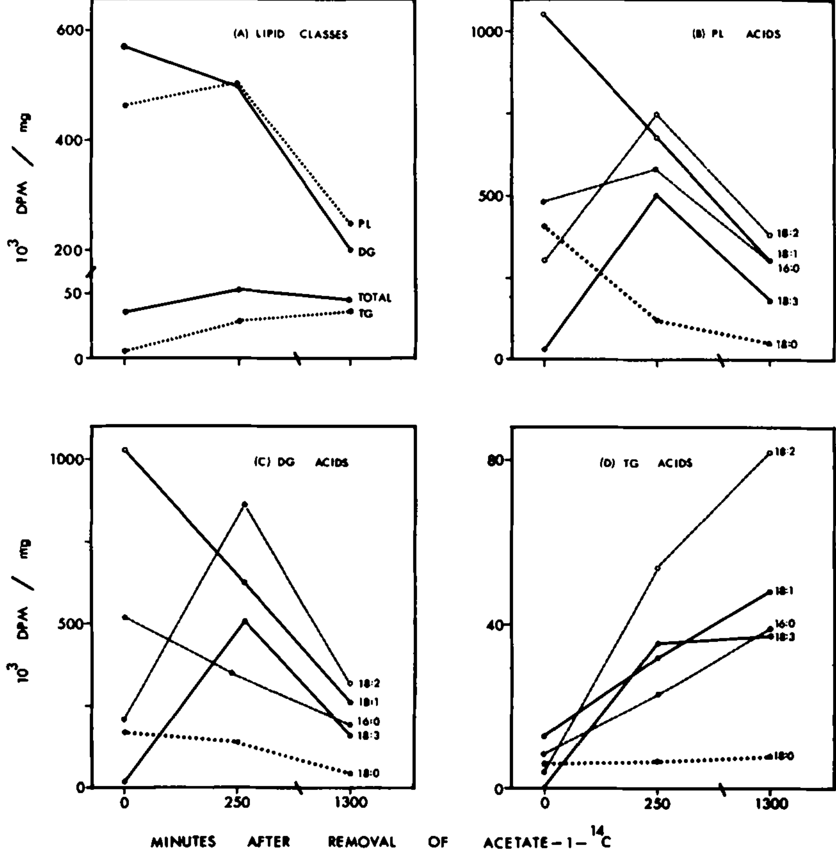

FIG. 2. Labeling of lipid classes and fatty acids within classes in flax embryos incubated in 1-^{14}C-acetate. Experimental treatment was 10 min exposure to labeled substrate (20 μc per 170 embryos) followed by water wash to remove unmetabolized substrate and then 0, 250, or 1300 min incubation on unlabeled acetate.

activity during the first 250 min after removal of the labeled substrate before the reduction in PL and DG activities became apparent. A possible explanation of this result is that non-lipid metabolites labeled during the 10 min exposure to ^{14}C were metabolized to lipid after removal of the labeled substrate.

Embryo Fatty Acid Labeling From Acetate

Embryos incubated in acetate for very short periods (5 and 10 min) had more than 80% of the radioactivity of the major lipid classes incorporated into oleic acid (Table IV). As the period of incubation was increased to 30 min, total radioactivity increased in all acids. The very large increase in labeling of linoleate and linolenate which occurred in this period was compensated, on a percentage basis, by a decline in labeling of oleate. Finally, oleate declined both in total and specific activity as the period of continuous exposure to acetate was lengthened from 30 to 90 min, and activity levels of linoleic and linolenic acids increased still further. This pattern of early labeling of oleate followed in longer periods by labeling of the polyunsaturated acids was verified in Experiment 2 (Table IV).

Changes in radioactivity in the saturated acids were less pronounced in both experiments than were the changes observed for unsaturated acids. Palmitic acid was labeled early resulting in specific activity values midway between oleate and other acids in 5 and 10 min exposures (Table IV). Prolonged exposure to labeled substrate increased total ^{14}C in palmitate but did not change the radioactivity level on a percentage basis. Pronounced accumulations of radioactivity in stearate were not observed and variations which occurred in stearate labeling with time were generally within experimental error. Calculation of the ratio of per cent radioactivity to per cent fatty acid composition

TABLE IV

Labeling of Fatty Acids in Summed PL, DG and TG Fractions From Immature Flax Embryos Incubated in 1-^{14}C-Acetate

Acid	Experiment 1, min[a]			Experiment 2, min[b]	
	5	30	90	10+0	10+1300
Total radioactivity: 10^3 dpm					
16:0	74	684	860	499	583
18:0	14	108	118	304	131
18:1	704	4890	3558	6517	4814
18:2	77	1887	3244	491	1578
18:3	8	277	2491	151	2497
Specific activity: 10^3 dpm/mg					
16:0	10	97	118	44	56
18:0	3	20	15	20	10
18:1	20	139	90	69	60
18:2	5	122	206	22	99
18:3	<1	4	46	2	44
Total radioactivity: %					
16:0	8.4	8.7	8.4	6.3	6.1
18:0	1.6	1.4	1.1	3.8	1.4
18:1	80.3	62.3	34.6	81.8	50.1
18:2	8.8	24.1	31.6	6.2	16.4
18:3	0.9	3.5	24.3	1.9	26.0
Ratio: per cent radioactivity-per cent composition					
16:0	1.44	1.53	1.44	1.22	1.02
18:0	0.35	0.31	0.18	0.57	0.18
18:1	2.76	2.18	1.09	1.92	1.10
18:2	0.68	1.93	2.49	0.62	1.82
18:3	0.02	0.07	0.56	0.05	0.81

[a]Experiment 1: 15 μc per 175 embryos for 5, 30 and 90 min.

[b]Experiment 2: 20 μc per 170 embryos for 10 min followed by 0 and 1300 min incubation on unlabeled acetate. Embryo age, 24 days in both experiments.

showed that stearate remained well below a ratio of one at all periods of analysis. In contrast, all other acids tended toward a ratio of unity.

Analysis of the fatty acids in each of the three major lipid classes revealed only minor differences among lipid classes although there were tendencies for maximum labeling of saturated acids in TG as compared to other classes and for maximum labeling of oleate in PL (Table V). One important difference was observed in Experiment 2 when the labeled substrate was removed at the end of 10 min exposure and the embryos were then transferred to unlabeled substrate for 250 to 1300 min (Fig. 2). In this experiment, all PL and DG acids declined in specific activity as the period on unlabeled substrate was prolonged to 1300 min, while all TG acids increased in activity in the same period.

Effects of Substrate and Seed Tissue

In the final experiment, effects of type of tissue and substrate on incorporation of ^{14}C into flaxseed lipids were determined. Immature flaxseeds were dissected prior to incubation or were tested intact. This procedure permitted incubation of the two lipid-accumulating seed tissues in separate dishes or in situ in undissected seeds. Embryo tissues were incubated on acetate, malonate and CO_2, endosperm tissues on acetate and malonate, and whole seeds on acetate alone. All three substrates were metabolized but definite differences between substrates and tissues were observed (Table VI).

Fatty acid specific activities in embryos incubated in acetate, as in previous experiments, were highest for oleic and lowest for stearic and linolenic acids. When malonate was the substrate for embryos, stearate was the acid with maximum specific activity, while with $^{14}CO_2$, linoleic acid activity was highest of all acids. If the data are examined on the basis of ratio of per cent radioactivity to per cent composition and allowance is made for time-course labeling patterns, CO_2 labeling of embryo fatty

TABLE V

Labeling of Fatty Acids in PL, DG and TG Lipid Classes From Immature Flax Embryos Incubated in 1-^{14}C-Acetate

Acid	Ratio: per cent radioactivity-per cent composition[a] PL	DG	TG
Palmitic	1.00 a	0.81 a	1.59 b
Stearic	0.29 a	0.26 a	0.45 a
Oleic	2.22 a	1.68 b	1.53 b
Linoleic	1.34 ab	1.09 b	1.51 a
Linolenic	0.37 a	0.37 a	0.48 a

[a]Values for a single acid which are followed by the same letter do not differ significantly at the 5% level. Data of experiments 1 and 2 (see Table IV) were combined for the statistical analysis presented in this Table. Tabulated values are means of treatments ranging from 5 to 90 min exposure to acetate at levels of 15 to 20 μc.

acids was most nearly representative of the preexisting fatty acid composition (ratio approaching unity for all acids) of the three substrates.

Total incorporation of ^{14}C from acetate into lipids of endosperm tissues and whole seeds was much lower than in embryos. Fatty acid labeling patterns were similar in endosperm and whole seeds in that incorporation of acetate resulted in very high specific activities for stearate. This pattern contrasted markedly with embryos where little labeling of stearate occurred from acetate.

DISCUSSION

These studies have revealed the presence in developing flaxseeds of small PL and 1,2-DG pools which are very active metabolically and label rapidly from acetate, malonate, or CO_2. The presence of a detectable DG pool in flaxseeds has been questioned in some work. Franzke and Stolz (17) detected not only DG but also MG and FFA. On the other hand, Zimmerman and Klosterman (13) were unable to detect DG in germinating flaxseeds. McKillican and Sims (18) claimed that their failure to detect FFA or partial glycerides in developing flaxseeds was evidence of a lack of artifacts in their techniques. We believe that the DG detected in the present work was not an artifact because tissue handling by other methods (13,18) failed to eliminate the highly labeled 1,2-DG fraction and because appreciable amounts of 1,3-DG, FFA, and phosphatidic acid were not found. Thus, the early labeling we observed in the DG pool followed by slower labeling of TG appears to be a signifi-

TABLE VI

Effects of Seed Tissue and Substrate on ^{14}C Labeling of Flaxseed Lipids[a]

	Embryo				Endosperm		Whole seeds
	Acetate	Malonate	CO_2	CO_2	Acetate	Malonate	Acetate
Lipid classes (10^3 dpm/mg)							
Phospholipids	850	63	20	--	323	40	157
Diglycerides	1110	50	14	--	260	13	176
Triglycerides	20	1	2	--	4	<1	4
Fatty acids (10^3 dpm/mg)							
Class analyzed	DG	DG	PL	TG	DG	DG	DG
Palmitic	911	42	24	3	276	16	187
Stearic	304	169	14	4	1803	154	801
Oleic	1764	72	43	3	774	66	314
Linoleic	918	43	59	9	50	7	18
Linolenic	220	11	10	2	14	2	5
Fatty acids (Ratio: per cent radioactivity-per cent composition)							
Palmitic	0.85	0.77	1.06	0.77	1.05	0.74	0.95
Stearic	0.27	3.12	0.62	1.08	6.72	7.83	3.98
Oleic	1.60	1.32	1.85	0.83	2.95	3.15	1.60
Linoleic	0.81	0.79	2.57	2.80	0.19	0.33	0.09
Linolenic	0.19	0.20	0.43	0.68	0.06	0.11	0.02

[a]Conditions of experiments: (a) 1-^{14}C-acetate, sp. act. 44.4 mc/mM, 15 μc per dish of 170 seeds or isolated seed tissues, 60 min incubation. (b) 2-^{14}C-malonate, sp. act. 18.3 mc/mM, 15 μc/170 tissues, 60 min. (c) $Ba^{14}CO_3$, 1.9 mc/g, 25 μc/110 embryos, 300 min. Tissue age, 23 to 26 days.

cant verification of the participation of a 1,2-DG as an intermediate in TG biosynthesis in plants (19). This conclusion is reinforced by the observation that when the tracer source was withdrawn, TG fatty acids increased in activity, apparently at the expense of PL and DG acids. The rapid labeling of fatty acids in DG and phosphatidyl choline indicates high turnover rates for these materials in flaxseeds. This observation agrees with reports by Nichols et al. (9) for *Chlorella* and may provide some support for their contention that such lipids function in transformations between members of a fatty acid series.

With regard to fatty acid labeling patterns, we believe that the following observations are significant: (a) In embryos, ^{14}C accumulated first in oleate; as treatment period increased, labeling of oleate declined while linoleate and linolenate increased. (b) Palmitate was labeled early, but significant accumulations of ^{14}C-stearate did not occur in embryos incubated in acetate. (c) As incubation time was lengthened for embryos in acetate, all acids approached a per cent activity-per cent composition ratio of unity except stearate which remained much less than one. (d) Use of malonate as the substrate enhanced incorporation into stearate in embryos. (e) Stearate labeling patterns differed in whole seeds and endosperm tissues as compared to embryos. These observations are consistent with the conclusions that oleate is sequentially desaturated to polyunsaturated acids (1) and that long chain saturated and unsaturated acids are formed by different metabolic pathways (7). The concept that stearate is desaturated to oleate (9,10) is inconsistent with the data for flax embryos because, as was observed in work with immature soybean seeds (20), absolute and specific activities for stearate remained low at all treatment periods. Very high turnover rates for the stearate desaturation reaction could keep total incorporation into this acid at a low level, of course, but then specific activity levels for stearate should rise to much greater levels than were observed in any of the lipid classes analyzed.

However, stearate activity levels appeared to be subject to regulation by several factors. Embryo tissues incorporated ^{14}C from acetate into lipids very rapidly, but they did not synthesize stearate at the equilibrium rate existing prior to seed dissection since per cent activity-per cent composition ratios were very low for this acid. This situation was reversed and rates of synthesis of stearate were greater than pre-existing proportions when malonate was the substrate. Only with $^{14}CO_2$ was the ratio nearly normal for stearate in embryos. These data might suggest that stearate was formed by chain elongation of palmitate by the malonate system (21).

Rate of stearate labeling was also influenced by the tissue studied. Both endosperm and whole seeds exhibited high labeling of stearate. This close agreement may indicate that stearate biosynthesis in the undissected seeds under the conditions of the experiment occurred primarily in the endosperm and not in the embryo. High specific activity of stearate in the endosperm may perhaps be accounted for on the basis of tissue senescence if it is assumed that the system by which unsaturated acids are formed is relatively inoperative in endosperm tissues older than 20 days, an age well beyond the period of maximum lipid accumulation in this tissue (8). Clearly, the numerous levels of regulation which were observed for stearate labeling in these studies indicate a need for further enumeration of major factors governing fatty acid composition in flaxseeds in vitro and in vivo.

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Isolation and Structural Determination of the C_{20} and C_{22} Unsaturated Fatty Acids of Rapeseed Oil[1]

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ABSTRACT

Polyunsaturated C_{20} and C_{22} fatty acids, which seldom are found in the triglycerides of higher plants, were isolated from rapeseed oil and their structures fully characterized. Pure 20:2 and 20:3 were identified as all-*cis* Δ-11,14 and all-*cis* Δ-11,14,17. Pure 22:2 and 22:3 were characterized as all-*cis* Δ-13,16 and all-*cis* Δ-13,16,19. Trienoic acids were found in very small amounts. Reinvestigation of the 20:1 acid showed that it is a mixture of 75% *cis* Δ-11 and 25% *cis* Δ-13, whereas the 22:1 is the *cis* Δ-13 isomer only. Evidence is also given for the presence of a 24:1 acid.

INTRODUCTION

Early investigations on the C_{20} and C_{22} fatty acids of Cruciferae seed oils showed besides the two saturated acids the existence of three unsaturated acids: a 20:1, a 22:1 and a 22:2. The 20:1 acid was first isolated by Hopkins (1) in 1946 from the seed oil of *Conringia orientalis* and was identified as the *cis* Δ-11 acid. The 22:1 acid (erucic) is a major component of most Cruciferae and the *cis* Δ-13 structure of this acid had been known for some time. Hilditch and Baliga (2) first indicated the existence of the 22:2 acid. Although this acid was not isolated in the pure state, they were able to show that it contained the methylene interrupted structure with Δ-13 and Δ-16 double bonds.

Mikolajczak et al. (3) were the first to show that several Cruciferae also contain 20:2 (0.2-6%) and 20:3 (0.3-2%) fatty acids. Although these investigators found a number of species of *Brassica* (including *Brassica napus* L.) to contain 20:2, only one species (*Brassica campestris*) was shown to contain a 20:3 acid. The structure of these polyunsaturated acids was not determined. In 1964 Kuemmel (4) also found that rapeseed oil (*Brassica napus* L.) contained the 20:2 acid, which he identified as the Δ-11,14 isomer, see also Miller et al. (5). Kuemmel further reported the occurrence of a mixed Δ-11 and Δ-13 20:1 acid with the ratio of the isomers being 13:1.

In our investigations of rapeseed oil we were able to isolate and determine the structure of a 20:3 and a 22:3 acid. These acids were present in very small amounts in the rapeseed oil. More recently Ackman (6) has also presented evidence for the existence of 20:3 and 22:3 acids in rapeseed oil based on retention time during gas liquid chromatography (GLC). A 20:3 acid containing Δ-5,11,14 double bonds has also been found in seed fats of non Cruciferae species (7,8,9).

MATERIALS AND METHODS

Rapeseed oil purchased from the Hospital and Wohlfahrtsapotheke in Cologne, Germany, was stored at 4 C under nitrogen and had the following characteristics: n_D^{20} = 1.4730; d_{20} = 0.910; saponification number 173 and average equivalent weight 116. Thin layer chromatography (TLC) according to Blank and Privett (10) showed mainly triglycerides with small amounts of sterol esters, free fatty acids, sterols and phospholipids. The fatty acid composition (%) as measured by GLC was as follows: 14:0, 0.2; 16:0, 3.0; 18:0, 0.9; 18:1, 10.0; 18:2, 16.0; 18:3, 11.0; 20:1, 6.0; 20:2, 0.4; 20:3, trace; 22:1, 50.4; 22:2, 1.3; and 22:3 trace.

Analytical Gas Chromatography

GLC analyses were made with Barber-Coleman (Model 10) and W. G. Pye gas chromatographs equipped with ionization detectors and using columns (1.2 m, Pye; 2.0 m, Barber-Coleman) packed with 10% or 20% diethylene glycol succinate (DEGS) on Celite or Chromosorb W (0.1-0.2 mm mesh). The columns were operated isothermally at 160-180 C for fatty acids, 140-160 C for long chain dicarboxylic acids and 100 C for malonic acid. The vaporizer temperature was 60 C above that of the column and the flow rate of the argon carrier gas was 50-60 ml/min.

Preparative Gas Chromatography

A Rumeo Type VGCH No. 1155 gas chromatograph equipped with a thermal conducti-

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vity detector from Rubarth & Co., made in Hannover, Germany, was used. A 130 x 1.1 cm stainless steel column packed with 20% Apiezon L on Chromosorb W (0.2-0.4 mm mesh) was used for the separation of the fatty acid esters according to chain length. The temperatures for column and vaporizer were 220 C and 230 C. Purified nitrogen was used as carrier gas with a flow rate of 500 ml/min. Up to 450 mg of material dissolved in ca. 1 ml of acetone could be separated in one run, at which the C_{22} acids had a retention time of about 3 hr. The separated fractions, collected in U-tubes, were obtained in yields of 70% after removal of some Apiezon L by silica gel column chromatography.

High Vacuum Distillation

Fatty acid methyl esters were distilled at 10^{-3} to 10^{-4} mm Hg through a 1730 mm column of the type described by Jantzen and Wieckhart (11). The esters distilled over between 96 and 160 C at silicon bath temperatures ranging from 200 to 265 C. Good separation of the fatty acids required very slow distillation. Quantities of 40 and 55 g of methyl esters yielded about 92% product; initial amounts below 10 g were recovered in yields of only 37%.

Thin Layer Chromatography

TLC plates, impregnated with silver nitrate, were prepared by dissolving 20 g of silver nitrate in 150 ml of water and adding these to 80 g of silica gel G (Stahl). This mixture was spread in a 0.6 mm thick layer on 20 x 20 cm glass TLC plates, which were activated at 110 C for 1 hr and then kept in a darkened dessicator. Of the fatty acid ester mixture, 70 to 80 mg/plate could be analyzed; the solvent system was petroleum ether-ethyl acetate (4:1). The R_f values of the unsaturated acids, observed under UV light after spraying the plates with Rhodamine B, were: 0.71 for the monoenes, 0.63 for the dienes, and 0.35 for the trienes. Using suction the individual bands were transferred to a special sintered glass funnel and the fatty acid esters extracted from the adsorbent with petroleum ether-ether (80:20). Rhodamine B was not eluted by this solvent. The yields of methyl esters were about 90%. All operations with the thin layer plates were carried out under nitrogen.

Oxidative and Reductive Ozonolysis of the Unsaturated Fatty Acids

Slight modifications of the procedures developed by Klenk and Bongard (12) and Klenk and Kremer (13) were used. The elution pattern of the dicarboxylic acids depends on the quality of the Isherwood gel. Because of the high adsorption effect of the gel in use, the butanol content had to be increased to elute the dicarboxylic acids. The C_{11} and C_{13} acids were eluted with 3% butanol in chloroform, and malonic acid with 50% butanol in chloroform. All dicarboxylic acids were identified by GLC using the methyl esters.

Column chromatography of the dinitrophenyl hydrazones of the aldehydes usually shows several other bands, which complicate the identification of the hydrazones. To overcome this problem, TLC on silica gel G (Stahl) was used to separate the dinitrophenyl hydrazones; the solvent system developed by Dhont and De Rooy (14) was applied. The plates were developed several times in order to get good separation of the aldehyde derivatives.

UV and IR Spectroscopy

The UV-spectra of the unsaturated fatty acid methyl esters before and after alkali isomerization (15) were obtained with a Beckman Instruments Model DK-2 spectrophotometer. The IR spectrum was run on a Perkin and Elmer Infracord spectrophotometer.

EXPERIMENTAL RESULTS

Purification of the C_{20} Fatty Acids

Rapeseed oil (450 g) was saponified in methanolic sodium hydroxide and the resulting reaction mixture crystallized at 5 C. The precipitate was converted to methyl esters (326 g), crystallized twice (-25 and -45 C) from acetone to give a soluble fraction (65.5 g) containing 11.4% C_{20} acids. This material was fractionally distilled and purified by preparative GLC to yield 3.8 g of C_{20} esters (79% 20:1, 19% 20:2 and 2% 20:3).

The mixture of C_{20} methyl esters was then resolved by degree of unsaturation by forming the mercuric acetate adducts (16) and separating them on a silicic acid column (17). The fractionation was monitored by TLC using the method of Wagner and Pohl (18). The monoene, diene and triene portions of the eluate were identified and isolated; however some overlapping of the three groups was evident. After regeneration of the original esters by addition of dilute HCl, the monoenes (3.25 g) and the dienes (0.7 g) were separately purified by preparative silver nitrate TLC. The trienes (70 mg) were purified twice by silver nitrate column chromatography using essentially the method of De Vries (19).

Characterization of the C_{20} Fatty Acids

Oxidative ozonolysis of the 20:1 (iodine value; 78.3 found, 81.8 theory) gave 0.75 mole C_{11} and 0.28 mole C_{13} dicarboxylic acids. Reductive ozonolysis gave 0.5 mole nonanal and 0.21 mole heptanal. This shows that the 20:1 consists of a mixture of 75% Δ-11 and 25% Δ-13 monoenoic acid. The percentage of the Δ-11 isomer in the original rapeseed oil is about 4.5% and that of the Δ-13 isomer about 1.5%.

The UV spectrum of the 20:2 acid after alkali isomerization showed the characteristic absorption at λmax, 230 mμ. Oxidative ozonolysis of this acid (iodine value; 160 found, 164.8 theory) gave 0.91 mole C_{11} dicarboxylic acid and 0.25 mole malonic acid. Reductive ozonolysis gave 0.79 mole hexanal. The 20:2 is therefore a Δ-11,14 dienoic acid and occurs in rapeseed oil at about 0.4%.

The 20:3 (iodine value; 240.6 found, 248.5 theory), which had the same GLC retention time as 22:1 was identified as C_{20} by hydrogenation with PtO as catalyst in methanol followed by GLC. UV-spectroscopy of the alkali isomerized substance showed the conjugated triene absorption, λmax, 268 mμ. Oxidative ozonolysis of this acid gave 1.0 mole C_{11} dicarboxylic acid and 0.81 mole malonic acid. Reductive ozonolysis gave 0.54 mole propanal, which was also identified by TLC, R_f = 0.37. The 20:3 is therefore a Δ-11,14,17 trienoic acid and occurs in rapeseed oil only in trace amounts.

Purification of the C_{22} Fatty Acids

Rapeseed oil (100 g) was saponified in methanolic sodium hydroxide, the unsaponifiables removed by petroleum ether extraction, and the fatty acids esterified with methanol. Fractional distillation of 95 g methyl esters yielded 37.3 g of over 90% pure C_{22} acids, which were then fractionated by low temperature crystallisation using the method of Kolb (20) with a slight variation. The material, dissolved in 1875 ml of acetone, was cooled to -20 C to yield a small precipitate (I), which was separated, and 36.8 g (iodine value; 76.5) of esters in the filtrate. These were dissolved in 940 ml of acetone and colled to -60 C to obtain 34.7 g (iodine value; 76.7) of mostly 22:1 in the precipitate (II) and 2.1 g (iodine value; 106.3) of diene and triene esters in the filtrate. Precipitate II was again dissolved in 940 ml of acetone and cooled to -60 C to yield 34.3 g (iodine value; 72.5) of esters in the precipitate (III) and 0.4 g (iodine value; 104) of material in the filtrate containing diene and triene esters. The fractions containing the C_{22} diene and triene acids (2.1 + 0.4 g) were combined and subjected to preparative GLC to obtain 1.1 g of pure C_{22} material.

The unsaturated C_{22} fatty acid methyl esters were separated by preparative silver nitrate TLC. From a mixture of 690 mg, 153 mg 22:1, 300 mg 22:2, 50 mg 22:3, and 6.8 mg (1% of the mixture) of an unidentified substance which had a R_f value between that of the diene and triene acid, were obtained. The total recovery was 73%. GLC analysis of the unknown material showed a double peak with a retention time similar to that of the triene acid. IR-spectroscopy of this compound showed an absorption band at 963 cm^{-1}, which is characteristic for a *trans* double bond. These analyses presented evidence that the unknown was probably a 22:3 acid containing *trans* double bonds. It remained to be determined whether this acid was a natural product or if it was formed during preparative TLC. The C_{22} rich distillation residue from the purification of the C_{20} acids was fractionated by forming mercuric acetate adducts (16). It is known that the formation of mercuric acetates from unsaturated fatty acids does not alter the configuration of the double bond. Neither GLC nor IR-spectroscopy indicated the existence of a *trans* acid. It is therefore likely that the 22:3 acid with *trans* bonds was an artificial product formed on the thin layer plate or elsewhere.

Characterization of the C_{22} Fatty Acids

Oxidative ozonolysis of the 22:1 (iodine value; 75 found, 75 theory) gave 1.05 mole C_{13} dicarboxylic acid. Reductive ozonolysis gave 0.72 mole nonanal. The 22:1, commonly known as erucic acid, has the double bond in Δ-13 position. There was no indication of an isomeric acid as in the case of 20:1. Erucic acid composes 50% of rapeseed oil.

Oxidative ozonolysis of the 22:2 (iodine value; 142.2 found, 151 theory) gave 0.96 mole C_{13} dicarboxylic acid and 0.31 mole malonic acid. Reductive ozonolysis gave 0.58 mole hexanal. Alkali isomerization of the 22:2 produced UV-absorption characteristic of a conjugated diene, λmax = 230 mμ. The 22:2 is therefore a Δ-13,16 dienoic acid and occurs in rapeseed oil at about 1.3%.

Oxidative ozonolysis of the 22:3 (iodine value; 219 found, 228 theory) gave 0.8 mole C_{13} dicarboxylic acid and 0.72 mole malonic acid. Reductive ozonolysis gave 0.40 mole propanal, which was also identified by TLC by comparison with a known standard, R_f = 0.37. Alkali isomerization of the 22:3 produced UV-absorption characteristic of a conjugated trienoic acid, λmax = 268 mμ. The 22:3 is

therefore a Δ-13,16,19 trienoic acid. This acid occurs in rapeseed oil only in trace amounts.

The existence of a 24:1 acid could be demonstrated by GLC analysis before and after hydrogenation of the distillation residue from the purification of the C_{20} acids, which contained, as already mentioned, mainly high boiling material. The position of the double bond was not elucidated.

DISCUSSION

The data presented for the oxidative and reductive ozonolysis of the C_{20} and C_{22} unsaturated fatty acids clearly shows the structure of these compounds. The yield of dicarboxylic acids in the oxidation reaction was close to the theoretical yield, i.e., one mole of dicarboxylic acid per mole of fatty acid. The yields of malonic acid (25-50%) were less than the theoretical yield due probably to the lability of this acid. The percentage yield of malonic acid increased with the number of double bonds of the fatty acid. Because of the occurrence of side products, the reductive ozonolysis results were less conclusive than the oxidation results. TLC was therefore used to aid in the identification of the aldehyde dinitrophenyl hydrazones. The recovery of the aldehydes was similar to that of malonic acid and increased with increasing carbon chain length.

The Δ-11 and Δ-13 20:1 acids, which occurred in large amounts, were unequivocally identified on the basis of column and gas chromatographic (GLC) analyses of the dicarboxylic acids. One of these peaks corresponded to azelaic acid. Fintelmann (21) has reported the existence of small amounts of Δ-9 C_{20} and C_{22} isomers in rapeseed oil. Ziegler et al. (22) however, have shown that dicarboxylic acids may be degraded during ozonolysis, and on the basis of this observation we were reluctant to postulate the existence of Δ-9 fatty acids.

Ackman (6) using open tubular gas liquid chromatography has recently presented evidence for the existence of various structural isomers of fatty acids including a Δ-15 22:1 in rapeseed oil. Our results on the GLC analysis of dicarboxylic acids from 20:1, 22:1, 22:2, 22:3 showed a peak with retention time similar to the C_{15} dicarboxylic acid, but upon saponification and rechromatography this peak disappeared. On the basis of these findings it was assumed that Δ-15 isomers do not occur in this rapeseed oil. Ackman's results should be interpreted with caution, as no attempt was made to isolate and purify the Δ-15 22:1 acid; also the peak corresponding to this acid was very small and may have possibly been an artifact.

The discrepancy between our findings and Kuemmel's results (4) concerning the 20:1 isomer may probably be explained by the observation of Appelqvist (23), who showed that the seed coat of *Brassica napus* contained considerable amounts of ω-7 acids. These differences in fatty acid composition may therefore be a reflection of different extraction procedures; presumably Kuemmel's sample of rapeseed oil was more completely free of contaminating seed coat oils.

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Glyceride Studies: Part IX: Intraglyceride Distribution of Vernolic Acid and of Five Conjugated Octadecatrienoic Acids in Seed Glycerides

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ABSTRACT

Vernolic (epoxyoleic) acid, present in six seed oils over the range 19-72%, appears to be preferentially attached to the secondary glycerol hydroxyl group. The distribution of five isomeric conjugated octadecatrienoic acids (8,10,12 and 9,11,13) in 18 seed oils has been examined by lipase-catalyzed deacylation. The results are not entirely consistent and more species must be examined before a rational distribution pattern becomes apparent.

INTRODUCTION

Recent studies of intraglyceride distribution by lipase-catalyzed deacylation have revealed a selective distribution of fatty acids in the lipid reserves laid down as glycerides in seeds. Such oils most commonly contain palmitic, stearic, oleic, linoleic and linolenic acids and it seems that the secondary hydroxyl group is acylated by the unsaturated C_{18} seeds in preference to the saturated acids. The higher unsaturated acids, eicosenoic and docosenoic, however, generally accompany the saturated acids in the 1 and 3 positions (1). Oleic, linoleic and linolenic acids do not, in fact, compete equally for the 2 position and linoleic acid is usually enriched at this position slightly more than oleic acid or linolenic acid (2,3). These widely accepted generalizations are based on results from many plant species and from investigations conducted in several laboratories.

Many unusual acids also occur in seed fats but the glyceride distribution of these acids has been studied less extensively because it is difficult to obtain enough species containing these acids in widely varying amount. It is, therefore, more difficult to arrive at satisfactory generalizations about their intraglyceride distribution. Attempts to do this have been made for hexadec-9 and 11-enoic acids (3), octadec-6-enoic (petroselinic) acid (3), octadeca-6,9,12-trienoic (γ-linolenic) acid (3), octadeca-6,9,12,15-tetraenoic acid (3), some conjugated octadecatrienoic acids (3,4), vernolic acid (4-6), dimorphecolic acid (4), coriolic acid (7), lesquerolic acid (6), ricinoleic acid (6), and 9-hydroxyoctadec-12-enoic acid (8). We report here further experiments with six seed oils containing vernolic acid and 18 seed oils (25 samples) containing five octadeca-9,11,13 and 8,10,12-trienoic acids (eleostearic, punicic, catalpic, calendic, jacaric).

EXPERIMENTAL PROCEDURES

All solvents were distilled before use. Petroleum refers to the fraction boiling between 40 C and 60 C.

Thin layer chromatography (TLC) was carried out on thin layers of silica (0.3 mm for analytical purposes, 1.0 mm for preparative

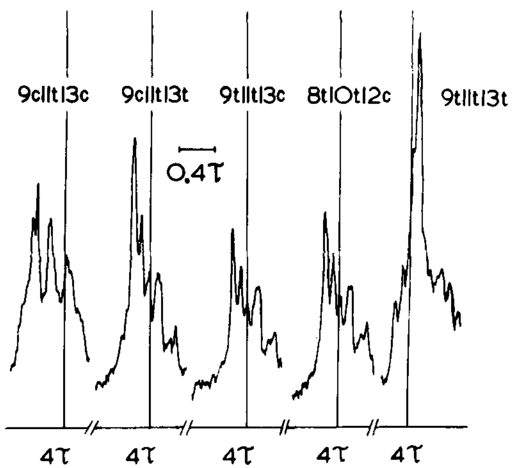

FIG. 1. NMR spectra of *M. balsamina* seed oil (9*c*11*t*13*c*), *M. charantia* seed oil (9*c*11*t*13*t*), *C. speciosa* seed oil (9*t*11*t*13*c*), *C. officinalis* (8*t*10*t*12*c*), and β-eleostearic acid (9*t*11*t*13*t*).

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TABLE I

Vernolic Acid-Containing Glycerides

Species	Family	V^a	$X_3{}^b$	X_2V^b	$XV_2{}^b$	$V_3{}^b$	X_2V^c	$XV_2{}^c$
Cephalocroton peuschelli (Cp)	Euphorbiaceae	72	3	14	43	40	35	79
Cephalocroton cordofanus (Cc)	Euphorbiaceae	67	4	18	44	34	39	77
Crepis aurea (Ca)	Compositae	60	9	18	59	14	56	96
Euphorbia lagascae[d]	Euphorbiaceae	58	10	15	56	19	20	92
Crepis aurea[d]	Compositae	54	13	18	59	10	57	94
Crepis vesicaria (Cv)	Compositae	52	14	15	60	11	54	96
Cephalaria joppica (Cj)	Dipsacaceae	36	31	38	25	6[e]	50	81
Cephalaria joppica[d]	Dipsacaceae	27	37	40	22	0	57	94
Cephalaria leucantha (Cl)	Dipsacaceae	19	55	32	10	3[e]	51	75

[a]Content (mole %) of vernolic acid in seed oil.

[b]Content (mole %) of glycerides containing 0,1,2 and 3 epoxyacyl chains (V, vernolic acid; X, all other acids).

[c]Content (mole %) of 2-monovernolin in the 2-monoglycerides resulting from lipolysis of these fractions.

[d]Result taken from Reference 5.

[e]These values are too high since the extracted glycerides contain less than 100% of vernolic acid (see Table III).

purposes) containing, where necessary, 15% of silver nitrate. Compounds were made visible by spraying with an ethanolic solution (0.2%) of 2,7-dichlorofluoroscein and viewing under UV light.

A Pye 104 was used for most of the gas liquid chromatography (GLC). Columns were packed with Gas Chrom Z (70-80 mesh) coated with 20% diethylene glycol succinate and operated at 190 C.

UV spectra were recorded in methanol solution on a Unicam SP 700 and NMR spectra were recorded in carbon tetrachloride solution using a Perkin-Elmer R10 spectrometer (60 Mc/sec).

Examination of Seed Oils Containing Vernolic Acid

Triglyceride Isolation and Separation. Seeds were ground in a mortar under petroleum and extracted with this solvent in a Soxhlet.

Triglycerides (~200mg) were separated into five fractions by preparative TLC (10 plates) using petroleum-ether (3:1) and the separated fractions recovered from the silica by extraction with ether in a Soxhlet.

The nonepoxy and monoepoxy glycerides (~6 mg) were each separated further by preparative silver ion chromatography using benzene-ether (9:1 and 3:1, respectively). The separated glycerides were recovered from the silica by stirring with methanol-ether-water (5:5:1) and methyl heptadecanoate was then added as internal standard (9). The mixture was extracted with ether and the product transesterified for GLC examination. A correction factor (1.26) was used with the peak due to methyl vernolate.

Methyl esters were prepared from the whole oil or from separated glycerides (~5 mg) by reaction at room temperature overnight with

TABLE II

Enrichment Factors of Oleic, Linoleic and Vernolic Acids in the X_3, X_2V and XV_2 Glyceride Fractions[a]

	X_3		X_2V			XV_2		
Species	Ol	Lin	Ol	Lin	V	Ol	Lin	V
Cp[b]	1.2	1.4	1.3	1.1	1.1	0.9	0.8	1.2
Cc	1.2	1.4	1.2	1.0	1.2	0.9	0.9	1.1
Ca	1.1	1.6	0.5	1.1	1.7	0.1	0.2	1.5
Cv	1.0	1.4	0.6	0.9	1.6	0.2	0.1	1.4
Cj	1.2	1.6	1.2	1.4	1.5	1.0	1.3	1.3
Cl	1.5	1.7	1.1	1.4	1.6	1.1	1.4	1.3

[a]Enrichment factor: Content of acid in the 2-monoglycerides/Content of the same acid in the triglycerides.

[b]Abbreviations detailed in Table I.

TABLE III

Component Acids (Mole %) of Triglycerides and 2-Monoglycerides Resulting From Lipase Hydrolysis of X_3, X_2V, XV_2 and V_3 Glyceride Fractions

	Triglycerides										2-Monoglycerides						
	12:0	14:0	16:0	18:0	18:1	18:2	18:3	20:0	V[a]	C[b]	14:0	16:0	18:0	18:1	18:2	18:3	V[a]
Cp[c] (29% oil)[d]																	
Whole oil			3.7	2.9	7.4	13.1	0.9		72.0								
X_3 (2.8)			16.0	9.7	30.4	40.7	3.2					1.9		36.5	57.4	4.2	
X_2V (13.6)			7.3	5.2	18.5	32.1	2.1		34.6			1.3		24.1	37.8	1.6	35.2
XV_2 (41.2)			4.4	3.3	8.7	15.2	1.0		67.4			0.5		7.8	13.0		78.7
V_3 (38.8)[e1]									100.0								
Cc (30% oil)																	
Whole oil			4.5	3.2	8.3	16.4	0.9		66.7			2.7		37.5	57.0	2.8	
X_3 (3.8)			13.5	10.5	31.1	41.8	3.1					1.4	1.2	20.3	36.1	1.8	39.2
X_2V (16.7)			6.6	5.4	17.0	35.6	1.8		33.6			0.8	0.4	6.3	15.5	0.5	76.5
XV_2 (40.6)			3.5	3.1	7.4	17.7	0.9		67.4								
V_3 (31.8)[e2]			0.3	0.1	0.4	0.7			98.5								
Ca (30% oil)																	
Whole oil			3.8	2.3	10.8	20.5			59.6	3.0							
X_3 (8.7)			11.4	6.7	22.0	47.1	1.7	1.1		10.0				24.8	73.1	2.1	
X_2V (17.2)			5.1	2.4	16.6	33.4			32.5	10.0				9.0	35.0		56.0
XV_2 (55.2)			3.9	2.0	8.8	18.6			66.2	0.5				1.2	2.8		96.0
V_3 (12.8)[e3]			1.1	0.5	1.6	3.0			93.3	0.5							
Cv (12% oil)																	
Whole oil			5.5	2.1	7.4	31.6	1.2		52.3	1.1							
X_3 (13.5)			13.1	5.5	16.6	59.1				4.5				15.8	82.5	1.2	
X_2V (14.6)			6.1	2.5	12.3	43.1			33.8	2.2				6.7	39.5		53.8
XV_2 (57.5)			3.8	1.6	4.5	22.4			67.7					1.0	2.9		96.1
V_3 (9.9)[e4]			1.3	0.6	1.1	5.8			91.2								
Cj (18% oil)																	
Whole oil		9.2	14.4	2.8	15.3	22.7			35.6								
X_3 (28.8)		10.1	18.2	3.5	28.6	39.6					0.6	0.9	0.4	35.0	63.1		
X_2V (35.4)		9.8	16.5	3.1	16.0	21.4			33.2		0.7	0.8	0.5	18.5	29.3		50.2
XV_2 (23.7)		7.3	12.2	2.2	6.9	7.5			63.9		0.6	1.0	0.4	7.1	9.9		81.0
V_3 (6.7)[e5]		3.1	6.2	1.2	6.6	7.6			75.3								
Cl (15% oil)																	
Whole oil	10.0	9.8	8.1	1.5	19.5	31.7			19.4								
X_3 (53.5)	13.5	11.4	9.2	1.6	23.0	41.3								34.1	65.9		
X_2V (30.7)	10.7	9.5	7.7	1.5	15.9	21.6			33.1					17.7	31.0		51.3
XV_2 (9.9)	6.8	7.1	5.3	1.0	9.1	10.9			59.8					10.1	15.4		74.5
V_3 (2.9)[e6]	5.5	6.2	5.5	1.0	10.3	13.4			58.1								

[a]V, vernolic acid.

[b]C, crepenynic acid.

[c]Abbreviations detailed in Table I.

[d]The lipolysis studies of these fractions were carried out on different preparations of the glyceride fractions from those reported here. Differences in triglyceride composition were insignificant.

[e]These oils also contain more polar fractions with a lower concentration of vernolic acid viz. 3.6% (31% vernolic acid)[1], 7.1% (38%)[2], 6.1% (60%)[3], 4.5% (52%)[4], 5.4% (48%)[5] and 3.0% (30%)[6].

TABLE IV

Glyceride Composition of X_3 and X_2V Fractions Determined by Silver Ion Chromatography

X_3 Glycerides	001[a]	011	002	111	012	112	022	122	222
Cp[b] (2.8%)[c]	6	7	11	6	20	13	19	13	5
	8	9	9	4	19	12	20	12	7
Cc (3.8%)	6	9	9	7	21	10	18	12	8
Cj (28.8%)	5	9	9	2	28	10	24	8	5
Cl (53.5%)	9	8	19	3	19	6	21	10	5
X_2V Glycerides	00V[a]	01V	11V	02V	12V	22V			
Cp[b] (13.6%)[c]	1	13	12	26	26	22			
	1	12	10	26	28	23			
Cc (16.7%)	1	10	9	28	26	26			
Cj (35.4%)	19	21	7	30	14	9			
Cl (30.7%)	17	23	8	31	12	9			

[a]These symbols indicate the three acyl chains in the glyceride (0, saturated; 1, monoethenoid; 2, diethenoid; V, vernolic) and include all possible isomers.

[b]Abbreviations detailed in Table I.

[c]Proportion of the total oil contained in this fraction.

anhydrous methanolic sodium methoxide (5 ml, 0.05%) and recovered without acidification of the reaction mixture.

Lipolysis. TRIS buffer was made by dissolving trihydroxymethylaminomethane (12.11 g) in distilled water (20 ml), titrating this with 1 M hydrochloric acid to pH 8.0, and diluting to 100 ml with water. Pancreatic lipase (15 mg) which had been purified by extraction with acetone, was dispersed in TRIS buffer (10 ml) and an aliquot (1 ml) of this was added to the triglyceride (5 mg) in a centrifuge tube. Calcium chloride solution (2.2%, 0.1 ml) and bile salt solution (0.05%, 0.3 ml) were also quickly added. After keeping the mixture at 40 C for 1 min it was stirred at this temperature for 8 min. The reaction mixture was then poured into water, extracted with ether, and the 2-monoglycerides isolated by preparative TLC with chloroform-acetone-0.880 ammonia (80:20:1).

Examination of Seed Oils Containing Conjugated Acids

Triglyceride Isolation. The seeds (full names are given in Tables I and VI) were ground in a mortar under petroleum with six different portions of solvent which was subsequently removed from the filtered solution at room temperature. If TLC (petroleum-ether, 4:1) showed the presence of partial glycerides and free acids, neutral triglycerides were isolated by column chromatography (10) or TLC.

TABLE V

Component Acids (mole %) of Triglycerides Before Lipolysis and of Unreacted Triglycerides Recovered After Lipolysis[a]

	16:0	18:0	18:1	18:2	18:3[b]
C. macrosiphon (9c11t13t)					
Original	5	3	5	35	52
Recovered	4	3	4	34	53
T. anguina (*9c11t13c*)					
Original	6	5	13	20	56
Recovered	6	5	13	20	55
C. ovata (9t11t13c)					
Original	3	3	8	38	44
Recovered	3	2	8	39	45
T. hyoseroides (8t10t12c)					
Original	5	3	8	41	40
Recovered	5	3	8	42	39

[a]Similar results were obtained with all the oils examined.

[b]This refers to conjugated octadecatrienoic acids; minor unsaturated acids are omitted.

TABLE VI

Component Acids (Mole %) of Triglycerides and 2-Monoglycerides Resulting From Lipase Hydrolysis of Seed Oils Containing Conjugated Octadecatrienoic Acids

Seed Oil	Triglycerides				2-Monoglycerides				Enrichment factors		
	sat.	18:1	18:2	18:3[a]	sat.	18:1	18:2	18:3[a]	18:1	18:2	18:3[a]
Eleostearic (9c11t13t)											
Tung oil[b]	5	11	15	69	--	9	31	60	0.8	2.1	0.9
Centranthus ruber[c]	7	4	36	52	1	6	91	1	1.5	2.5	0.0
C. ruber	7	3	37	52	1	6	90	3	2.0	2.4	0.1
C. macrosiphon[d]	8	5	35	52	1	10	83	5	2.0	2.4	0.1
Momordica charantia[e]	20	9	10	61	5	5	14	76	0.6	1.4	1.2
M. charantia	22	8	7	63	1	3	9	88	0.4	1.3	1.4
M. charantia	26	6	6	61	3	3	10	79	0.5	1.7	1.3
M. dioica[f]	22	10	10	57	2	7	10	81	0.7	1.0	1.4
Valeriana officinalis[g]	7	3	41	48	3	10	81	5	3.0	2.0	0.1
Punicic (9c11t13c)											
Momordica balsamina[h]	15	7	10	68	1	6	21	72	0.9	2.1	1.1
Punica granatum[i]	4	4	4	86	1	4	12	83	1.0	3.0	1.0
Tricosanthes anguina[j]	11	13	20	56	4	28	49	19	2.2	2.5	0.3
Apodanthera undulata[k]	14	14	38	33	2	17	79	3	1.2	2.1	0.1
Cucurbita palmata[l]	12	20	38	29	2	20	73	4	1.0	1.9	0.1
Catalpic (9t11t13c)											
Catalpa speciosa[m]	7	8	38	45	3	12	70	12	1.5	1.8	0.3
C. speciosa	7	9	47	33	1	15	74	9	1.7	1.6	0.3
C. ovata[n]	5	10	40	41	--	17	67	13	1.7	1.7	0.3
C. ovata	6	8	38	44	--	17	72	8	2.1	1.9	0.2
C. bignonoides[o]	4	10	45	41	--	15	76	9	1.5	1.7	0.2
C. bignonoides	6	9	45	40	--	16	79	5	1.8	1.8	0.1
Chilopsis linearis[p]	8	9	44	26[t]	4	14	67	11[t]	1.6	1.5	0.4
Calendic (8t10t12c)											
Calendula officinalis[q]	3	4	29	64	1	5	14	80	1.3	0.5	1.3
C. officinalis	4	5	31	59	--	3	16	80	0.6	0.5	1.4
Tripteris hyoseroides[r]	8	8	41	40	1	10	41	44	1.3	1.0	1.1
Jacaric (8c10t12c)											
Jacaranda mimosifolia[s]	9	12	38	39	1	17	76	6	1.4	2.0	0.2

[a]This refers to conjugated octadecatrienoic acids; some minor unsaturated acids have been omitted.

[b-s]Plant families: Euphorbiaceae (b), Valerianaceae (c,d,g), Cucurbitaceae (e,f,h,j-l), Punicaceae (i), Bignoniaceae (m-p,s), Compositae (q,r).

[c-s]Structure of octadecatrienoic acids: species c, reference (16), d (17), e (16), f (27), g (20), h (16), j (19), l (20,21), m (16), n (24), p (24), q (18 and 23), r (17), s (25).

[t]Also 18:2 (10*t*12*t*) 12% in triglyceride, 4% in 2-monoglyceride.

TABLE VII

Component Acid (mole %) of Triglycerides and 2-Monoglycerides Resulting From Lipase Hydrolysis of Seed Oils Containing Conjugated Dienoic, Trienoic and Tetraenoic Acids

	16:0	18:0	18:1	18:2	18:2[a]	18:3[a]	18:4[a]
Parinarium laurinum (Chrysobalaneae)							
Triglyceride	3.4	3.9	1.9	1.8	17.8[b]	30.3[c]	40.9[d]
2-Monoglyceride	1.5	0.9	2.6	3.5	28.2	32.8	30.5
Enrichment factor	---	---	---	---	1.6	1.1	0.7
Impatiens gladuligera (Balsaminaceae)							
Triglyceride	8.8	11.8	14.7	29.8	10.3[b]	6.6[b]	17.4[d]
2-Monoglyceride	3.0	9.4	16.5	43.1	10.8	5.4	11.0
Enrichment factor	0.3	0.8	1.1	1.4	1.0	0.8	0.6

[a]These acids have conjugated unsaturation.

[b]Detailed structure not known.

[c]Eleostearic acid (9c11t13t).

[d]Parinaric acid (9*c*11*t*13*t*15*c*).

Extraction and purification was carried out on the same day as the glycerides were required for analysis.

Lipolysis and Separation and Analysis of Glycerides. The glycerides (400 mg) were subject to lipolysis as described by Desnuelle and Savary (11) and Coleman (12). After 4-5 min the lipolysis mixture was acidified and extracted with ether.

Aliquots of the lipolysis product (~30 mg) were placed on a TLC plate and developed with chloroform-acetone-0.880 ammonia (80:20:1) [a mixture of ether-petroleum (15:85 was used as developing solvent to isolate unreacted triglycerides]. The separated components were extracted from the silica with ether (six extractions) and converted to methyl esters by reaction with sodium methoxide (13) in a nitrogen atmosphere at room temperature for 2 hr.

The methyl esters were examined by GLC and, in some cases, by UV spectroscopy also. These two procedures gave similar results for the proportion of conjugated octadecatrienoates and the following examples are typical: tung (*Aleurites montana*), 69.0% by GLC and 70.5% by UV spectroscopy; *M. charantia*, 61.2% and 59.5%; *C. ruber*, 52.3% and 48.0%; *C. ovata*, 40.6% and 40.2%; *C. bignonoides*, 40.1% and 40.0%. The GLC procedure which was necessary for the nonconjugated components of the mixtures was thus used for the conjugated esters also.

NMR Spectra. The examination of a number of the oils available to us and, in some cases, of the isolated conjugated octadecatrienoic acids, along with β-eleostearic acid formed through isomerization of the α-isomer in tung oil, showed that there was a marked difference in the signals produced by the six olefinic protons (Fig. 1). All gave complex signals with the most intense signal at 4.1τ for the all *trans* isomer (9*t*11*t*13*t*), at 3.8τ for *ttc* isomers (9*c*11*t*13*t*; 9*t*11*t*13*c*; and 8*t*10*t*12*c*), and at 3.7τ for a *ctc* isomer (9*c*11*t*13*c*).

RESULTS AND DISCUSSION

Vernolic Acid

The proportion of glycerides containing three, two, one and no vernolic acid chain is summarized in Table I which contains our results along with those previously reported by Tallent et al. (5). Our separations were effected by TLC and theirs by column chromatography but the results are in general agreement. The figures do not, in general, agree with values calculated according to a 1,2,3-random or to a 1,3-random-2-random distribution pattern as already reported by Tallent et al. (5).

The present lipolysis studies of the mono- and divernolin fractions confirm and extend the earlier results (5). The 2-monoglycerides resulting from lipase-catalyzed deacylation are generally enriched in vernolic acid both in the monovernolins (mainly 50-75% compared with 33% in the triglycerides) and the divernolins (75-96% compared with 67% in the triglycerides). In the divernolins from *E. lagascae*, *C. aurea*, *C. vesicaria*, and *C. joppica* the 2 position is acylated almost entirely by vernolic acid (92-96%). Tallent et al. (5) have already drawn attention to the unusually low concentration of vernolic acid (20%) in the 2-monoglycerides from *E. lagascae* monovernolins. Our results with two other species of the Euphorbiaceae family, both with high proportions of vernolic

acid (58-72%), give values for vernolic acid in the 2-monoglycerides from the monovernolins which are a little higher (35% and 39%), but not markedly above the random value (33%).

In Table II we summarize the enrichment factors (14,15) for oleic, linoleic and vernolic acid in the X_3, X_2V and XV_2 glyceride fractions (V, vernolic acid; X, any other acid). The factors for oleic and linoleic acid are generally a little lower in the X_2V and XV_2 glycerides than in those glycerides with no vernolic acid but in the divernolins from *C. aurea* and *C. vesicaria* these values are exceptionally low. These results again indicate that vernolic acid competes successfully with oleic and linoleic acid for the C_2 position. Full results are given in Table III.

Some of the X_3 and X_2V glyceride fractions have also been examined by silver ion thin layer chromatography (9) and the results are given in Table IV. Both fractions from *C. peuschelli* were analyzed twice and all values are quoted to show the reproducibility of the results.

Conjugated Octadecatrienoic Acids

It has been possible to examine 18 species (25 samples) whose seed oils contain, between them, the five known conjugated octadecatrienoic acids. When these oils are hydrolyzed in the presence of pancreatic lipase, reaction occurs as quickly as with oils as typical as cottonseed oil. Since, in addition, unchanged triglycerides had virtually the same composition as the original oils, lipolysis occured with no undesirable selectivity arising from the unusual acid present. Some typical results are given in Table V. The proportion of conjugated ester can be determined by UV spectroscopy or by GLC. In the latter, some isomerization probably occurs on the column but the total area of peaks for conjugated octadecatrienoates is easily measured. These two methods gave similar values in some test cases and we find the GLC method simpler.

It is possible to distinguish *ctt* or *ttc* isomers (eleostearic, catalpic, calendic) from *ctc* isomers (punicic, jacaric) and both of these from *ttt* isomers by NMR spectroscopy (see Fig. 1). We therefore examined each oil and found results consistent with the structures proposed for the acid present in each oil (references are included in Table VI). Isomeric conjugated acids could be detected by this means at a 10% level and we found our sample of *V. officinalis* to contain 10-15% of the all *trans* isomer. This was also apparent in several old samples of seed oils containing conjugated acids (not used in this investigation) and probably arises from degradative changes in storage. It should not affect the results of lipolysis.

The results of this investigation are given in Table VI. Different samples of some species were examined but these gave consistent results and in the following discussion we refer to the number of species examined rather than to the number of samples. The discussion is conducted in terms of the enrichment factors listed in Table VI. Low values indicate that the acid in question is concentrated at C_1 or C_3 or both rather than C_2. A value of 1.0 signifies random distribution of the acid in question, values greater than 1.0 indicate a preference for the C_2 position. In assessing the significance of the enrichment factor, account must be taken of the total proportion of the acid and the nature of the other acids also present.

Catalpic acid (9*t*11*t*13*c*) gives uniformly low enrichment factors (0.1-0.4) for the four species examined, all belonging to the family Bignoniaceae. The single jacaric (8*c*10*t*12*c*) acid-bearing species belongs to this same family and also has a low enrichment factor (0.2). Calendic acid (8*t*10*t*12*c*) could be examined in only two species of Compositae and in both the enrichment factor (1.1-1.4) is comparable with that normally observed for oleic and linoleic acid. This leaves punicic acid (9*c*11*t*13*c*) and eleostearic acid (9*c*11*t*13*t*) for which the results are somewhat confusing since both low (0-0.3) and high (0.9-1.4) enrichment factors are observed. In three species of Valerianaceae eleostearic acid has a low enrichment factor but the same acid shows a high enrichment factor in one Euphorbiaceae (tung oil) and in two Cucurbitaceae species. Punicic acid shows low factors for three Cucurbitaceae species and high factors for one Cucurbitaceae and for the single Punicaceae species examined. Of the six Cucurbitaceae species which we have been able to examine, three (containing punicic acid) have low enrichment factors. The remainder are members of the *Momordica* genus and have high enrichment factors; two contain eleostearic acid and one contains punicic acid.

Table VII shows some interesting results with oils containing conjugated dienoic, trienoic and tetraenoic acids. In *P. laurinum* where these three acids comprise 89% of the total there is evidence that they compete for attachment at the C_2 position in order of decreasing effectiveness: diene>triene>tetraene. The same order is apparent in *I. glanduligera* (26), but here oleic and linoleic compete even more effectively than the conjugated acids.

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SHORT COMMUNICATION

Spectrophotometric Determination of Molar Amounts of Glycosphingolipids and Ceramide by Hydrolysis and Reaction With Trinitrobenzenesulfonic Acid

ABSTRACT

Three spectrophotometric procedures for determination of chromatographically separated glycosphingolipids (cerebroside, sulfatide, ganglioside) are described and compared. Hydrolysis to release long chain base followed by reaction of the amino group with trinitrobenzene sulfonic acid (TNBS) and measurement at 340 mμ was shown to give values in good agreement with those obtained by reaction of the carbohydrate moiety with α-naphthol or anthrone and measurement at 550 and 625 mμ respectively. The TNBS method is also applicable to ceramide.

Glycosphingolipids have been determined by weighing or by reaction with a reagent, such as anthrone, that produces a color with the carbohydrate portion of the molecule, and trinitrobenzenesulfonic acid (TNBS) has been used previously for determination of lipids with free amino groups (1). When reacted with different amino acids and amines, TNBS yields colored derivatives with the same or closely similar molar extinction coefficients in contrast to the highly variable color yield with ninhydrin. In this report we describe a procedure for determination of molar amounts of chromatographically separated glycosphingolipid (cerebroside, sulfatide, ceramide polyhexosides, gangliosides) and ceramide by hydrolysis, and the spectrophotometric determination of long chain base with TNBS, which gives values that correspond closely to those obtained with the anthrone and α-naphthol procedures which are also described.

Lipid (10-120 μg) is heated at 105-120 C for 90 min in 1.5 ml of methanol-concentrated (12 N) hydrochloric acid (9:2 or 8:3) or boron trifluoride in methanol (14 g/100 ml) in a screw-capped Teflon-lined tube. After cooling, 1.5 ml of water is added, and lipid is extracted into three portions of chloroform with care to obtain complete separation of phases. The chloroform solution, in a tube with a Teflon-lined screw cap, is evaporated to dryness under a stream of nitrogen; 1 ml of 4% aqueous sodium bicarbonate and 1 ml of 1% aqueous TNBS solution is added, and the mixture is incubated in the dark for 1 hr at 40 C. Methanolic hydrochloric acid (1 N, 1 ml) is then added and the solution is extracted three times with 2 ml portions of *n*-hexane, quantitative extraction being insured by vigorous manual shaking (about 200 times) or by use of an automatic shaker. The hexane solution is evaporated under a stream of nitrogen, 4 ml

TABLE I

Comparison of Human Brain Ganglioside Values[a] Determined by the TNBS and α-Naphthol Procedures

	Age				
	Fetus[b]	3 Weeks	6 Months	22 Months	8 Years
α-Naphthol[c]	0.132	0.277	0.323	0.234	0.244
TNBS	0.143	0.275	0.337	0.272	0.242

[a]Gram per 100 g fresh weight.

[b]Twenty-five week gestation.

[c]Optical density was read at 520, 550 and 580 mμ making possible correction for interfering substances. Corrected OD at 550 mμ was obtained by subtracting 50% of the value of the OD difference between 520 and 580 mμ from the OD at 550 mμ.

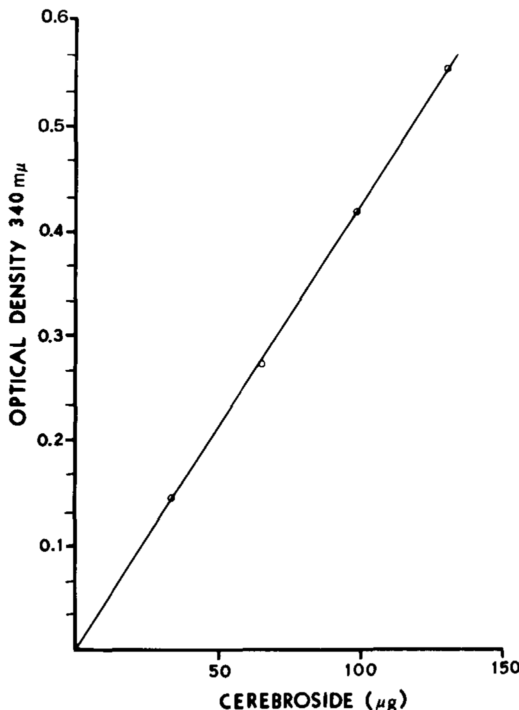

FIG. 1. Optical density values obtained for different amounts of pure cerebroside with the trinitrobenzene sulfonic acid procedure.

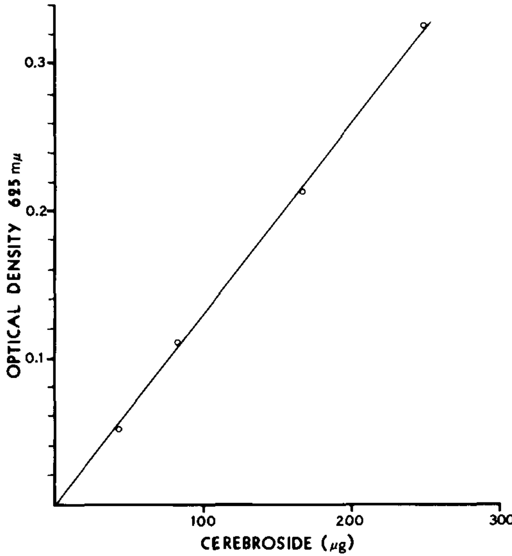

FIG. 2. Optical density values obtained for different amounts of pure cerebroside with the anthrone procedure.

95% ethanol is added, and the optical density determined at 340 mμ. The amount of glycolipid is determined by comparison with OD values obtained from pure glycolipid standards processed in the same way as samples (Fig. 1). The same molar color yield is obtained from sphingosine, dihydrosphingosine and the *o*-methyl derivatives produced during hydrolysis of sphingolipids.

Recoveries of 98-100% are obtained with the method described, and recoveries are not changed by the presence of thin layer chromatography (TLC) adsorbent. Spots separated by TLC are visualized by spraying with water, scraped into tubes, dried in a desiccator and processed as described. Low recoveries are obtained with several hydrolytic procedures. Thus, recoveries for cerebroside after heating at 105-120 C were 17.6% with 3 N aqueous hydrochloric acid (5 hr), 48% with 6 N aqueous hydrochloric acid-methanol (1:1, 5 hr), and 90% with 2 N aqueous sulfuric acid.

Values obtained by the TNBS procedure were found to agree closely with those obtained using modified anthrone and α-naphthol procedures (Tables I and II). The TNBS procedure gives accurate molar values for complex glycolipids (ceramide polyhexosides, gangliosides) of unknown structure without use of a pure preparation of each lipid as a reference standard. Pure reference standards are required with the other methods because color yields depend upon the type and number of carbohydrate residues in the molecule. Determination with anthrone is as follows. Lipid (250 μg or less) from a column fraction or a TLC spot is transferred to a 10-12 ml glass-stoppered tube, dried, and 1 ml of N,N-dimethylformamide is added. The solvent is then warmed gently for about 10 sec for rapid solution of lipid and cooled in an ice bath. A stock solution (10 ml) of 2% anthrone in 98% reagent grade sulfuric acid (stored at 4 C for three weeks or less) is diluted with 90 ml of 87.5% sulfuric acid, and 4 ml (ice-cold) of this reagent is added to the sample. The solution is then mixed thoroughly and brought to room temperature. Tubes are placed in a boiling water bath for exactly 4 min or in a water bath at 90 C for 6 min, removed, cooled under running tap water, the TLC adsorbent is removed by brief centrifugation, and the optical density read at 625 mμ using a reagent blank and standards of glucose, galactose and/or glycolipid run in parallel with samples. A straight line passing through the origin is obtained with standards (Fig. 2) in contrast to the curve obtained with previous anthrone procedures. The cuvette must be very clean, otherwise the solution becomes turbid.

Determinations with α-naphthol are performed by scraping TLC spots (containing no

TABLE II

Comparison of Human Brain Cerebroside Values[a] Obtained by the Anthrone and TNBS Procedures

	Age	
	8 Months	6 Years
TLC, Anthrone[b]	7.05	12.96
TLC, TNBS[b]	6.95	13.12
DEAE column, Anthrone[c]	6.58[d]	13.21[d]

[a]Per cent total lipid.

[b]Cerebroside separated from other lipids by two-dimensional TLC with chloroform-methanol-28% aqueous ammonia (65:25:5 followed by chloroform-acetone-methanol-acetic acid-water 3:4:1:1:0.5.

[c]Cerebroside eluted with chloroform-methanol (9:1).

[d]Variable values are obtained in the presence of interfering substances which in this case were phosphatidyl choline and sphingomyelin.

more than 250 μg of lipid) into 10-12 ml glass-stoppered tubes, drying in a dessicator over potassium hydroxide pellets, addition of 2.0 ml of dimethylformamide, warming (10 secs) for solution of lipid, cooling in an ice bath, addition of 6 ml of ice-cold sulfuric acid (98% sulfuric acid-water 9:1 v/v), mixing, cooling again in the ice bath, and then bringing to room temperature. The stoppers are then removed from the tubes, the solution heated for exactly 5 min, cooled quickly to room temperature, TLC adsorbent removed by centrifugation, 4 ml of the solution transferred to a cuvette and a control reading at 550 mμ obtained. The solution is then cooled (10 min) in an ice bath, 0.20 ml of ice-cold 2% aqueous α-naphthol solution (prepared from α-naphthol recrystallized from hexane-chloroform) is added, the tube contents mixed thoroughly, cooled (10 min) in an ice bath, brought to room temperature and allowed to stand for 1 hr. The optical density (550 mμ) is read against a reagent blank prepared with adsorbent scraped from an area of the TLC plate free of glycolipid, the control reading subtracted, and the amount of glycolipid determined from values obtained with standards run with each set of samples.

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Association of Phospholipid-Cholesterol Micelles With Rat Heart Mitochondria: Stimulators and Inhibitors

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ABSTRACT

The uptake of emulsified labeled lipids by rat heart mitochondria was studied. Divalent cations greatly stimulated uptake of cholesterol in emulsions containing phospholipids; the effect increased with increasing atomic number. Epinephrine and norepinephrine were also stimulatory. Lipid-depleted mitochondria were less effective in taking up cholesterol unless lecithin or serum lipids were included in the emulsion. Addition of iodoacetamide did not inhibit uptake, while use of heated mitochondria or extremes of pH augmented lipid uptake. Thus the process appears to be nonenzymatic. While lecithin emulsions showed no visible change on addition of calcium ions, phosphatidylethanolamine emulsions became turbid and the turbidity was largely removed by addition of heparin. Heparin as well as chondroitin sulfate B and nonionic detergent did inhibit lipid uptake by mitochondria. The possible role of such nonenzymatic lipid uptake in membrane formation and cholesterol accumulation is discussed.

INTRODUCTION

As recently reported (1), brain myelin and mitochondria can take up emulsified lipids (cerebroside, lecithin, and lecithin-cholesterol mixtures) by a nonenzymatic process. The uptake is greatly increased by divalent cations and reduced by partial delipidation of the membrane. The suggestion was offered that the uptake process may be similar to the process by which membranes are made in vivo. Rodbell (2) has found a similar uptake of triglycerides by adipose tissue and Borgstrom (3) has indicated that the uptake of lipids by intestinal wall is a nonenzymatic process depending on the lipid concentration in the adjacent medium. Much recent work on the self-assembly of membranes and restoration of enzyme activity within membranes has shown that significant combination of lipids with membrane components is nonenzymatic and does not require a chemical energy source (4-6).

This paper presents a further investigation of factors influencing lipid uptake by membranes. Cholesterol emulsions containing detergent, lecithin (PC), phosphatidylethanolamine (PE), or serum lipids were examined for uptake by native and lipid-depleted mitochondria. Stimulation of uptake was observed in the presence of Ca^{++}, Mg^{++}, Cd^{++} and Hg^{++}, epinephrine and norepinephrine, while heparin, chondroitin sulfate B, and a nonionic detergent were found to inhibit uptake.

MATERIALS AND METHODS

Materials

^{32}P-labeled PC (0.3 mc/mmole) and PE (0.6 mc/mmole) were prepared by a modification of a biosynthetic procedure (7,8). Nonradioactive

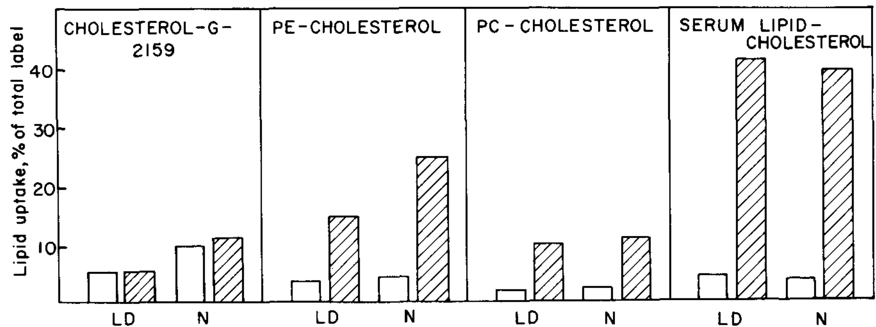

FIG. 1. Uptake of lipids by native (N) and lipid-depleted (LD) rat heart mitochondria, and the effects of added calcium. Medium contains 1 mg of mitochondrial protein in 1 ml and 0.3 ml of radioactive emulsion, as indicated. Shaded bars show effect of 4 μmoles Ca^{++}.

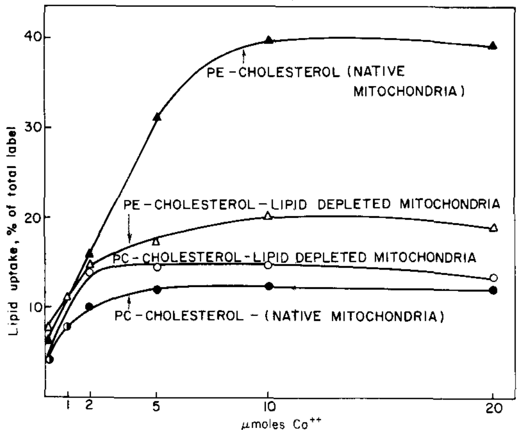

FIG. 2. Effect of Ca^{++} concentration on uptake of labeled PC-cholesterol or PE-cholesterol emulsions. Conditions as in Figure 1.

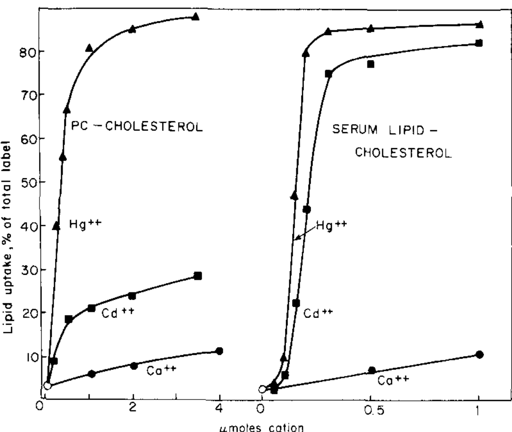

FIG. 3. Comparison of heavy metal cations with Ca^{++} on uptake of cholesterol microemulsions. Conditions as in Figure 1.

PC and PE were isolated from rat liver using the same procedure as for labeled compounds. All phospholipid preparations and cholesterol were tested by TLC and radioautography and were found pure. 4-^{14}C-Cholesterol (30 mc/mmole) was obtained from the Radiochemical Center, Amersham and *l*-norepinephrine *d*-bitartrate, epinephrine hydrochloride, heparin (5,000 U.I./ml), and chondroitin sulfate B were products of Spofa, CSSR. The detergent G-2159 (a polyoxyethylene stearate, now called MYRJ 59) was purchased from Atlas Chemical Industries, Wilmington, Delaware. Chondroitin sulfate A and hyaluronic acid were from Koch-Light Laboratories Ltd., England. Divalent cations were added as their chlorides.

Lipid microemulsions were prepared by ultrasonication of a lipid-water suspension in an ice bath with an MSE Model 60W ultrasonic disintegrator for 15 min. The lipids and detergent, when used, were evaporated from chloroform solution prior to addition of the water. Four emulsions were used: (a) 10 μg cholesterol + 1 mg detergent; (b) 10 μg cholesterol + 40 μg detergent + 200 μg PC; (c) 10 μg cholesterol + 40 μg detergent + 200 μg PE; and (d) 266 μg human serum lipids, estimated (9) to contain 26 μg cholesterol, 100 μg cholesteryl esters, 40 μg triglycerides, and 100 μg phospholipids (5 μg of ^{32}PE or 10 μg of ^{32}PC were added). The weights refer to the amounts present per 0.3 ml emulsion. Incubated samples contained about 20,000 cpm of ^{14}C-cholesterol (ca. 0.25 μg), 10,000 cpm of ^{32}P-PC (10-15 μg) or ^{32}P-PE (5-10 μg).

Mitochondria

A 10% homogenate of rat heart in 0.25 M sucrose containing 10 mM Tris (pH 7.4) was filtered through cheese cloth and centrifuged twice (at 0-4 C) for 5 min at 750 x *g* in a refrigerated Janetzki centrifuge. The supernatant liquid was then centrifuged twice at 10,000 x *g* for 10 min, suspending the pellet each time in Tris-sucrose. Lipid-depleted mitochondria were prepared with cold 85% acetone (10). The treatment removed 80-85% of the phospholipids.

Methods

Mitochondrial protein was determined by the Lowry procedure (11), lipid phosphorus by a combustion method (12-14). Radioactivity was determined in toluene containing PPO and POPOP and using a Mark I liquid scintillation counter (Nuclear Chicago, Des Plains, Ill.). Densitometric determinations were made with a Model 1100M Eppendorf recording photometer (Eppendorf Gerätebau, Netheler & Hinz GMBH, Hamburg).

Incubation Conditions

Emulsion (0.3 ml) was placed in strong walled test tubes, salt or other solution (0.05 ml or less) were added, and then 1 ml of mitochondrial suspension was added. The mitochondrial suspension contained 1 mg of protein or an integral multiple of this. Following gentle shaking at 37 C, usually for 15 min, 4 ml of cold sucrose-Tris were added and the mitochondria were centrifuged 30 min at 4,500 x *g*. The particles were resuspended in 4 ml of sucrose-Tris and again centrifuged. The washed pellets were suspended in 1 ml of water, 4 ml of chloroform-methanol (2:1 v/v) was added, and the extraction carried out by agitation for 1

min. After centrifugation at low speed, the chloroform layer was evaporated to dryness in scintillation vials and the radioactivity determined.

RESULTS

Composition of Emulsions Taken up by Mitochondria

When doubly-labeled emulsions were used (^{14}C-cholesterol and ^{32}P-lipids), it was found that the isotopic ratio in the mitochondria was the same as that in the initial emulsion. The presence of stimulating materials (Fig. 1-4) and the time of sampling (after 5, 10 or 15 min of incubation) did not affect this ratio. Thus, it is evident that mixed lipid micelles were taken up by the membrane as a whole, without preferential uptake of one of the components. This is in agreement with our previous findings that cerebroside-lecithin micelles (in certain ratios) were taken up similarly by myelin and brain mitochondria (1).

Effect of Ca^{++} on Uptake by Native and Depleted Mitochondria

Ca^{++} ions have a marked stimulatory effect on the uptake of labeled cholesterol when the micelle includes phospholipid, but not when only detergent is present (Fig. 1). Emulsions containing detergent alone or PE plus detergent were taken up to a somewhat lesser extent by lipid-depleted mitochondria, but the presence of lecithin or serum lipids made the uptake normal. The capacity of depleted membranes to take up cholesterol was in large part restored by preincubating the delipidated mitochondria (1 mg protein) with 0.5 mg of a lecithin dispersion and 4 μmoles Ca^{++}, or with emulsified serum lipids and 3 μmoles Ca^{++}. When the treated, presumably relipidated mitochondria were washed twice to remove excess lipid and then incubated with cholesterol-detergent emulsion, the uptake was 9.7% as contrasted with 5% for depleted mitochondria. Thus, an important factor for the association of cholesterol with the mitochondrial membrane is apparently the amount of lipid present in the membrane. This point may be related to the observation (Fig. 1) that cholesterol-serum emulsions were taken up more completely than the other emulsions. However, the total weight of lipid in the former incubations was somewhat higher.

Increasing the amount of Ca^{++} in the medium (Fig. 2) further emphasizes differences between micelles containing PC and PE, with respect to the uptake by delipidated mitochondria. Inclusion of PE and detergent in the cholesterol micelles results in much greater uptake by normal mitochondria than the inclusion

TABLE I

Uptake of Lipids by Mitochondria and Lipid Depleted Mitochondria[a]

Microemulsion	Mitochondria				Lipid depleted mitochondria			
	Ca^{++} 4 μmoles	Mg^{++} 4 μmoles	Cd^{++} 0.2 μmole	Hg^{++} 0.2 μmole	Ca^{++} 4 μmoles	Mg^{++} 4 μmoles	Cd^{++} 0.2 μmole	Hg^{++} 0.2 μmole
Cholesterol-detergent	5.55	4.8	7.6	8.8	2.7	2.6	2.7	2.5
PE-cholesterol	15.4	14.0	24.0	29.6	12.5	11.0	17.0	15.4
PC-cholesterol	11.8	10.6	13.4	22.1	10.0	9.8	25.4	67.0
Serum lipids-cholesterol	36.2	34.0	41.2	77.0	38.3	34.3	85.2	95.0

[a]Medium: 1 ml mitochondrial suspension (1 mg protein), 0.3 ml microemulsion (doubly labeled in experiments with native mitochondria, ^{14}C-cholesterol in depleted ones), chlorides of divalent cations; 15 min incubation at 37 C. Values (averages of 4-7 estimations) are per cent of initial activity added.

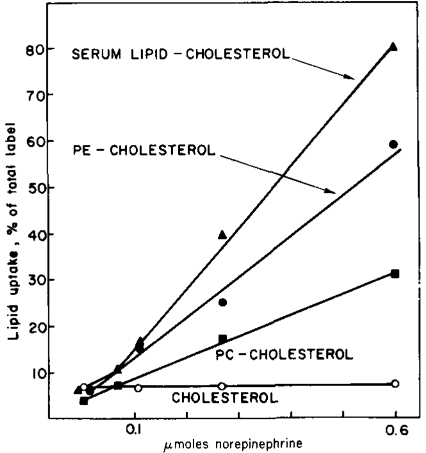

FIG. 4. Effect of norepinephrine on uptake of lipid emulsions by native mitochondria.

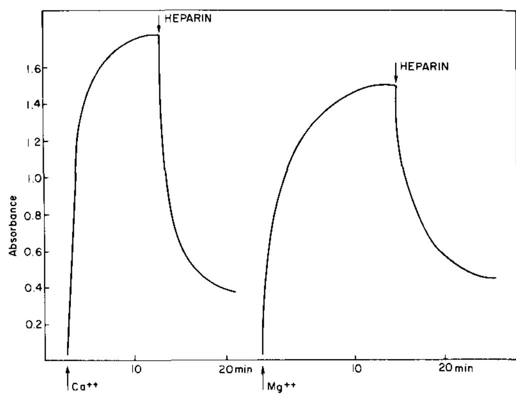

FIG. 5. Turbidity of micellar PE as affected by Ca^{++}, Mg^{++}, and heparin. Absorption cells contained 1 ml of microemulsified PE (0.7 mg). At zero time 8 µmoles of Ca^{++} or 12 µmoles of Mg^{++} were added and at time of maximal turbidity 0.3 mg heparin in 0.1 ml was added.

of PC; with lipid-depleted mitochondria, however, the effect is somewhat less, especially at higher Ca^{++} levels. A similar effect was found with Cd^{++} and Hg^{++} ions (see below).

Comparison of Ca^{++}, Mg^{++}, Cd^{++} and Hg^{++} Ions

The affinity of labeled cholesterol micelles to membranes increases with atomic number of the cation in the medium (Fig. 3). The effect was seen with both PC and serum lipid emulsions, although Cd^{++} was more effective with the latter than with the former.

Table I shows the uptake of cholesterol in detergent alone is less affected by these cations. The stimulatory effect of Cd^{++} and Hg^{++} with PC and serum emulsions is observed again in this experiment, but the stimulation is even greater with delipidated mitochondria. The PE emulsions, in contrast, show a reverse reaction to Cd^{++} and Hg^{++} with depleted membranes,

TABLE II

Competitive Effect of Ca^{++} on Phospholipid Uptake Stimulated by Hg^{++}

Microemulsion					Uptake %
PC-cholesterol					2.8
PC-cholesterol	+	Hg^{++}			80.0
PC-cholesterol	+	Hg^{++}	+	Ca^{++}	21.2
PE-cholesterol					3.4
PE-cholesterol	+	Hg^{++}			85.2
PE-cholesterol	+	Hg^{++}	+	Ca^{++}	50.1

[a]One milliliter suspension of normal mitochondria (1 mg protein), 0.3 ml microemulsion (labeled ^{14}C-cholesterol); 1 µmole Hg^{++}, 2 µmoles Ca^{++} incubated 15 min at 37 C.

although these ions are still more effective than Ca^{++} or Mg^{++}.

A competitive effect of Ca^{++} on the uptake stimulation by Hg^{++} could be demonstrated (Table II). High uptake of PC-cholesterol and PE-cholesterol micelles by native mitochondria induced by Hg^{++}, was considerably reduced, if Ca^{++} in addition to Hg^{++} was present.

Effect of Additional Factors

With normal mitochondria norepinephrine increased the uptake of cholesterol micelles containing phospholipids but not those containing detergent alone (Fig. 4). As with Ca^{++}, serum lipids proved most effective and PE somewhat less so. Epinephrine was slightly less effective than norepinephrine. The stimulatory effect is not likely to have physiological significance because of the high concentrations required.

Disodium EDTA (1 mM) did not depress Ca^{++} stimulated uptake but, on the contrary, itself stimulated uptake in all cases studied to about twice the normal value with Ca^{++} alone. A similar effect of EDTA has been described for the binding of tetracycline to collagen (15).

Heating mitochondria for 15 min at 90 C increased the affinity of the membrane although metallic cations were still stimulatory. A similar effect had been seen with cerebroside uptake (1). Perhaps heating exposes additional binding sites.

A similar stimulation was seen in a study of the effect of pH on uptake of serum lipids, labeled with cholesterol and PC. At pH 7.4, in the absence of cations, the PC-cholesterol

uptake was 5.8%; at pH 4.5 it was 88.3% and at pH 10.0 it was 21.6%.

Iodoacetamide and puromycin had no effect on lipid uptake, but heparin, chondroitin sulfate B and nonionic detergent, G-2159 were inhibitory with all emulsions containing phospholipids (Table III). All three substances suppress the stimulatory effect not only of metallic cations but also of epinephrine, norepinephrine and EDTA. However, chondroitin sulfate A and hyaluronic acid, in the same concentration (500 μg in medium) had no effect.

In a more detailed examination of the mucopolysaccharides (Table IV), we found chondroitin sulfata A to be much less inhibitory than the B form. Hyaluronic acid, which does not contain sulfate groups, enhanced lipid uptake at higher levels.

The Clearing Effect of Heparin

Even though heparin inhibited lipid uptake regardless of which stimulator was used (Table III), it appears that different mechanisms were involved in the effects. When Ca^{++} and Mg^{++} were added to PE dispersions, slight turbidity was induced (Fig. 5). This turbid suspension did not yield any sediment after centrifugation for 30 min at 4,500 x *g*. Addition of heparin quickly produced almost complete clarification. On the other hand, Hg^{++}, Cd^{++}, epinephrine, and norepinephrine had no effect on the turbidity of the emulsion.

Like PE emulsions, serum lipid emulsions also become turbid with addition of Ca^{++}; but added heparin did not clear the suspension. In contrast, PC emulsions were not made turbid by any of the cations used in this study.

Stability of the Lipid-Membrane Association

Mitochondria which had taken up labeled serum lipids were incubated with unlabeled serum lipid emulsion, Ca^{++}, heparin, G-2159, or distilled water. After washing the particles in the usual way, we determined the protein and radioactivity content in the pellets or repeated the incubation and washing (Table V). Progressive loss of mitochondrial protein was seen in all incubations with the greatest loss in the presence of heparin or detergent. Calcium ions prevented loss of labeled lipid, except for a 12% loss in the second incubation with unlabeled lipids. Heparin and detergent caused appreciable loss of lipid. In all cases, protein content decreased more rapidly than lipid content as measured by radioactivity. As shown with brain mitochondria (1), PE was lost into the medium more rapidly than PC.

The physicochemical processes leading to the binding or absorption of lipids to subcellu-

TABLE III

Inhibition of Lipid Uptake[a]

Microemulsion	5 μmoles Ca^{++}				0.2 μmole Cd^{++}				0.2 μmole Hg^{++}				0.5 μmole norepinephrine			
	-	He	CHS B	D	-	He	CHS B	D	-	He	CHS B	D	-	He	CHS B	D
Cholesterol	6.0	5.9	6.2	6.1	7.6	5.6	5.6	7.3	8.8	6.4	4.8	8.0	5.9	4.9	4.7	5.3
PE-cholesterol	19.6	6.5	6.8	-	24.6	2.8	4.0	5.2	27.8	3.2	4.6	7.6	21.8	3.1	4.2	4.7
PC-cholesterol	10.1	4.9	5.4	-	13.4	2.2	2.3	2.5	22.1	1.9	2.5	2.9	5.7	2.4	2.7	2.5
Serum lipids-cholesterol	36.0	7.6	12.2	-	41.2	2.1	3.6	6.0	77.0	3.9	3.9	-	37.5	4.4	4.1	4.1

[a]One milliliter suspension of normal mitochondria (1 mg protein), 0.3 ml microemulsion (doubly labeled PE-cholesterol and PC-cholesterol), stimulators and inhibitors (500 μg heparin, chondroitin sulfate B or detergent). Incubation 15 min at 37 C.

TABLE IV

Effect of Heparin, Chondroitin Sulfate B, Chondroitin Sulfate and Hyaluronic Acid on the Uptake of PC-Cholesterol and Serum Lipid-Cholesterol Micelles by Normal Mitochondria[a]

Substance	μg	Uptake of PC-^{14}C-cholesterol %	Uptake of serum lipid-^{14}C-cholesterol %
None		11.1	35.0
Heparin	250	7.2	34.0
	500	6.5	10.6
	1,000	4.1	13.6
Chondroitin sulfate B	250	6.5	28.4
	500	5.4	
	1,000	6.8	15.0
Chondroitin sulfate A	250	11.1	38.1
	500	10.2	
	1,000	9.4	35.4
Hyaluronic acid	250	8.2	32.8
	500	11.0	39.9
	1,000	18.1	47.5

[a]One milliliter mitochondrial suspension (1 mg protein), 0.3 ml microemulsion, 4 μmoles of Ca^{++}; other substances added and incubated 15 min at 37 C.

lar particles are complex and have had limited study. Yet it appears that they are of fundamental physiological importance. Fleischer et al. (16) were the first to reassociate phospholipid to lipid deficient lipoproteins and thereby reactivate some enzyme systems. They concluded that there are two types of binding between phospholipids and the protein matrix: (a) an ionic bond between basic proteins such as cytochrome c and acid phospholipids and (b) an apolar interaction of structural proteins with essentially all phospholipids. The uptake phenomena in our study seem to involve only the latter type of binding, since the lipids used were nonacidic (or only slightly so). In addition, as suggested previously (1), there appears to be lipid-lipid binding, since delipidated mitochondria were found to exhibit decreased capacity for exogenous nonionic lipid. This decreased capacity was nearly restored by

TABLE V

Radioactivity and Protein Loss From Mitochondria During Repeated Incubations

Incubation conditions	Loss in per cent after incubation	Loss in per cent after incubation
Radioactivity		
Serum lipids-cholesterol		
+ 4 μmole Ca^{++}	5	12
4 μmole Ca^{++}	4	4
Water	10	15
500 μg Heparin	14	27
500 μg Detergent	29	58
Protein		
Serum lipids-cholesterol		
+ 4 μmole Ca^{++}	20	40
4 μmole Ca^{++}	20	40
Water	30	50
500 μg Heparin	40	55
500 μg Detergent	40	60

[a]A 10 ml suspension of normal mitochondria (1 mg protein/ml) was incubated 15 min at 37 C with 3 ml of serum lipid-^{14}C-cholesterol-^{32}P-PC microemulsion and 40 μmoles Ca^{++}. Aliquots of labeled mitochondria (1 ml) were incubated again 15 min with 0.3 ml of one of the following: serum lipid emulsion (nonradioactive) + Ca^{++}; Ca^{++}; water; heparin; detergent. Values estimated after repeated incubations are expressed as loss in per cent of initial values.

rebinding of lecithin, suggesting that zwitterionic lipids are bound to membrane proteins more strongly than are nonionic lipids.

Since uptake of Ca^{++} into membranes is not affected by PC or PE in the media (unpublished results) it is unlikely that phospholipids are associated with membranes through ionic bridges. The presence of Ca^{++} changes the surface potential of phospholipid micelles (17) and tends to solidify lecithin monolayers (18), which may explain the stabilizing effect of the divalent cation on membrane stability and lipid binding (Table V). Slight interaction between phosphate groups of phospholipids and Ca^{++} may increase the apolar affinity between the lipid micelles and the mitochondrial membrane.

The effect of Cd^{++} and Hg^{++} on lipid uptake seems to be more complicated. Preliminary treatment of mitochondria with these metal cations considerably enhanced membrane affinity to lipids in media not containing divalent cations or stimulators. In addition to electrostatic effects on lipid microemulsions, there may also be involved rearrangement of the mitochondrial membrane. Electrostatic effects were not involved in the uptake of the nonionic cholesterol micelles, in contrast to the phospholipid-cholesterol micelles where phospholipids mediated the interaction of cholesterol with the membrane. Uptake of cholesterol seems to be influenced by the concentration of lipid in the membrane, as shown by the several fold higher uptake by brain myelin (1) in comparison with heart mitochondria; the lipid-protein ratio is much higher in myelin.

Prevention of lipid uptake by nonionic detergent may be the result of solubilization of membrane components and decrease of lipid micelle size. However, this explanation would not apply to the effects of heparin and chondroitin sulfate B. These could be ascribed partly to the high binding capacity of acid mucopolysaccharides for cations, confirmed and correlated with anticoagulant effects by Mathews (19) as well as to electrostatic effects on microemulsions and an increased degree of dispersion of the micellar structures, as assumed by Bianchini (20) and Capraro et al. (21). Finally, a direct interaction of the acid polysaccharides with the mitochondrial membrane is possible; here the former would compete with the lipid micelles for free sites.

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Quantitative Determination of Heparin Released Lipoprotein Lipase Activity in Human Plasma[1]

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ABSTRACT

A method for quantitative determination of heparin released lipoprotein lipase (LPL) activity has been developed. The importance of standardization of substrate and albumin in the assay system has been investigated. Determination of glycerol release in the assay system has been shown to have advantages compared with titration of fatty acids released. Enzyme plasma samples frozen for four months decreased in activity. The method seemed to be specific for emulsified triglycerides and the LPL activity was 98% inhibited by 1 M NaCl. However, there was a lack of reproducibility of the substrate when different lots were used. Analytical error of the method was approximately 5% and there was good reproducibility.

INTRODUCTION

Lipoprotein lipase (LPL) activity appearing in the blood after parenteral administration of heparin was first described by Hahn (1). Since then many methods to determine LPL activity both in blood plasma and in different tissues have been described (2,3). However, it has been difficult to find a specific method for LPL activity with good reproducibility, allowing comparison of activities in samples determined at different times.

This paper describes a quantitative method to determine heparin released LPL activity in human plasma. It is essentially a modification of a method described earlier (4,5) where materials and procedures have been further standardized.

MATERIALS

The substrate, supplied by AB Vitrum, Stockholm, was a soybean oil emulsion (containing 10% triglycerides and 1.2% egg phosphatides) made in the same way as Intralipid (AB Vitrum, Stockholm) (6) with the exception that no free glycerol had been added. The emulsion was stored at 4 C in 10 ml sterile portions (7). A new portion was broken each experimental day.

Egg phosphatide emulsion, the emulsifier of the substrate mentioned above, was supplied by AB Vitrum and prepared in the same way as the ordinary substrate with the exception that no triglyceride was added.

Albumin was lyophylized and prepared from human blood serum using donor blood and retroplacental blood (AB Kabi, Stockholm).

Heparin (AB Vitrum, Stockholm) was given intravenously (100 IU/kg body weight) to overnight fasting adult men.

DETERMINATIONS

Fatty acids were determined by microtitration according to the method of Dole (8). Following each extraction, titrations were done in duplicate. Glycerol was determined by pipetting 0.2 ml of incubation mixture into 1 ml 12% trichloroacetic acid. The protein was precipitated by centrifugation, glycerol was determined on the supernatant by periodic acid oxidation and colorimetric estimation in triplicate of the formaldehyde, as previously described (4).

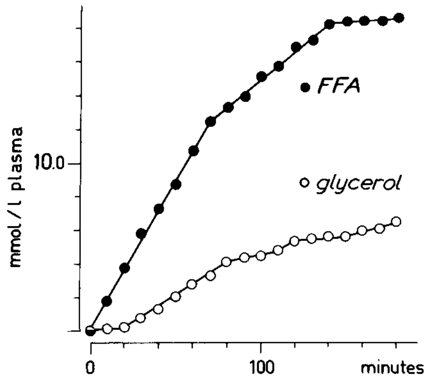

FIG. 1. Release of fatty acids and glycerol in the lipoprotein lipase reaction. Albumin concentration in the medium was 6%. Unless otherwise stated all other incubation conditions are as described under General Procedure.

[1]Presented in part at the AOCS-AACC Joint Meeting, Washington, D.C., April, 1968.

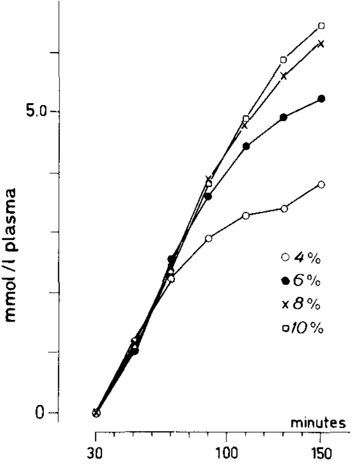

FIG. 2. Release of glycerol at different albumin concentrations.

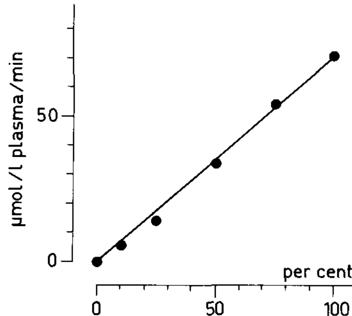

FIG. 3. Release of glycerol with different amounts of lipoprotein lipase using postheparin plasma mixed with different amounts of preheparin plasma. Postheparin plasma without addition has been called 100%.

GENERAL PROCEDURE

Plasma Samples

Nine volumes of blood were added to glass tubes containing 1 vol of 0.1 M trisodium citrate. The tubes were stored in ice water. Blood was centrifuged at 5,000-10,000 *g* x min and plasma was removed either for immediate use or put into a freezer (-15 C) for later determination of LPL activity.

Substrate Mixture

One milliliter of substrate mixture was prepared by dissolving 133 mg albumin (10% in the final incubation mixture) in an ammonium buffer (31 vol of 0.1 M NH_4OH + 30 vol of 0.1 M NH_4Cl). Of the substrate 0.093 ml was added to the albumin solution. Finally this mixture was made up to 1 ml with the ammonium buffer. If necessary the pH was adjusted to 8.68-8.72 with the above mentioned solutions of NH_4OH and NH_4Cl.

Incubation

One volume of plasma was added to 3 vol of substrate mixture and the incubation was performed at 37 C in a Gallenkampf metabolic incubator (80-100 cycles/min). Incubation time was dependent on expected amount of LPL activity in the actual plasma sample. Generally aliquots for glycerol determinations were removed at 10 min intervals after 30 to 90 min incubation and immediately extracted.

Calculations of LPL Activity

The amount of glycerol in the incubation medium was plotted against incubation time and the slope of the linear part of the curve that was taken as LPL activity. Measurement was expressed in µmoles or mmoles of glycerol released per liter of incubated blood plasma per minute.

RESULTS

Release of Fatty Acids and Glycerol in the Lipoprotein Lipase Reaction

A typical study in which there was a linear release of fatty acids from zero time to 70 min incubation is shown in Figure 1. For glycerol there was a lag period of 20 min followed by a linear release up to 80 min. The ratio between the slopes of the linear part of fatty acid and glycerol release in postheparin plasma from 16 healthy individuals was 3.25±0.45 (mean ± standard deviation). This ratio is compatible with triglycerides being the essential substrate for the enzyme system studied.

In plasma samples with high LPL activities the linear part of glycerol release was short but could be extended when the albumin concentration of the incubation mixture was increased (Fig. 2). With 4% albumin the curve started to

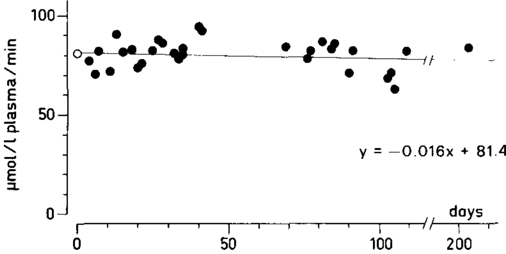

FIG. 4. LPL activity in a postheparin plasma obtained 40 min after i.v. heparin (100 IU/kg body weight) kept in a freezer for 203 days. Zero time value is determined on unfrozen plasma.

TABLE I

Errors in Determination of Fatty Acid and Glycerol Release in the Lipoprotein Lipase Reaction[a]

FFA	Concentration after incubation 20 min, μmole/liter plasma		Concentration after incubation 40 min, μmole/liter plasma		LLA in μmole/ liter plasma/min	
	FFA	Glycerol	FFA	Glycerol	FFA	Glycerol
Mean	11607	1442	16054	2920	223	73
SD	921	27	1027	33	32	2
	(7.9%)	(1.9%)	(6.4%)	(1.1%)	(14.4%)	(2.7%)
n	30	15	30	15	30	15

[a]Activity determined by fatty acid and glycerol release in one postheparin blood plasma sample. SD, standard deviation and n, number of incubations.

level off when the glycerol concentration in the medium was approximately 0.5 mmoles/liter. If the albumin concentration was increased to 10% the release was linear up to a medium concentration of glycerol of about 1.2 mmoles/liter.

The lag period for glycerol release in the beginning of the incubation was longer with lower LPL activity. For this reason plasma samples with low activities require incubation for longer periods than samples with high activities. If LPL activities were measured from the linear part of the curve the release of glycerol was directly proportional to the amount of postheparin plasma present in the incubation medium, which is evident from Figure 3.

Table I shows the analytical errors for the fatty acid and the glycerol determinations to be approximately 7% and 1.5%, respectively. These results demonstrate that measurement of glycerol release is preferable for evaluation of LPL activity.

Studies on the Characteristics of the Test System

Table II shows plasma LPL activity in 28 men, 40 min after a single intravenous injection of heparin. The heparin released LPL activity found in these men was 98% inhibited by 1 M NaCl, in good agreement with the studies by Korn (9) and with our previous results using Intralipid as a substrate (4,5).

TABLE II

Blood Plasma LPL Activity in Healthy Men 40 Min After Intravenous Injection of Heparin

	Plasma glycerol release, μmole/liter plasma/min	
	Standard conditions	1 M NaCl
Mean	112	2
SEM	5	0.2
Range	76-181	1-4
n	28	28

[a]NaCl was added to the substrate mixture 15-30 min before the incubation was started. SEM, standard error of the mean and n, number of subjects.

Table III suggests that triglycerides in the fat emulsion are the only substrate for lipoprotein lipase in the test system. With ordinary substrate LPL activity was 123±3 μmole/liter/min, and this activity was 98% inhibited by 1 M NaCl and 47% by heparin (10 IU/ml added in vitro). However, with no triglycerides in the emulsion and only egg phosphatides, the emulsifier of the ordinary substrate emulsion, no LPL activity was found in the test system. This suggests that the phospholipase which is also released or activated by heparin injection (10) is not measured in this test system.

Factors Affecting Reproducibility of the Method

Various batches of the fat emulsion used resulted in different LPL activity. Two postheparin plasma samples incubated with two different substrate lots resulted in LPL activity of 108, 106 and 51, 45 μmole/min, respectively. The same variability in LPL activity occurred with different batches of albumin (Table IV) and the activity obtained did not seem to be related to the FFA content of the albumin (Table IV). Postheparin plasma obtained after centrifugation at 2200 *g* x min (platelet rich) and 24,000 *g* x min (platelet poor) had the same LPL activity. Also, the LPL activity was present only in plasma since whole blood activity corrected for blood cell volume reached the same value as the activity determined on plasma. During low temperature storage of postheparin plasma the LPL activity decreased (4). Table V shows that the LPL activity had decreased 10-30% during storage at -15 C for four months.

Statistical Analysis, Analytical Error and Reproducibility of the Method

Plasma from a healthy young man obtained

TABLE III

Postheparin Plasma LPL Activity Incubated With Different Substrates[a]

	Plasma glycerol release, μmole/liter/min				
	Standard conditions[b]	Addition of NaCl[b]	Addition of heparin 10 IU/ml[b]	No substrate	Egg phosphatide emulsion
Mean	123	3	65	1	1
SEM	3	2	6	1	1
n	5	5	5	5	5

[a]The inhibitors NaCl and heparin were added in vitro to the substrate solution 15-30 min before the incubation was started. For "No substrate" the solution consisted of only buffer and albumin. The egg phosphatide emulsion was prepared by AB Vitrum in the same way as the fat emulsion with the exception that no triglycerides (soybean oil) were added. SEM, standard error of the mean and n, number of incubations.

[b]Standard substrate.

40 min after an intravenous injection of heparin was pipetted into several tubes and kept in a freezer at -15 C. Over a period of 200 days LPL activity was determined in duplicate (the same batch of emulsion and albumin was used). Mean activity for each day has been plotted versus time in Figure 4. The data have been subjected to linear regression analysis (11) and the equation of the regression line is $y = -0.016x + 81.4$ (where x is storage time in days and y is LPL activity in μmoles per liter plasma per minute. Standard deviation for y is ± 7.2. The analytical error calculated from the differences of the duplicates $(d^2/2n)^{1/2}$ is ± 3.9 corresponding to 5%. From the regression line this plasma lost an activity of 0.016 μmoles/liter/min/day, corresponding to about 0.02%/day of storage.

DISCUSSION

The results suggest determination of glycerol release rather than FFA release is preferable to use for in vitro assay of heparin released LPL activity. The determination of glycerol has much lower analytical error than titration of fatty acids, and with the substrate used here there seems to be no risk to include heparin released phospholipase activity in the assay procedure. Furthermore, determination of glycerol is less tedious than titration of fatty acids by the Dole method. One point of major importance in determination of heparin released LPL activity is to standardize the substrate and the albumin used in the assay system. This study shows that even different batches from one and the same company can differ considerably, i.e., assay of one and the same enzyme plasma sample results in different values for the activity. This causes serious doubt as to the validity of comparing LPL activity values determined by different laboratories. Our method for quantitation of heparin released LPL activity appears to measure an enzyme system which hydrolyzes triglycerides to glycerol and fatty acids. It has recently been

TABLE IV

LPL Activity of a Single Plasma Sample Using Three Different Batches of Albumin

Albumin concentration, %	μmole/liter plasma/min		
	Batch A	Batch B	Batch C
4	144	112	72
6	156	120	72
8	148	128	76
10	152	128	64
mmole FFA/g albumin	0.022	0.022	0.023

TABLE V

Effect of Four Months of Plasma Storage at -15 C on Plasma LPL Activity in 21 Men 40 Min After Heparin[a]

	μmole/liter plasma/min			
	Initial	Four months storage	Change %	Significance
135-185 (n=4)	162	111	-52±14	$p < 0.05$
100-134 (n=8)	110	101	-10± 8	$p < 0.05$
70-99 (n=9)	89	68	-22± 7	$p < 0.02$

[a]LPL activity was determined directly on unfrozen plasma and later on plasma frozen for four months. Plasma samples were ranked in three groups according to the initial levels of LPL activity.

shown that this enzyme system consists of at least one triglyceride lipase and one monoglyceride hydrolase (12). However, the rate limiting step for complete lipolysis has been shown to be the triglyceride lipase activity (13). This observation is supported by the constant relationship between free fatty acid and glycerol release found in the system presented in this report.

LPL activity measured as described in this study is inhibited 98% by 1 M NaCl. This inhibitable activity is heat-sensitive in contrast to the activity not inhibited by 1 M NaCl (Boberg, Gustafsson and Kiessling, unpublished data). Since the triglyceride lipase is described as considerably more heat-sensitive than the monoglyceride hydrolyase (12) there is good evidence to believe that the activity inhibited by 1 M NaCl in this study is a measurement of triglyceride lipase activity.

The suitability of Intralipid as substrate for determination of postheparin LPL or better triglyceride lipase activity was recently described by Biale and Shafrir (14). They also showed that Ediol, which is a substrate widely used for determination of LPL activity, gave too high activities. This was due to hydrolysis of partial glycerides, present in the emulsion both as monostearin and as the emulsifier Tween 60, induced not by LPL activity but by a monoglyceridase.

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Studies on the Nuclear Magnetic Resonance Spectra of Olefinic Protons of Conjugated Fatty Acid Methyl Esters

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ABSTRACT

The NMR spectra of olefinic protons in the four representative conjugated fatty acid methyl esters, methyl *cis*-9,*trans*-11-octadecadienoate, methyl *trans*-9,*trans*-11-octadecadienoate, methyl α eleostearate, and methyl β eleostearate, were studied. The chemical shift of each olefinic proton in these compounds was determined by considering their intramolecular environment. Coupling constants were also obtained as the results of spectral analysis.

INTRODUCTION

The NMR spectra of many types of fatty acids or their esters have been investigated by many workers in regard to the relationship between chemical shifts and chemical environment of protons (1,4-6,11). However, only a few studies on chemical shifts and coupling constants of olefinic protons in conjugated fatty acid esters relating to their structures have been reported.

Recently, Purcell et al. (10) investigated the NMR spectra of various unsaturated fatty acids using high resolution NMR at 60 MHz. In this report, particular attention was focused on the changes in NMR spectra caused by *cis-trans* isomerism and conjugation, but the complex spin-spin coupling patterns of olefinic protons in conjugated fatty acids have not been analyzed. Tallent et al. determined the geometry of the double bonds of methyl corioleate by the complete first order analysis of the conjugated olefinic multiplets from the 100 MHz NMR spectrum (14).

We have already examined the NMR spectra of fatty acid methyl esters by using a high resolution 100 MHz spectra and demonstrated the characteristic spectral patterns of the olefinic protons in unsaturated fatty acid methyl esters, i.e., methyl oleate, methyl elaidate, methyl linoleate and methyl linolenate (2). In the present work, we have shown the olefinic proton signals of four conjugated fatty acid methyl esters, which include methyl *cis*-9,*trans*-11-octadecadienoate, methyl *trans*-9,*trans*-11-octadecadienoate, methyl α eleostearate, and methyl β eleostearate. In order to interpret them, the double resonance method has been used. The chemical shifts and coupling constants are also obtained for each olefinic proton by analyzing the spectra, and discussed in relation to the geometric structures of the compounds.

EXPERIMENTAL PROCEDURES

Measurement of NMR Spectra

The NMR spectra were obtained by a Varian HA-100 spectrometer operating at 100 MHz in the field sweep mode except in the double resonance experiment, in which case the frequency sweep mode was adopted. The spectra of fatty acid esters were obtained at about 30 C with the solutions of approximately 20% of the esters in CCl_4 with TMS added as internal reference. The τ-value is used to report chemical shift.

Preparation of Conjugated Fatty Acid Methyl Esters

Methyl Cis-9,trans-11-octadecadienoate

The commercial dehydrated castor oil fatty acids containing 49.0% of *cis,trans*-conjugated octadecadienoic acid, which is considered to be formed by the dehydration of ricinoleic acid, were converted to methyl esters by refluxing for 1 hr with methanol containing about 1% of *para*-toluenesulfonic acid. Methyl *cis*-9,*trans*-11-octadecadienoate was separated from the methyl esters by the urea-adduct method, and it was purified by solvent crystallization at -60 C, using a mixture of methyl alcohol and acetone (7). The ester separated was further refined by adsorption chromatography on alumina column, using petroleum ether as eluant. The product was analyzed by GLC using a Hitachi KGL-2B chromatograph with butanediol succinate (BDS) as the stationary liquid at a column temperature of 210 C (3), and proved to be 96% pure including methyl linoleate and trace of *trans,trans*-conjugated octadecadienoate.

Methyl Trans-9,trans-11-octadecadienoate

Ricinelaidic acid was dehydrated by heating at 235 C under vacuum for 3 hr and the crude dehydrated acids were obtained by vacuum distillation. *Trans*-9,*trans*-11-octadecadienoic acid was obtained by crystallization from 95%

TABLE I

Identification of the Chemical Shifts of Various Olefinic Protons and Observed Coupling Constants

Compound	Proton	Chemical shifts (ppm)	Coupling constants (Hz)	
(Y) (C) $CH_3(CH_2)_4CH_2$ C=C H (X) H C=C $CH_2(CH_2)_6COOCH_3$ (D) H H (B) (A)	A	4.79_3	J_{AX}, J_{DY}	7.2
	B	4.15_9	J_{AB}	10.5
	C	3.81_0	J_{BC}	10.9
Methyl *cis*-9, *trans*-11-octadecadienoate	D	4.37_8	J_{CD}	14.5
			J_{BX}	1.2
			J_{CY}	1.0
(Y) (B') $CH_3(CH_2)_4CH_2$ C=C H (A) H C=C H (A') H $CH_2(CH_2)_6COOCH_3$ (B) (X)	A	4.54_3	$J_{AB}, J_{A'B'}$	15.4
	A'		$J_{AX}, J_{A'Y}$	6.5
	B	4.13_3	$J_{BB'}$	9.5
Methyl *trans*-9, *trans*-11-octadecadienoate	B'		$J_{AB'}, J_{A'B}$	-0.4
			$J_{BX}, J_{B'Y}$	1.0
(Y) (E) $CH_3(CH_2)_2CH_2$ C=C H (C) H C=C H (X) (F) H C=C $CH_2(CH_2)_6COOCH_3$ (D) H H (B) (A)	A	4.69_2	J_{AX}, J_{FY}	7.5
	B	4.11_0	J_{AB}	10.5
	C	3.81_5	J_{BC}, J_{DE}	10.6
	D	3.96_5	J_{CD}, J_{EF}	15.0
Methyl α eleostearate	E		J_{AC}	-0.4
	F	4.40_8	J_{BX}	1.0
(Y) (B4) $CH_3(CH_2)_2CH_2$ C=C H (B2) H C=C H (A) (A') H C=C H (B3) H $CH_2(CH_2)_6COOCH_3$ (B1) (X)	A	4.61_9	J_{AB}	15.0
	A'			
	B1B1			
	B2	4.04_7		
Methyl β eleostearate	B3			
	B4			

aqueous ethanol solution of the crude acids at -15 C (12). The methyl ester of the acid was analyzed by GLC and proved to be 95% pure with methyl stearate.

Methyl α Eleostearate

The ester was prepared from tung oil by the method described by O'Connor et al. (8). After saponification of tung oil, the fatty acids were liberated by adding diluted sulfuric acid. The α eleostearic acid was separated by crystallization from 95% aqueous ethanol and then from petroleum ether solution of the acids. The product was esterified in N_2 atmosphere, and refined by adsorption chromatography with a similar method to that used for the methyl *cis*-9,*trans*-11-octadecadienoate. The ester obtained was found to be 98% pure by GLC analysis.

Methyl β Eleostearate

The β eleostearic acid was prepared from α eleostearic acid by isomerization with 0.3% iodine at room temperature, and purified by successive recrystallization from petroleum ether, a mixture of petroleum ether and acetone, and acetone. The ester was obtained

TABLE II

Coupling Constants in Conjugated Fatty Acid Methyl Esters

Structure	Coupling constants (Hz)	
>C = C(H (A))–CH2- (X)	J_{AX}	7.5 – 5.5
= C(H (D))–C(H (B)) = C(H (A))–CH2- (X)	J_{AB}	15.5 – 14.5
	J_{AD}	0.4 (negative)
(C) H>C = C(H (A))–CH2- (X)	J_{BX}	1.2 (negative)
	J_{AC}	10.5
(B) H–C = / = C–H (D)	J_{CX}	1.0 (negative)
	J_{BD}	11.0 – 9.5

through esterification of β eleostearic acid. It was analyzed by GLC, and proved to be 98% pure.

RESULTS AND DISCUSSION

The observed chemical shifts of olefinic protons of the fatty acid methyl esters, and their spin coupling constants are listed in Table I together with the structural formulas of the compounds. The results of the analysis of the olefinic proton signals will be described later in detail. Table I clearly shows that the chemical shifts of olefinic protons are different depending on chemical environment arising from the position of the protons in conjugated double bonds. These chemical shifts are graphically summarized in Figure 1, where the chemical shift ranges of several types of olefinic protons are shown by filled rectangles. The data for the NMR spectra of methyl oleate, methyl elaidate, methyl linoleate, methyl linolenate, methyl ricinoleate and methyl ricinelaidate (2) are also included in the correlation chart.

From Figure 1, it is apparent that the olefinic proton signals of unsaturated fatty acid

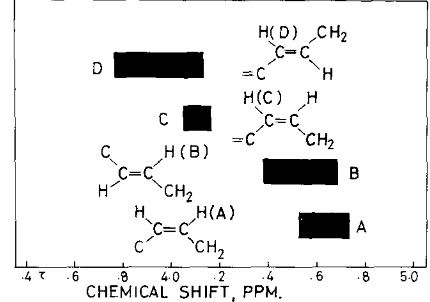

FIG. 1. Chemical shifts of olefinic protons in unsaturated fatty acid methyl esters.

esters with the *trans* configuration generally appear at the lower field than those of *cis* configuration. However *cis* and *trans* isomers of the unsaturated fatty acid esters cannot be identified by the chemical shifts of olefinic protons. As will be shown later, the olefinic proton signals of conjugated fatty acid methyl esters give complicated broad bands. This may be due to the fact that each olefinic proton is in magnetically different environment. The analysis of these complicated signals shows that the chemical shifts of interior protons in conjugated double bonds are observed at a lower field than those of terminal protons. The signals of the interior protons with the *trans* configuration are also observed at the lower field than those with the *cis* configuration.

The spin coupling constants are given in Table II. The coupling constants in Table II are generally in good agreement with those given for aliphatic monoolefin hydrocarbons by Stheling and Bartz (13).

The results of these interpretation of the complicated olefinic proton signals in conjugated fatty acid esters may give much information in analyzing the structure of unknown unsaturated fatty acids and their esters.

Analysis of Spectra

Methyl Cis-9,trans-11-octadecadienoate. The olefinic proton signal of methyl *cis*-9,*trans*-11-octadecadienoate is shown in Figure 2. This signal is a multiplet consisting of 18 observable lines. The structural formula in Table I indicates that four olefinic protons are chemically nonequivalent with each other. Therefore, each olefinic proton will give a different chemical shift, and also have a different spin-spin interaction with each other.

In this case the olefinic proton signal is expected to give rise to a pattern of $ABCDX_2Y_2$ spin system including the spin-spin interaction with the four protons of α-methyl-

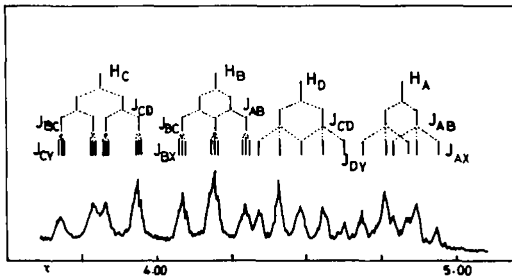

FIG. 2. Olefinic proton signal of methyl *cis*-9,*trans*-11-octadecadienoate at 100 MHz, with lines assigned to each proton.

ene groups adjacent to double-bonded carbons. From this point of view, the spectrum was analyzed, and it has been found that the internal chemical shifts of four protons are large enough to give the nearly first-order spectrum. Considering the coupling of each olefinic proton, each line of the signal could be assigned to each olefinic proton by first order approximation.

The result of the analysis is shown in Figure 2. For instance, the signal of H_A observed at the highest field is split into two lines with the spacing of 10.5 Hz by the interaction with H_B, and then each of these lines is split into three lines separated from each other by J_{AX} = 7.2 Hz by the interaction with two equivalent H_X protons to give a sextet. The signal of H_B appears at 4.15 ppm with three lines, which is split by spin-spin interaction with H_A and H_C at J_{AB} = 10.5 and at J_{BC} = 10.9 Hz, respectively. Furthermore, these three lines are observed to be split into three lines by long range coupling with H_X at 1.2 Hz. Signals of H_C and H_D were assigned in a similar manner. The spin coupling constant, for example, J_{AB}, was determined from the repeated spacing corresponding to J_{AB} in H_A and H_B signals. The chemical shifts were determined by taking the center of gravity of area intensity of each olefinic proton signal. The observed chemical shifts and coupling constants are given in Table I.

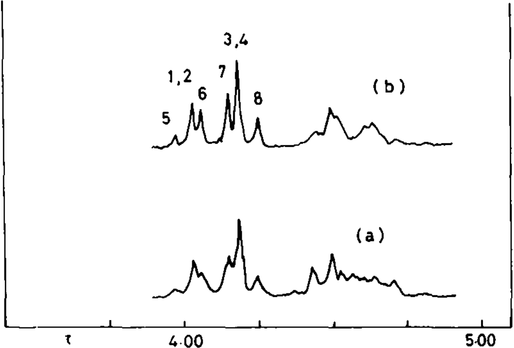

FIG. 4. The 100 MHz spectra of olefinic protons of methyl *trans*-9,*trans*-11-octadecadienoate. (a) The single resonance spectrum. (b) The double resonance spectrum of olefinic proton signal, in which the upper field signal is perturbed by irradiating with the second R_f frequency of 205.0 Hz at the position of the α-methylene protons.

In order to confirm the above spectral analysis, the spectral pattern of olefinic protons obtained by means of a 60 MHz spectrometer was compared with the calculated one by using parameters obtained from the 100 MHz spectrum. They are shown in Figure 3, where both patterns are in good agreement with one another. This fact further supports the interpretation of olefinic proton signal of methyl *cis*-9,*trans*-11-octadecadienoate mentioned above.

Methyl Trans-9,trans-11-octadecadienoate. The olefinic proton signal of methyl *trans*-9,*trans*-11-octadecadienoate is shown in Figure 4a. This signal is characteristically split into two

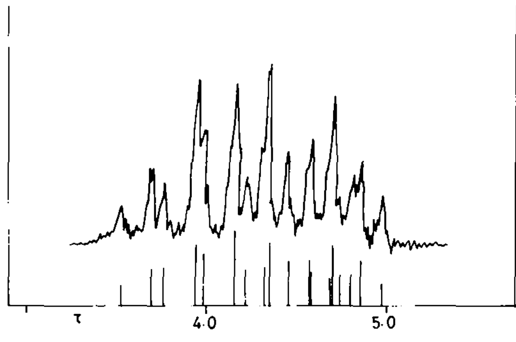

FIG. 3. Observed and calculated spectra of olefinic protons of methyl *cis*-9,*trans*-11-octadecadienoate at 60 MHz.

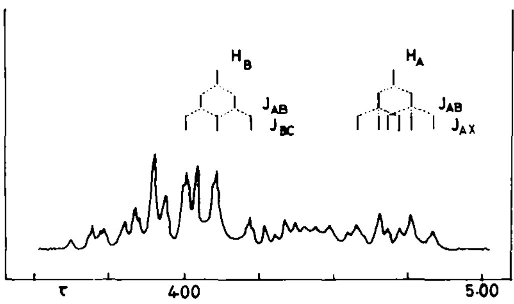

FIG. 5. Olefinic proton signal of methyl α eleostearate at 100 MHz, with lines assigned to H_A and H_B.

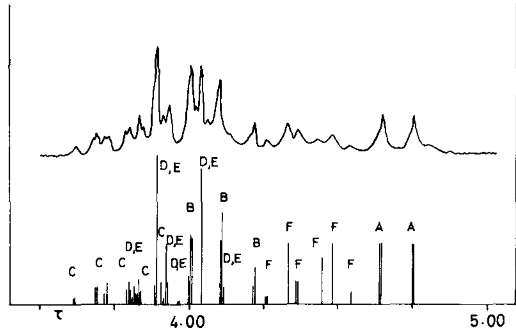

FIG. 6. The double resonance spectrum of olefinic proton signal of methyl α eleostearate at 100 MHz, with the theoretical spectrum by a digital calculation.

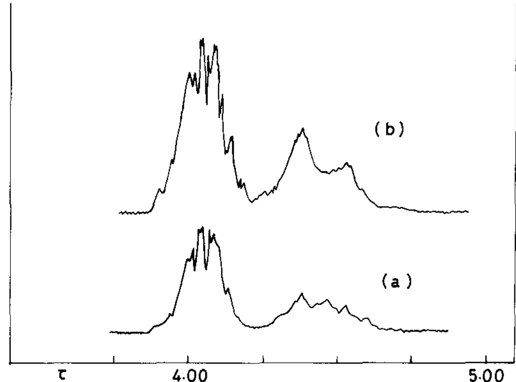

FIG. 7. Olefinic proton signal of methyl β eleostearate at 100 MHz. (a) The single resonance spectrum. (b) The double resonance spectrum.

parts; one is a complex pattern at the upper field, and the other at the lower field is a multiplet consisting of six observable lines. The four olefinic protons of this compound, as shown in Table I, consist of two equivalent protons (H_A,$H_{A'}$) adjacent to the methylene groups and two equivalent protons (H_B,$H_{B'}$) at internal positions in the conjugated double bonds. Apparently, H_A and H_B are nonequivalent. Thus it is considered that either of the two parts of the signal corresponds to the protons H_A or H_B, but these complicated signals do not give any useful information. Accordingly, the double resonance spectrum which was partially decoupled by irradiating with the second R_f frequency of 205.0 Hz at the position of α-methylene proton signal was measured as shown in Figure 4b. In this spectrum, the upper field signal is observed to be symmetrical compared with that of the lower field, although the former is much more broadened than the latter. The spectrum certainly gives rise to a pattern of A_2X_2 spin system as far as the olefinic protons are concerned. So the spectrum was examined as A_2X_2 pattern by using the Table published by Pople et al. (9). An A_2X_2 spectrum generally gives a symmetrical pattern with respect to the center position of the signal. The expressions for the frequency of signal A are given in Tables 6-18 of Reference 9, which may be considered the same as those of X. Here A and X correspond to H_A,$H_{A'}$ and H_B,$H_{B'}$, respectively. It is clearly noticed that H_A and $H_{A'}$ are separated by four carbon atoms so that the spin-spin coupling between them may be neglected in the A_2X_2 approximation. So $J_{AA'}$ is equal to zero. From this approximation, K is equal to M. Therefore, the signals of the total 10 transitions decrease to 6 transitions, where signals 5, 6, 7 and 8 agree with signals 9, 10, 11 and 12, respectively. The calculated pattern agrees with the measured one in number of lines. By calculating the function in the Table with the values of chemical shifts from actual measurement, each peak can be assigned as indicated in Figure 4b. At the same time, the spin coupling constants are determined by the calculation. Besides, it is concluded that the signal at the upper field is assigned to H_A and $H_{A'}$, for the signal is perturbed apparently by irradiating at the position of the signal of α-methylene protons. The partial decoupled signal at upper field seems to be broadened by the spin-spin interaction of H_A and $H_{A'}$ with β and more remote methylene protons. Thus, the spectrum in Figure 4b was interpreted. The spectrum in Figure 4a was also analyzed. Here, each signal of the decoupled spectrum is split into three lines by J_{AX} = 6.5 Hz at the upper field side, and also by J_{BX} = 1.0 Hz at the lower field side.

Methyl α Eleostearate. The olefinic proton signal of methyl α eleostearate is shown in Figure 5. This spectrum is a very complicated pattern. In order to obtain the additional information from the spectrum, the double resonance spectrum shown in Figure 6 was measured by irradiating with the second R_f frequency of 216.0 Hz at the position of the α-methylene proton signal. Referring to the structural formula in Table I, it is found that the three olefinic protons H_A, H_B and H_F are almost in the same chemical environment with H_A, H_B and H_D in methyl *cis*-9,*trans*-11-octadecadienoate, respectively. So the spectrum was analyzed by using the results obtained for the signal of methyl *cis*-9,*trans*-11-octadecadienoate, and the partially decoupled spectrum. The signals assigned to H_A and H_B are satisfactorily interpreted, as shown in Figure 5, by

the standard first order treatment. However, the signals of the protons, H_C, H_D and H_E, which are considered to be chemically equivalent, cannot be distinguished by the first order approximation. Therefore, the partially decoupled spectrum was analyzed using a LAOCOON II program modified for a FACOM 270-30 digital computer.

A theoretical spectrum was calculated by using the spin coupling constants resulting from the analysis of the spectra of methyl *cis*-9,*trans*-11-octadecadienoate and methyl *trans*-9,*trans*-11-octadecadienoate and the assuming chemical shift of each olefinic proton from Figure 6. Then the comparison with the experimental spectrum allowed a rough assignment of the signal. By means of the explicit relations between line positions and spectral parameters, further adjustment was made until the calculated spectrum agreed with the experimental one. The spectrum is observed to be a pattern of $ABCD_2E$ spin system. The theoretical lines of the spectrum are given in Figure 6.

The spectrum in Figure 5 was also analyzed. Here, each signal assigned to H_A, H_B and H_F in Figure 6 is split into three lines by J_{AX}, J_{FY} = 7.5 Hz and J_{BX} = 1.0 Hz, respectively. The chemical shifts and spin coupling constants determined by calculating the theoretical spectrum are listed in Table I.

Methyl β Eleostearate. The olefinic proton signal of methyl β eleostearate and the decoupled one are shown in Figure 7a and 7b, respectively. As shown in Table I, the olefinic protons of this compound consist of two chemically equivalent protons (H_A, $H_{A'}$) adjacent to α-methylene groups and four almost equivalent protons (H_{B1}, H_{B2}, H_{B3}, H_{B4}) at the internal positions in conjugated double-bonded structure. Roughly speaking, the spectrum in Figure 7a is split into two parts, in which the signal at lower field has twice the area intensity as that at upper field. Hence it is reasonable to conclude that the signal at upper field is assigned to H_A and $H_{A'}$ and that at lower field to H_{B1}, H_{B2}, H_{B3} and H_{B4}. Furthermore, the decoupled spectrum of Figure 7b, which was obtained by irradiating at the position of signal of α-methylene protons, shows the remarkable change of the signal at the upper field side, and confirms this assignment. The interval between the two peaks of the signal of the upper field side in Figure 7b corresponds to the coupling constant J_{AB}, which was determined to be 15.0 Hz.

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Preparation of ^{3}H-Phosphatidylmyoinositol With *Kloeckera brevis*

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ABSTRACT

The growth of *Kloeckera brevis* in the presence of uniformly tritiated myoinositol is described. The major radioactive lipid isolated from the cells is ^{3}H-phosphatidylmyoinositol, labeled exclusively in the myoinositol moiety. Chromatographic methods for purifying ^{3}H-phosphatidylmyoinositol, free from other labeled lipids, are described, and the use of this labeled lipid as a precursor in studying enzymatic synthesis of complex inositol lipids is demonstrated.

INTRODUCTION

Lester and Steiner (1) have identified labeled glycerylphosphorylmyoinositol and glycerylphosphorylmyoinositol phosphate from the deacylated lipids of *Saccharomyces cerevisiae*, grown in the presence of ^{14}C- and ^{3}H-labeled myoinositol. The yeast *Kloeckera brevis* (ATCC 9774) is known to require exogenous myoinositol when grown on glucose as the sole carbon source (2). Dawson et al. (3) have studied the fate of ^{3}H-myoinositol incorporated by *K. brevis* and reported that the major labeled lipid resembled phosphatidylmyoinositol. Since uniformly tritiated myoinositol, produced commercially by the Wilzbach technique (4), is both cheap and readily available it was thought worthwhile to study the growth of *K. brevis* in the presence of tritiated myoinositol in order to follow the metabolism of the label within the cell, in general, and its incorporation into phosphatidylmyoinositol, in particular. This paper describes a simple method of preparing ^{3}H-myoinositol-labeled phosphatidylmyoinositol of very high specific activity, which can serve as a valuable tool in studying complex inositol-lipid metabolism in living systems.

MATERIALS AND METHODS

^{3}H-Myoinositol

Myoinositol, uniformly tritiated by exposure to tritium gas (4), was obtained from New England Nuclear Corporation as a brown powder. An excess of unlabeled myoinositol was added and the mixture was recrystallized three times from hot aqueous ethanol, when a constant specific activity was obtained.

The white, crystalline product was dried in vacuo at 100 C and then made up to a 50 mM solution, 1 μmole of myoinositol containing 2.5 x 10^6 cpm. Radioactivity was determined with a Packard Tri-carb Liquid Scintillation Spectrometer using a toluene-base phosphor. Tritium was counted with about 30% efficiency.

Growth of Kloeckera brevis

In addition to myoinositol, *K. brevis* (ATCC 9774) requires exogenous biotin, pyridoxine, pantothenic acid, niacin and thiamine when glucose is the carbon source. Therefore, in this study, Difco Inositol Medium KB was used, which contains all of the essential growth factors except myoinositol (5). The concentration of Difco medium used was 0.5 g/10 ml of culture. Unlabeled or ^{3}H-labeled myoinositol was added in amounts as described under Results. All cells were grown at 25 C for seven days, with vigorous shaking. Turbidity was measured at 600 mμ by diluting a 1 ml portion of the culture to 7.5 ml with water and reading against a reference blank culture, similarly diluted, but which contained no added myoinositol.

Chromatographic Methods

Column chromatography of lipids was performed on Whatman DE-32 ion-exchange cellulose (acetate form) in chloroform-methanol-water (20:9:1 v/v/v) with ammonium acetate gradients, as described previously (6,7). Lipid extracts were desalted by filtration over Sephadex G-25 (8).

Thin layer chromatography (TLC) of lipids was performed on Silica Gel H (Merck), containing 10%, by weight, of magnesium silicate, as described by Rouser et al. (9). The following solvent systems were used (all ratios are by volume): Solvent A, chloroform-methanol-water (19:7:1); Solvent B, chloroform-methanol-acetic acid-water (30:15:4:2).

Paper chromatography was performed on Whatman No. 1 paper, using the following solvent systems: Solvent C, *n*-butanol/pyridine/water (10:3:3); Solvent D, ethylacetate-

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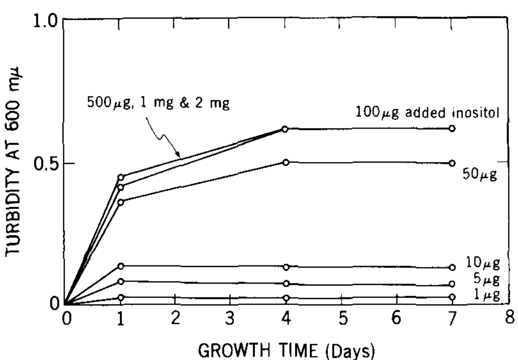

FIG. 1. Growth of *Kloeckera brevis* at different concentrations of myoinositol.

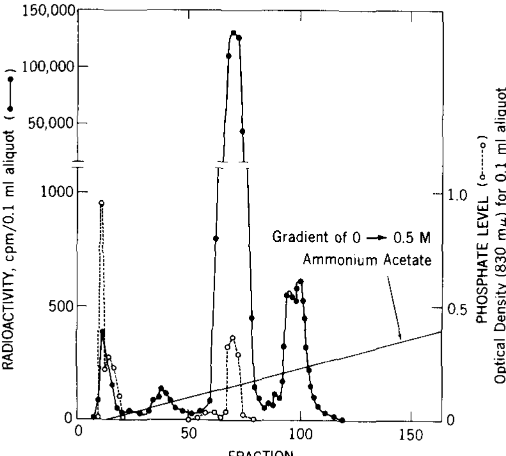

FIG. 2. Ion-exchange (DEAE-cellulose) column chromatography of the lipids of *K. brevis* grown on ^{3}H-myoinositol. The solid line is radioactivity, the dotted line is total phosphate.

pyridine-water (5:3:2); Solvent E, *n*-propanol/ NH_4OH/water(5:4:1). Sugars were located by the alkaline-$AgNO_3$ dip (10). Highvoltage paper electrophoresis was performed in 0.1 M oxalate buffer, pH 1.5. Phosphorus-containing compounds were revealed by the perchloric acid-ammonium molybdate spray (11). Phosphorus was determined by the method of Bartlett (12).

Extraction of Lipids

After growth, *K. brevis* cells were harvested by centrifugation at 4 C (30,000 x *g* for 20 min). The cell-paste was then extracted with chloroform-methanol (2:1 v/v) for 3 hr. This step was repeated for 3 hr. A final extraction was made for 3 hr with 'acidic' chloroform-methanol (2:1 v/v, containing 0.3% conc. HCl v/v) to insure complete extraction of very acidic lipids (13). The three extracts were each washed with 1/5 vol of sodium EDTA solution, buffered at pH 7.0, and the aqueous phases were discarded. After determination of radioactivity in the extracts, they were combined, evaporated to a small volume and filtered over Sephadex G-25 to remove any salt (8). The resultant straw-colored lipid solution was stored at -10 C, under nitrogen, prior to chromatography.

Purification of ^{3}H-Labeled Lipids

The total lipid extract, after Sephadex filtration, was dissolved in 5 ml of chloroform-methanol-water (20:9:1 v/v/v), applied to a 25 x 2.5 cm column of DEAE-cellulose and eluted with a linear gradient of 0-0.5 M ammonium acetate in the same mixture of solvents. Fractions of 7.5 ml were collected of which 0.1 ml aliquots were taken for analysis.

Metabolic use of ^{3}H-Phosphatidylmyoinositol

Enzymatic conversion of ^{3}H-phosphatidylmyoinositol to ^{3}H-diphosphoinositide followed the method of Kai et al. (14). A total volume of 14 ml contained 30 mM $MgCl_2$, 20 mM tris buffer, pH 7.4, 7 mM ATP, 10 mM glutathione, 4 μmoles of unlabeled phosphatidyl myoinositol from *S. cerevisiae*, and 0.43 μmoles of ^{3}H-phosphatidylmyoinositol from *K. brevis* (6.3 x 10^6 cpm). The enzyme preparation was a sucrose homogenate of one half of a rat brain. The mixture was incubated at 37 C for 20 min. The labeled product was purified by ion exchange cellulose column chromatography (6).

^{3}H-Phosphatidylmyoinositol monomannoside was prepared enzymatically from ^{3}H-phosphatidylmyoinositol by the method of Brennan and Ballou (15), with modifications. A 10 ml incubation contained 40 mM tris buffer, pH 7.4, 10 mM $MgCl_2$, 0.1% cutscum, 3 mM GDP-mannose, 10 mM glutathione and 1.3 μmoles of ^{3}H-phosphatidylmyoinositol (14 x 10^6 cpm). The enzyme source was 0.5 g (wet weight) of *Propionibacterium shermanii* (ATCC 9614) cells which had been suspended in 2 ml of 40 mM tris buffer, pH 7.4, and disrupted for 3 min at 4 C in a MSE 60W ultrasonicator. Incubation was for 1 hr at 37 C. The lipid products of the reaction were extracted and chromatographed on a 21 x 1 cm column of silicic acid, using a gradient elution from pure chloroform to pure methanol. Fractions of 5.0 ml were collected and 0.1 ml aliquots were taken for analysis.

RESULTS

Growth of K. brevis at Different Concentrations of Myoinositol

Cultures of *K. brevis* (10 ml) were grown, in

duplicate, with increasing amounts of added myoinositol. The growth-time curves are shown in Figure 1. For levels of myoinositol up to 10 μg/10 ml culture, the growth of cells reached a plateau after 24 hr. For levels of 50 μg to 2 mg, no difference in growth was observed after four days. After this time, no greater growth was obtained with 500 μg of myoinositol than that produced by 100 μg. Increasing the myoinositol concentration from 50 to 100 μg/ml gave only a 30% increase in growth. For the large-scale cultures of *K. brevis* with labeled myoinositol, two 2.5 l cultures contained 125 g of Difco Inositol Assay Medium KB and 250 μmoles of ^{3}H-myoinositol (2.6 x 10^6 cpm/μmole). This level of inositol corresponds to 180 μg/10 ml of culture. After seven days of growth the cultures were centrifuged, yielding 27 g wet-weight of pale yellow cells. The radioactivity in a portion of the supernatant indicated that 54% of the tritiated myoinositol had been taken up by the cells.

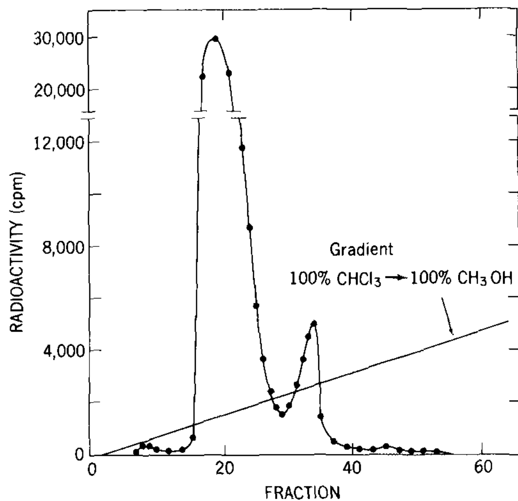

FIG. 3. Silicic acid column chromatography of the products of the mannosylation of ^{3}H-phosphatidylmyoinositol by *P. shermanii*.

Extraction of Lipids from the Cells

The first chloroform-methanol extract removed about 98% of the labeled lipid from the cells. No radioactivity was detected in the third extract. A total of 1.3 x 10^9 cpm of labeled myoinositol was added to the cultures, of which 54% was taken into the cells during growth (700 x 10^6 cpm). Only 65 x 10^6 cpm were found in the combined lipid extracts, which accounts for less than 10% of the label taken up by the cells. After extraction of lipids, the cells were stored at -10 C. About 2.7 mg of phospholipid phosphorus was obtained.

Chromatography of the ^{3}H-Labeled Lipid on DEAE Cellulose

The column chromatography of the labeled lipid from *K. brevis* is shown in Figure 2. Four peaks of radioactivity were observed. The first was eluted before the salt gradient was applied and corresponded with a yellow pigment. Neutral lipid, free fatty acid and nonacidic phospholipid (e.g., lecithin) have this property on DE-32 cellulose. A second, very small peak was eluted at low salt concentration and was not further investigated. The major radioactive peak was eluted with a gradient of 0-0.16 M ammonium acetate. Fractions 60 through 80 were pooled and desalted by Sephadex G-25 filtration. TLC of a portion of the combined fractions showed only one radioactive spot, which co-chromatographed with phosphatidylmyoinositol prepared from *S. cerevisiae*. A portion of the third radioactive peak was deacylated (11) and the water-soluble fragment examined by paper chromatography in Solvent D. Glycerylphosphorylmyoinositol was the major radioactive compound, together with a small amount of inositol phosphate. After deacylation it was found that 94% of the radioactivity was water soluble, suggesting that very little, if any of the tritium label was metabolized by the cells to long chain fatty acids and then incorporated into the phosphatidyl moiety of the phosphatidylmyoinositol.

A portion of the third radioactive peak was hydrolyzed with 2 N H_2SO_4 at 100 C for 4 hr and the hydrolysate examined by paper chromatography in Solvent C. The only radioactivity detected corresponded to a marker of myoinositol. None was detected in the glycerol moiety. These data show that the major ^{3}H-labeled phospholipid extracted from *K. brevis* was phosphatidylmyoinositol, in which at least 94% of the label was located in the myoinositol moiety.

A fourth peak of radioactivity, eluted from the DE-32 column after the ^{3}H-phosphatidylmyoinositol, had chromatographic characteristics which suggested a phosphorylated phosphatidylmyoinositol (6). This was confirmed by deacylation of the lipid from the combined fractions 91 through 110. On examination of the water-soluble fragments by paper chromatography (Solvent D for 48 hr) with markers of glycerylphosphorylmyoinositol, glycerylphosphorylmyoinositol 4-phosphate and glycerylphosphorylmyoinositol 4,5-diphosphate, the radioactivity corresponded solely with glycerylphosphorylmyoinositol 4-phosphate, the expected deacylation product of diphospho-

inositide. This result suggests that *K. brevis* contains diphosphoinositide, as has been reported for the yeast *S. cerevisiae* (1).

The specific activity of the ^{3}H-phosphatidylmyoinositol obtained from the column was 1.45 x 10^6 cpm/μmole of lipid phosphorus. Thus, unlabeled phospholipids, which co-chromatograph with the ^{3}H-phosphatidylmyoinositol, were present only in small amounts.

Use of ^{3}H-Phosphatidylmyoinositol in Studying Lipid Metabolism in vitro

Two previously reported enzymes, which use phosphatidylmyoinositol as substrate, were used to test the metabolic activity of the ^{3}H-phosphatidylmyoinositol purified by the above procedures. ^{3}H-Phosphatidylmyoinositol 4-phosphate was synthesized from ^{3}H-phosphatidylmyoinositol using a rat-brain homogenate as the enzyme source (14). A total of 6.3 x 10^6 cpm of ^{3}H-phosphatidylmyoinositol was used as substrate. After ion-exchange cellulose column purification of the product, a total of 12,500 cpm of diphosphoinositide was recovered, representing about 0.2% conversion of the substrate to product. The product was deacylated (11) and examined by paper chromatography (Solvent D for 48 hr). The radioactivity corresponded to marker glycerylphosphorylmyoinositol 4-phosphate, the deacylation product of diphosphoinositide.

^{3}H-Phosphatidylmyoinositol monomannoside was synthesized from ^{3}H-phosphatidylmyoinositol using an enzyme from *P. shermanii* and the lipid products of the incubation were separated on a silicic acid column, using a gradient of pure chloroform to pure methanol (7). The chromatogram is shown in Figure 3. Fractions 30 through 39 were combined and contained about 40,000 cpm, representing almost 3% conversion of the substrate to product.

A portion of the product was examined by TLC in Solvent B, when it was found that 87% of the radioactivity traveled with an R_f of 0.21. The remaining 13% of radioactivity had an R_f of 0.48, which was identical to the ^{3}H-phosphatidylmyoinositol substrate and to marker phosphatidylmyoinositol from *S. cerevisiae* in this system. The chromatographic characteristics of the product closely resembled ^{14}C-phosphatidylmyoinositol monomannoside, which has been synthesized from yeast phosphatidylmyoinositol and ^{14}C-GDP-mannose (15).

The remainder of the product was hydrolyzed with 10 N NH_4OH for 18 hr at 150 C and then acetylated with acetic anhydride-pyridine (1:1 v/v). Carrier myoinositol 2-monomannoside nonaacetate was added and the mixture was recrystallized a total of five times, when constant specific activity of the acetate was achieved. This indicated that mannosylation of the ^{3}H-myoinositol moiety of the labeled lipid substrate had occurred and that the linkage was to position 2 of the myoinositol ring.

Metabolism of Myoinositol by K. brevis

Since only about 10% of the radioactivity incorporated into the cells was converted to phosphatidylmyoinositol, the nature of the remainder of the tritium in the cells was studied. A portion of the *K. brevis* cells, after removal of lipids, was extracted overnight at 25 C with 10% trichloroacetic acid. The trichloroacetic acid was removed by washing with ether five times. The solution was examined by paper chromatography (Solvent A) with markers of glycerol, glucose and myoinositol. Of the radioactivity recovered from the paper, 61% co-chromatographed with free myoinositol and the remainder stayed at the origin, indicative of a charged compound. No radioactivity was detected in other regions of the chromatogram.

The trichloroacetic acid solution was also examined by paper electrophoresis. It was observed that 62% of the radioactivity remained at the origin, as did myoinositol, and 38% migrated to the position of inositol monophosphate and glucose 6-phosphate (8.5 cm), which were not separated by this system. No radioactivity corresponded to inositol hexaphosphate (19.5 cm).

On paper chromatography of the extract in solvent E, radioactivity co-chromatographed both with inositol 2-phosphate (22.5 cm) and glycerylphosphorylmyoinositol (29 cm). The identity of the latter radioactive component was not further confirmed, although it was unaffected by digestion with alkaline phosphatase.

A portion of the lipid-extracted *K. brevis* cells was hydrolyzed with 6 N HCl for 18 hr at 100 C. The hydrolysate was treated with mixed-bed resin and then examined by paper chromatography in Solvent C. Almost all of the radioactivity co-chromatographed with myoinositol, although a very small amount of radioactive glucose was detected.

DISCUSSION

This study shows that the yeast *Kloeckera brevis* will grow at high concentrations of added myoinositol, although the dose response is not linear for amounts above about 2 μg of myoinositol per 10 ml. Although maximum growth was obtained at 100 μg of myoinositol per 10

ml, 180 μg/10 ml was used in the large-scale experiments with ^{3}H-myoinositol. About 54% of the available myoinositol was taken up by the cells. That growth did not increase above this concentration or with extended incubation times suggests the exhaustion of other essential factors in the medium. The radioactivity remaining in the supernatant after harvesting the cells was confined to myoinositol, suggesting that this substance is not metabolized to other sugars and secreted back into the medium.

The radioactive lipids in the cells were removed by two extractions with neutral chloroform-methanol. A subsequent extract made with acidic chloroform-methanol, which is used for complete extraction of polyphosphoinositides (13), contained no radioactivity. No inositol-containing lipoprotein was detected in this study, in disagreement with a previous report (3). Our work confirms that ^{3}H-phosphatidylmyoinositol is the predominant labeled lipid that is extracted. The presence of labeled diphosphoinositide in this yeast is consistent with its detection in *S. cerevisiae* by Lester and Steiner (1), although the level in both yeasts is very low.

That the major labeled lipid was phosphatidylmyoinositol was suggested by co-chromatography with authentic standards of the lipid and its degradation products. The isolated labeled lipid was converted to complex inositol lipids using enzyme preparations from rat brain and *Propionibacterium shermanii*. Previous work has shown (14,15) that phosphatidylmyoinositol isolated from liver, soyabean or yeast is the acceptor lipid in complex lipid metabolism. Thus, it is highly probable that the absolute configuration of the labeled lipid from *K. brevis* is identical to that of all species of phosphatidylmyoinositol reported, namely, 1-phosphatidyl-D (1)-myoinositol. Kai et al. (14) prepared ^{32}P-labeled phosphatidylmyoinositol by growing *Schizosaccharomyces pombe* in the presence of ^{32}P-orthophosphate and showed that it was converted to ^{32}P-diphosphoinositide using rat-brain preparations.

Although two of the minor labeled lipids shown in Figure 2 were not identified, it is reasonable to suppose that little, if any, of the ^{3}H-myoinositol was degraded to other compounds and incorporated into fatty acid. It is possible that myoinositol could be degraded to glucuronic acid, or oxidized to 2-inosose, both of which reactions would generate ^{3}H-pyridine nucleotides coenzymes which could then incorporate tritium into glycolytic intermediates or long-chain fatty acids. If this occurred in *K. brevis*, one would expect to find radioactivity in the glycerol or the fatty acid moieties, or both, of ^{3}H-phosphatidylmyoinositol, which was not the case. In contrast to this, we have grown *Mycobacterium phlei* and *Propionibacterium shermanii* in the presence of ^{3}H-myoinositol and detected large amounts of label in the fatty acid moieties of the inositol lipids isolated from these bacteria (C. Prottey, unpublished work).

The metabolism of ^{3}H-myoinositol to nonlipid products by *K. brevis* shows a similarity to previous work (3). Myoinositol phosphate and the tentatively identified glycerylphosphorylmyoinositol could come from phospholipase action on ^{3}H-phosphatidylmyoinositol. Dawson et al. (3) reported "...a neutral derivative of mesoinositol...not identified as any known naturally occurring form of combined inositol...". We have found no evidence for this, although a large intracellular pool of ^{3}H-myoinositol was found, unreported by the above-mentioned workers. In this study, a higher level of ^{3}H-myoinositol was used than by Dawson et al. (3) (180 μg/10 ml compared with 10 μg/10 ml), and the differences observed may result from this fact.

The ready availability of tritium-labeled myoinositol, coupled with its specific incorporation into the lipid of *K. brevis*, make this method of preparation of isotopically labeled phosphatidylmyoinositol very straightforward. Although the production of ^{32}P-phosphatidylmyoinositol using a myoinositol-requiring yeast has been reported (14), that procedure is complicated by the facts that a low concentration of inorganic phosphate is necessary to avoid dilution of the isotope and extensive purification of the ^{32}P-phosphatidylmyoinositol is required to remove other labeled lipids. In the present study, ion-exchange chromatography effects a good separation of the ^{3}H-phosphatidylmyoinositol from the small amounts of other radioactive lipids. The enzymatic reactions of ^{3}H-phosphatidylmyoinositol described in this paper demonstrate the value of this labeled substrate for studies on the metabolism of complex lipids.

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Liver Lipids of the Polar Bear, *Thalarctos maritimus*

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ABSTRACT

The distribution of total and free fatty acids as well as the acids from the glycerides, sterol esters and phospholipids of polar bear liver lipids was ascertained and found to contain somewhat higher levels of unsaturated components as compared to those of such mammals as the pig. Saponification of the liver lipids yielded the hydrocarbons, alcohols and sterols which were analyzed by GLC. The hydrocarbons occurred at an overall level of 55 mg/kg liver or 57.9 mg/100 g total lipids, of which pristane and other saturated hydrocarbons, mainly normal homologs, comprised 2.6 and 5.3 mg/100 g, respectively; the remainder contained squalene (37.7 mg/100 g) and other unsaturated types (12.3 mg/100 g). As based on the total lipids, the levels of fatty alcohols, sterol and glyceryl ethers amounted to 1.65%, 5.9% and 0.03%, respectively. The fatty alcohols displayed about 31 peaks of C_{12} to C_{30}, of which the hexadecanol and a branched C_{20} component were prominent.

INTRODUCTION

The presence of small amounts of saturated hydrocarbons has been reported in various animal lipids and special attention has been directed to 2,6,10,14-tetramethylpentadecane or pristane. This hydrocarbon occurs in significant levels in marine lipids, notably those of the basking shark and owes its origin principally to plankton on which the animal feeds (1-3). Aside from special coal tar and petroleum sources, the presence of pristane has been demonstrated in wool wax (4), several human and animal tissues (5) and in human sebaceous lipids (6). The distribution of hydrocarbons occurring together with pristane and squalene has been recently surveyed in liver lipids of the basking shark (7), dogfish and cod and in whole herring oil (8) and the presence of several homologous series noted. As the opportunity presented itself for a study of the liver lipids of the polar bear, *Thalarctos maritimus*, it was thought timely to screen the hydrocarbons of this mammal in addition to other components, considering that certain similarities might exist in relation to the above marine lipids. In this conjunction, the distribution of fatty acids in depot fats has been investigated by Brockerhoff et al. (9,10) for seals, a whale and a polar bear and the findings with the latter were consistent with those generally encountered with mammals consuming marine fats.

EXPERIMENTAL PROCEDURES

All glassware and allied pertinent equipment employed in this study were thoroughly defatted with chloroform-methanol and ethyl ether and with no exceptions, the solvents were of AR grade and redistilled before use. The petroleum ether boiled at 30-60 C and all solvent compositions are expressed on a volume basis. Anhydrous AR sodium sulfate comprised the desiccant. Concentration of extracts and filtrates was carried out in all glass rotating vacuum evaporators. A nitrogen atmosphere was maintained to minimize oxidation during various procedures and in the removal of the last traces of a solvent. The reference fatty acid methyl esters, alcohol acetates and batyl, chimyl and selachyl alcohols originated from Applied Science Laboratories; hydrocarbons to C_{29} and higher were obtained from the latter source as well as from special collections.

Liver was removed from a polar bear shot during a winter hunt in the Canadian Arctic, chilled and shipped frozen to the laboratory. A total of 925 g of the tan-colored tissue was thawed, blended with chloroform-methanol, (2:1), filtered and the residue repeatedly extracted with the solvent. The latter was removed under vacuum and the residual material taken up in petroleum ether, washed with several portions of water and the extract dried; recovery of lipids: 88.07 g or 9.5%.

Fractionation of Lipids by Chromatography Over Florisil

A sample of the total lipids in hexane was chromatographed over Florisil by the method of Carroll (11). Elution of the column with hexane as such and containing 5%, 15%, 25% and 50% ethyl ether, 98% ethyl ether + 2% methanol and 96% ethyl ether + 4% acetic acid, gave rise to the hydrocarbons, sterol esters, triglycerides, sterol, diglycerides, monoglycerides and free fatty acids. The phospholipids occurred in a final fraction eluted with absolute methanol. The mono-, di- and triglycerides, also

TABLE I

Fatty Acid Composition of Polar Bear Liver Lipid Fractions[a]

Acid[b]	Total[c]	Free	Glycerides Mono-	Glycerides Di-	Glycerides Tri-	Sterol esters	Phospholipids
10:0	0.3	1.0	Trace	0.4	0.6	0.2	Trace
11:0	0.3	1.2	Trace	0.3	0.6	0.6	Trace
12:0	2.9	4.0	5.1	3.8	4.4	4.3	0.3
13:0	0.7	2.0	1.7	1.1	0.6	0.8	Trace
14:0	1.5	4.5	3.4	1.9	3.3	2.0	0.6
14:1	Trace					Trace	
15:0	0.7	0.5	1.7	0.8	1.0	1.1	0.1
16:0	9.6	10.0	13.7	15.7	10.7	6.5	3.9
16:1	13.5	12.7	11.1	11.7	13.1	18.6	6.5
17:0	0.7	1.0	1.7	1.1	1.3	1.1	
18:0	13.8	9.5	8.5	7.0	8.4	1.4	21.0
18:1	21.4	19.7	18.8	21.4	20.8	23.4	16.6
18:2	1.3	3.0	2.6	1.6	2.0	1.1	1.9
18:3	0.3	Trace	Trace	Trace	Trace	0.5	0.3
20:1	4.8	7.0	5.9	9.4	6.7	3.5	1.8
20:2	Trace	Trace	Trace	Trace	Trace	0.5	
20:4	0.3	0.5	0.8	1.1	0.6	0.5	0.6
22:1	5.2	4.1	4.3	3.8	5.0	1.7	16.6
20:5	8.6	6.2	6.8	5.1	6.7	11.5	12.8
24:0	Trace	1.5	Trace	Trace	Trace	0.6	0.6
24:1	0.8	0.2	0.8	0.7	Trace	1.2	0.6
22:5	2.5	2.0	2.6	2.8	2.7	4.2	3.0
22:6	10.4	9.1	10.2	9.8	11.1	14.2	12.8
Saturated	30.5	35.2	35.8	32.1	30.9	18.6	26.5
Unsaturated	69.1	64.5	63.9	67.4	68.7	80.9	73.5
Mono-	45.7	43.7	40.9	47.0	45.6	48.4	42.1
Poly-	23.4	20.8	23.0	20.4	23.1	32.5	31.4

[a]All GLC values in the Tables are relative (area) percentages.

[b]Number of C atoms: number of double bonds.

[c]Acids obtained from saponification of the lipids by refluxing with 10% aqueous sodium hydroxide for 16 hr.

checked by the infrared spectra, were transesterified by the use of sodium methoxide and methanol according to the procedure of Luddy et al. (12). The phospholipid fraction was hydrolyzed by heating with 5% methanolic hydrochloric acid for 2 hr (13), a procedure also applied to the sterol esters. The free fatty acids were esterified by means of methanol containing hydrogen chloride.

Separation and Analysis of Unsaponifiable Fractions

Yields of 6.59 g (8.9%) unsaponifiables (UNS) and 57.09 g of saponifiable material were obtained from 73.87 g lipids using procedures (7,14-16) which involve saponification with alcoholic sodium hydroxide, ether extraction of UNS, followed by acidification and ether extraction of the saponifiable portion.

The UNS in petroleum ether was fractionated (7) on alumina (Alcoa F-20) which had been determined by prior tests to be essentially free of adsorbed lipid, eluting with successive portions of petroleum ether alone, petroleum ether plus 5%, then plus 10% chloroform, 100% chloroform, and absolute methanol; Fractions 1-5 amounted to 4.7, 0.2, 28.0, 53.7 and 5300 mg (small losses), respectively. Infrared spectrophotometry indicated hydrocarbons in Fractions 1, 2 and 3 and to a lesser extent, in 4. Fraction 5 contained alcohols and sterol as did Fraction 6 (315 mg) which was obtained by repeated extraction of the spent adsorbent with chloroform. Additional separations were made by chromatographing 1% solutions of Fractions 1 and 3 in petroleum ether on Davison's silica gel (200-236 mesh) and eluting with petroleum ether, benzene, ethyl ether, acetone and methanol.

The alcohols and sterol of Fractions 5 and 6 were acetylated with acetic anhydride in pyridine preparatory to GLC analysis; sterol was also determined by way of the digitonide. For check purposes, fractions high in the fatty alcohols were crystallized from methanol and analyzed. By use of acetic anhydride, the

TABLE II

Hydrocarbon Composition of Alumina- and Silica Gel-treated Mixtures (Temperature-Programmed; SE-30)

Relative C-No.	Alumina Fraction 1	Gel cuts, Fraction 1[a]		Gel cuts, Fraction 3[b]		
		Cut 2	Cut 4	Cut 5	Cut 7	Cut 9
14.0	0.6	0.3			0.6	2.2
15.0	0.9	0.3			0.6	1.2
16.0	0.6	0.6	0.8	0.6	0.6	1.2
17.0	1.3	0.8	0.8	0.6	0.6	1.2
17.5 + Pristane	40.3	30.1	46.8	0.3		
18.0	12.4	12.0	9.4	0.8	0.8	1.2
18.5	2.6	1.1	0.8	Trace	0.5	Trace
19.0	12.4	8.7	9.4	0.8	0.5	1.2
19.5	0.9	1.1	0.8		2.6	3.4
20.0	3.9	5.5	7.8	0.6		
20.5					0.5	1.2
21.0	1.9	2.2	3.1			
21.5				1.6	0.5	1.2
22.0	1.6	2.2	3.1			
22.5				0.6	0.5	1.2
23.0	1.6	2.5	3.1			
23.5				0.8	2.3	3.4
24.0	1.9	2.5	2.3	0.3		
25.0	1.9	2.7	2.3			
25.5					0.8	Trace
26.0	1.6	3.0	2.3	0.6		
26.5					1.1	1.2
27.0	1.6	3.0	1.6			
28.0 + Squalene	1.9[c]	2.7[c]	1.6[c]	75.6	62.8	69.7
29.0	1.6	3.0	1.6			
29.3				11.2	18.5	8.1
29.5				4.2	3.4	1.2
30.0	1.3	2.5	0.8			
30.1				1.1	1.4	1.2
30.5					0.8	
31.0	1.3	2.2	0.8			
32.0	0.9	1.9	0.8			
33.0	0.9	1.6	Trace			
34.0	0.9	1.6	Trace			
35.0	0.9	1.4	Trace			
36.0	0.6	1.4	Trace			
37.0	0.6	1.1	Trace			
38.0	0.6	0.8	Trace			
39.0	Trace	0.6				
40.0	Trace	0.3				
41.0	Trace	0.3				

[a]A sample of 4.0 mg Fraction 1 from alumina-treatment as a 1% petroleum ether solution was passed over 157 mg silica gel and the column eluted with volumes of 0.25 ml each of petroleum ether (four cuts) and then benzene (three cuts), the weights being 1.4, 1.3, 0.9, 0.1, 0.2, 0.1 and 0.0 mg, respectively. Lipid was absent in the eluates from ethyl ether, acetone and methanol. Cuts 1 and 3 were similar to Cut 2 in composition, the pristane, C_{18}, C_{19} and C_{20} levels amounting to 36.8%, 6.4%, 6.4% and 6.9% and 41.1%, 8.8%, 7.1% and 7.1%, in the order stated. Cuts 5 + 6 contained 51.5% pristane, 17.2% C_{18}, 17.2% C_{19} and 13.8% C_{20}.

[b]Alumina Fraction 3 (28.0 mg) was treated with silica gel (825 mg) and the latter eluted with 0.90 ml volumes each of petroleum ether (four cuts), benzene (three cuts), ethyl ether, acetone and methanol, two cuts being taken with each of the latter three media. Lipid was absent in Cuts 1-4 and 11-13, inclusive and Cut 10 was high in squalene. The weights of Cuts 5-10 were: 3.9, 4.9, 10.8, 6.7, 1.0 and 0.6 mg, in the order given. Cuts 6 and 8 paralleled Cut 7 in composition, squalene, $C_{29.3}$ and $C_{29.5}$ being present at levels of 62.7%, 18.4% and 5.5% and 54.3%, 21.7% and 2.7%, in the order stated.

[c]Squalene is absent.

glyceryl ethers were also converted to the acetates, an approach employed for their analysis by Bloomstrand and Gürtler (17). As with the fatty acid esters, aliquots of the acetate mixtures were hydrogenated in a Parr apparatus at 40 psi and in the presence of the Adams platinum oxide catalyst. The glyceryl ethers of the UNS were also purified by chromatography according to the procedure of Hallgren and Larsson (18), followed by TLC as described by Ramachandran et al. (19). GLC analysis was carried out with the isopropylidene derivatives prepared by acetonation (20) as such and following hydrogenation.

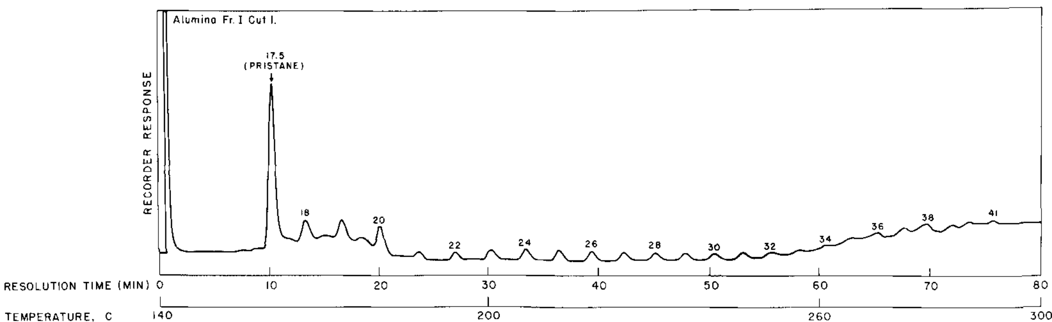

FIG. 1. Gas liquid chromatogram of alumina chromatographed Fraction 1 saturated hydrocarbons following treatment with silica gel (Cut 1). Column: 3% SE-30 on Gas Chrom P; temperature-programmed from 140 to 300 C.

GLC Analysis

A Barber Colman model 5000 gas chromatograph equipped with a hydrogen flame detector was used with the conditions essentially the same as previously described (7). Peaks were identified by use of references and the logarithmic plots of relative retention times vs. chain length and degree of unsaturation (21). Pristane and squalene eluted at $C_{17.5}$ and $C_{28.0}$, respectively, on the SE-30 packing (16).

RESULTS AND DISCUSSION

The composition of free, glyceride, sterol ester and phospholipid fatty acids derived from the Florisil-chromatographed fractions and the total acids from saponification of the liver lipids with 10% aqueous sodium hydroxide is presented in Table I. Essentially the same peaks occurred among the samples and the unsaturated acid levels exceeded those of the saturated members. Polyunsaturated acids were prominent in the phospholipid and sterol ester fractions, with the 20:5 constituent at 11.5% and 12.8%, respectively, compared to 8.6% for the total acids and an average of 6.2% for the remaining products. The acids from the sterol esters were also high in hexadecenoic acid but very low in octadecanoic acid in contrast to the other mixtures. The ratios of olefinic to saturated acids ranged from 1.8 to 2.3 but amounted to 2.8 for the phospholipids and even higher, 4.4, for the sterol ester components. Except for the acids from the sterol ester fraction, these ratios lie far under those of marine lipids such as basking shark, dogfish or cod liver oils (7,8) but are in the range of the fatty acids of the liver of the fin whale, *Balaenoptera physalus* (23) and possibly are higher than those of the total, free and glyceride acids of the liver lipids of such terrestrial mammals as the pig [1.2-1.4; (22)].

Of the hydrocarbons isolated from the polar bear lipids, pristane together with other saturated components occurred in Fraction 1 to the exclusion of any squalene as shown in Table II. Of the 31 peaks (C_{14}-C_{41}) obtained by temperature-programmed GLC, the pristane level was 40.3%, and the remaining members were primarily normal, C_{18}, C_{19} and C_{20} amounting to 12.4%, 12.4% and 3.9%, respectively. On silica gel chromatography, the lipid occurred in the cuts eluted with petroleum ether, all of which were quite similar in composition (Table II). As with some of the marine lipid mixtures, the pristane peak was well defined as can be noted from Figure 1. Fraction 2 (0.2 mg) presented several peaks including pristane and squalene but the latter hydrocarbon was especially prominent in Fraction 3 together with lesser amounts of $C_{29.3}$ and $C_{29.5}$, also observed in the past in marine and pig liver lipids. Squalene made up 18% of Fraction 4.

The overall hydrocarbon content was 57.9 mg/100 g of total lipids of which the saturated components comprised 2.6 mg pristane and 5.3 mg/100 g other types and the unsaturated hydrocarbons, 50.0 mg/100 g (composition: squalene, 37.7; $C_{29.3}$, 6.6; $C_{29.5}$, 1.4 and additional homologs, 4.3 mg/100 g). The hydrocarbons occurred at a level of 55 mg/kg polar bear liver and in the range observed with such mammals as the pig [30-45 mg/kg; ratio of unsaturated to saturated members, 1.3; (7)]. Although the presence of pristane in the porcine source was equivocal, a small amount is probable in view of the findings of Avigan et al. (5) with several human, bovine and rat tissues. The saturated hydrocarbons of the polar bear liver lipids were simpler in composition and the branched homologs, far lower than those of such marine sources as basking shark liver oil which displayed at least two homologous series in addition to the normal paraffins.

TABLE III

Alcohol and Sterol Distribution in Polar Bear Liver UNS-Fractions 5 + 6 From Alumina Chromatography (DEGS)

Alcohol	Sterol-free	With sterol
12:0	3.8	0.8
12:1	3.0	0.7
13:0	3.2	0.7
14 Br[a]	2.3	0.5
14:0	2.8	0.6
14:1	3.1	0.7
15:0	1.4	0.3
16 Br	1.6	0.4
16:0	9.5	2.1
16:1	0.8	0.2
17:0	1.2	0.3
17:1	1.8	0.4
18:0	1.8	0.4
18:1	5.6	1.2
19:0	Trace	Trace
20 Br	18.3	4.0
20:0	3.0	0.7
20:1	2.3	0.5
22 Br	5.3	1.2
22:0	1.9	0.4
22:1	3.8	0.8
24 Br	1.8	0.4
24:0	3.9	0.8
24:1	1.4	0.3
25:0	Trace	Trace
Chimyl	6.4	1.4
26:1	2.4	0.5
28 Br	3.5	0.8
Batyl	0.4	0.1
Selachyl	0.8	0.2
29 Br	2.1	0.5
30 Br	0.4	0.1
30:0	0.1	Trace
Sterol		(77.9)

[a]Br, branched.

Fractions 5 and 6 from alumina chromatography contained 81.3% and 20.6% sterol, respectively, and the fatty alcohol composition of both mixtures was similar on a sterol-free basis except that the branched C_{20} and C_{29} components and chimyl alcohol of Fraction 6 were present at 1.6%, 24.3% and 9.7%, in the order stated. The findings for Fractions 5 + 6 both as such and on a sterol-free basis are advanced in Table III. Of the fatty alcohols, hexadecanol and the branched C_{20} compound were quite prominent. In terms of the total liver lipids, sterol occurred at 5.9%, the fatty alcohols at 1.65% and the alkyl glyceryl ethers, principally chimyl alcohol, at 30 mg/100 g. The latter was somewhat higher but with a similar relative distribution by procedures employing TLC and analysis of the isopropylidene derivatives. The fatty alcohols occur at levels above those ascertained for pig liver lipids (7) and the glyceryl ether contents are in the range reported for several human and bovine lipid sources (18) but are far below those observed for some of the fish liver oils (8,18).

In spite of the marine dietary habits of the polar bear, its liver lipids show several similarities to those of an animal like the pig but the occurrence of pristane is quite remarkable although the overall saturated hydrocarbon content is low. This view had been anticipated earlier by Ackman et al. (23) in even a more extreme case of a marine animal, the fin whale, with a liver fatty acid composition approaching those reported for the human and rat. However, as based on depot lipids, polar bear fat has been regarded as a marine oil (9). Glyceride structures have been compared in relation to the position of the polyenoic acids by Brockerhoff et al. (24) and a difference noted between the polar bear on the one hand and whales and the rat, on the other. It must be emphasized that tissue lipid make-up is dependent on the nutrition of the animal and wide variations in lipid and vitamin A contents have been described for the polar bear (25). Thus, of 14 animals, the liver oil ranged from 8.9% to 28.5% with a mean of 14.4%, as compared to a recovery of 9.5% in the present study. Few generalizations can be made relative to lipid distribution in relation to nutritional status, details of which are lacking for the single animal involved in the current report. Also, no special attention was directed to the isolation and analysis of vitamin A which figures prominently in the toxicity of polar bear liver (25).

ACKNOWLEDGMENTS

J. B. Haeberlin, Jr. donated the polar bear liver.

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Factors Affecting Incorporation of Precursors Into Body Constituents: A Review of Common Sense Considerations With Glycolipids as Examples

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ABSTRACT

The dynamics of cellular growth are of prime importance to the biochemist. The dynamic state of lipids can be studied by employing radioactive substrates or stable isotope-labeled substrates. This paper illustrates major factors which may affect the incorporation of precursor substances into body constituents. These factors are: (a) age and species; (b) molar size of the body's pool of precursor; (c) metabolic activity of the lipid being synthesized; (d) metabolic pathways; (e) route of administration of the precursor; (f) the nature of the precursor, i.e., molecular size, ionic or nonionic, water soluble vs. lipid solubility, and micelle formation; and (g) the influence of hormones and drugs. The synthesis and turnover of cerebrosides and gangliosides in the rat are used to illustrate these seven factors.

INTRODUCTION

The dynamics of cellular growth, anabolism and catabolism have long been major points of interest to the biological chemist. One of the first methods used to investigate the dynamics was the balance study in which input and output of substances were measured as a function of time. The addition of the powerful tool of labeled substrates to the balance study permitted the clever chemist to investigate the rate of uptake, the extent of metabolism, the rate of excretion and deductions of possible mechanisms of metabolism. The classic example is the elucidation of β-oxidation of fatty acids by Knoop in 1905 (1). Knoop prepared labeled fatty acids by adding a phenyl radical to the ω-methyl carbon of fatty acids. The labeled fatty acids were given to dogs and the metabolites isolated from their urine. The fatty acids with an even number of carbon atoms always produced phenyl acetic acid and never benzoic acid; while fatty acid with an odd number of carbon atoms always produced benzoic acid and never phenyl acetic acid. Hence β-oxidation must occur. The use of chemically-labeled substrates is still in wide use, despite the many disadvantages such as the employment of unnatural substrates and the associated risk of only studying the metabolism of the unnatural substrate rather than the natural pathways. The introduction of isotopes as a means of labeling natural substrates has provided a method for very detailed analysis of metabolic pathways and the dynamics of many body constituents (2). While some biological discrimination does occur between the molecules containing the natural element and the molecules containing the isotope, this discrimination is not, in general, a problem. Of course it is most apparent when the mass of the element and its isotope are very different, e.g., hydrogen (mass 1) vs. deuterium (mass 2) or vs. tritium (mass 3). One of the more useful isotopes is carbon-14 used to replace carbon-12; the mass differences are relatively small being about 16-17% (3).

The study of the factors which may influence the incorporation of precursor substances into cellular constituents in vivo has been investigated by radioactive isotope labeled substrates. A new tool introduced by Rynhagen (4,5), coupled gas liquid chromatography-mass spectrometry, permits the use of stable-isotope labeled substrates thereby expanding the scope of dynamic, in vivo studies to normal, healthy humans without the dangers of radiation injury (6). Such stable isotope studies on blood glycolipids are currently in progress (C. Sweeley, personal communication).

While there is a host of examples in the literature of factors affecting incorporation, the following discussion will be confined to the glycolipids of the central nervous system since two glycolipids, cerebrosides and gangliosides, will serve to illustrate the various points. For the most part, these glycolipids are characteristic of the central nervous system. They are relatively easy to isolate and are composed of small molecular weight components both charged and uncharged. The neonatal rat readily synthesizes and deposits these glycolipids in the brain.

The primary factors that can affect the in vivo incorporation of precursors into lipids of the central nervous system are: (a) Age and

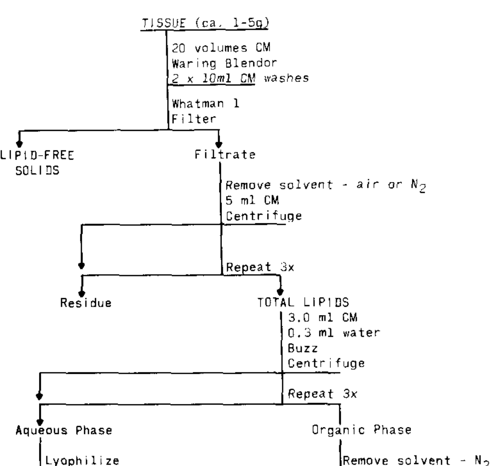

FIG. 1. Lipid isolation flow chart.

species. (b) The moles of precursor administered relative to the molar size of the body's pool of precursor: specific radioactivity. (c) Metabolic activity of the lipid being synthesized: used as fuel or energy reserve, as a metabolite or as a precursor of other cellular constituents and structurally. (d) Metabolic pathways: the position of the isotope label in the compound synthesized, and the position of the isotope label in the precursor compound administered. (e) Route of administration. (f) Nature of the compound: molecular size, ionic or nonionic and water-lipid solubilities and micelle formation. (g) Influence of hormones and drugs.

The text that follows and the examples cited will illustrate most of these factors and will discuss their qualitative and quantitative importance to experiments conducted in vivo. While many of these factors are apparent from common sense considerations, errors of interpretation still do occur which may lead to faulty conclusions. Therefore, it should be useful to consider these factors in some detail.

Most of the examples cited that illustrate these factors are from the author's laboratory to provide a consistency of the products labeled and to facilitate comparison of the factors. Primary references are cited specifically in the References section or can be found in the bibliography of the references cited.

TABLE I

Incorporation Into Gangliosides

Components	Dose[a]	Specific radioactivity	Radioactivity incorporated
U-^{14}C-D-glucose	15 μc	1.0 mc/mM	1536 cpm/g ww brain
1-^{14}C-D-galactose	15	0.31	9150

[a]The radioactive hexoses were given ip to 13-day-old rat litter mates. After 24 hr the rats were killed, the ganglioside fraction isolated, and radioactivity determined.

TABLE II

Incorporation of Radioactive Substances Into Gangliosides

Precursor	Incorporation into gangliosides[a]
	dpm/g ww brain
1-^{14}C-Glucosamine	65,600
1-^{14}C-Galactose	38,400
1-^{14}C-Glucose	18,350
3-^{14}C-Serine	5,760
4-^{14}C-Aspartic acid	769

[a]The radioactive substrates were administered ip to male rats 13 days old. After 24 hr the rats were killed and their brain gangliosides isolated as described for Figure 3, in Figure 1 and Methods section.

MATERIALS AND METHODS

Sprague-Dawley rats were obtained from either Charles River or Holtzman Animal colonies. In general, only male rats were used and their birthdates are accurate to within 12-16 hr. The radioactive compounds were purchased from either New England Nuclear Corporation or Tracerlab and administered by intraperitoneal or intracerebral injections. The intracerebral injections were made using a number 25 1/4 in. needle and a 1/4 ml tuberculin syringe. The needle was inserted at the base of the skull, slightly to the right of the midline, and directed towards the third ventricle space. While there was no noticeable effect of the intracerebral administered 0.05 ml volume in the young rats, most of the rats over four weeks of age showed a transient paralysis of the left fore and hind limbs. Within an hour or two, the paralysis disappeared and there was no visible residual impairment. Only in rare incidents, did death of the rat follow the intracerebral injection and this was due to subdural hemorrhage.

The animals were killed by decapitation and the brains rapidly removed and blended with at

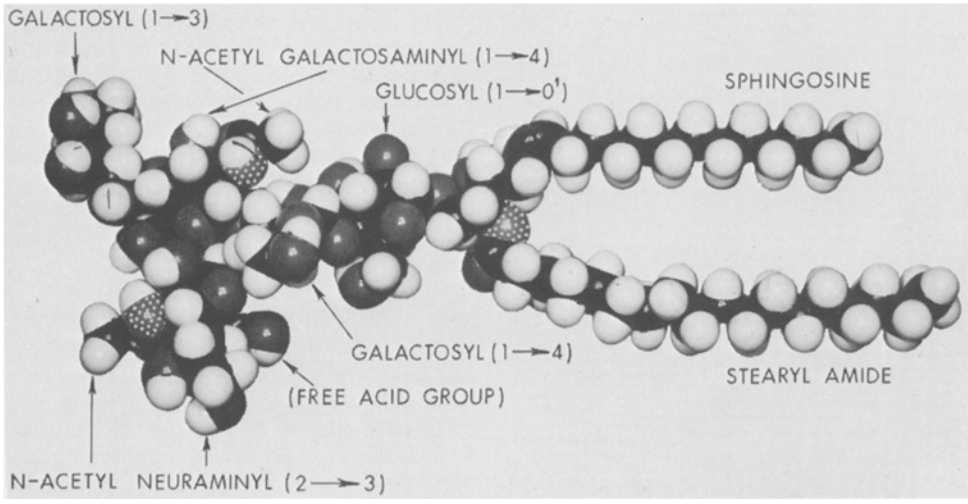

FIG. 2. Ganglioside structure as represented by space-filling models. The ganglioside shown is gal-galNAc-(neuNAc)-gal-glc-cer. The lipophillic parts are to the right, i.e., sphingosine and stearic acid, and the hydrophillic moiety is to the left side of the photograph. The individual components are indicated by arrows. Black color indicates carbon atoms, grey indicates oxygen atoms, white indicates hydrogen atoms, and the stippling shows the nitrogen atoms. The numbers indicate the position of the bonding between the appropriate moieties.

least 100 ml of chloroform-methanol (CM) (2:1 v/v) or 20 vol CM whichever is larger (time to CM was approximately 5-8 sec). The flow sheet (Fig. 1) presents the steps involved in isolation of the lipids. This is essentially the Folch-Pi et al. procedure (7-9). Cerebrosides were isolated by the Radin column (10) and both cerebrosides and gangliosides were examined by thin layer chromatography (9,11,12) and silicic acid loaded paper chromatography (12a). Gangliosides were estimated by the Warren thiobarbituric acid procedure (13), the modified Svennerholm resorcinol method (14,15), or the Kishimoto and Radin stearic acid measurement (16). Radioactivity was measured with the Packard 314EX liquid scintillation spectrometer using toluene, dioxane-toluene, or impregnated filter paper in toluene. Scintillators employed were 2,5-diphenyloxazol (DPO) and *p*-bis 2-(5-phenyloxazolyl)-benzene (POPOP).

Other experimental procedures are described or referred to in the text, Tables and Figures. Post-mortem changes of the glycolipids have been evaluated and appear to be of no significance in these experiments (Burton et al., submitted for publication).

RESULTS AND DISCUSSION

The gangliosides are a family of sphingosine-containing glycolipids discovered in brain tissue by Klenk (17). They contain sphingosine (sph), stearic acid (ste) (85-95% of total fatty acids), glucose (glc), galactose (gal), N-acetyl neuraminic acid (neuNAc), and often contain N-acetyl galactosamine (galNAc). Their structures have been determined (18,19) and the structural studies recently reviewed (20-23). The structure of one of the dineuraminyl gangliosides is as follows:

```
Sph - glc
 |     |
Ste   gal - neuNAc
       |
      galNAc
       |
      gal - neuNAc
```

Other gangliosides have more or less neuNAc; in some forms they contain neuNAc (2→8) neuNAc bonds. The relative size of the hydrophillic oligosaccharide portion of a ganglioside compared to the lipophillic hydrocarbon moiety may be visualized by space-filling molecular models (Fig. 2). It is this amphipathic property which gives these glycolipids their relatively unique solubility properties and their ready ability to form micelles (24).

Age and Species

The brain of the late embryo and the neonate undergoes rapid and remarkable maturation. This is reflected by a number of parameters such as the increase in dry weight, formation of synapses, myelination and the increase in lipids including the glycolipids (22,25). The data represented in Figure 3 illustrates the accumulation of gangliosides in the

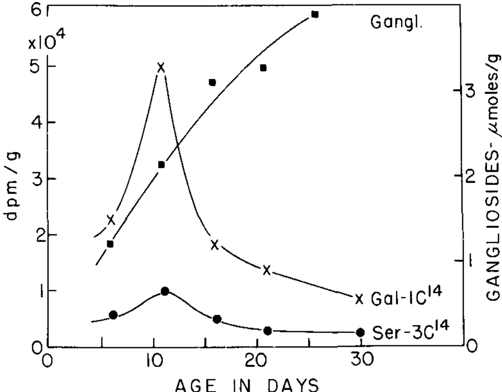

FIG. 3. Effect of age upon the incorporation of 1-^{14}C-D-galactose and 3-^{14}C-DL-serine into rat brain gangliosides (8). The serine (3 mc/mmole) and galactose (0.9 mc/mmole) were injected into rats (ip; 1.8 μc/g body weight). After 24 hr the rats were decapitated and their brain lipids extracted and processed as indicated by the flow sheet (Fig. 1) and Methods section.

developing brain of young rats. Adult concentrations of gangliosides are reached in rats at about 40-50 days of age. The fastest rate of incorporation of radioactive galactose into the oligosaccharide moiety of gangliosides and of radioactive serine into the sphingosine and fatty acid moieties occurs in rats of 11-12 days postpartum (8). This maximal rate of incorporation of the radioactive substrates into gangliosides occurs approximately two days prior to the inflection point of the ganglioside accumulation curve. This difference must be the reflection of the biosynthesis and deposition of gangliosides combined with the biosynthesis and turn-over of gangliosides. Similar data have been published for rat brain cerebrosides (26,27) and mouse brain cerebrosides (25,28).

Different species undergo myelination and maturation of their brains at different ages. This can be demonstrated in mice whose brains incorporate radioactive substrates into cerebrosides and gangliosides at an earlier age than rats (28). The guinea pig undergoes a large fraction of its brain myelination during fetal life. The different rates of lipid accumulation by the central nervous system of rats, mice and humans have been compared (22).

Specific Radioactivity and Pool Size

The administration of either glucose or galactose to young rats results in the same extent of incorporation into gangliosides by the intraperitoneal as by the intracerebral route. These neutral hexoses can cross the blood-brain barrier with ease, from either direction. Table I presents results which show a greater extent of incorporation into gangliosides by 1-^{14}C-galactose even though its specific activity is one-third that of the glucose. This can be interpreted to mean, (a) that the pool sizes of the body's glucose and galactose are not of equal size, and (b) that the glucose-galactose equilibrium via the nucleotides UDP-glc and UDP-gal is sufficiently slow, inhibited by galactose (or its phosphate ester) or saturated, or both, that preferential incorporation of galactose can occur. To some extent this relates to the metabolic demands for glucose which is much greater than for galactose.

The relative incorporation of radioactive precursors into ganglioside is illustrated by the data reported in Table II. It may be seen that glucosamine radioactivity is incorporated to a much greater extent than that of either the hexoses or the amino acids. This certainly reflects differences in pool sizes, in metabolic demands upon the pool, and in the directness of the routes of incorporation.

Galactose —ATP→ gal-1-P ⇄(UDPglc / glc-1-P) UDPgal

UDPgal ⇅ UDPglc

Glucose —ATP→ glc-6-P ⇄ glc-1-P ⇄(UTP / PP) UDPglc

glc-6-P ⇅ fru-6-P

fru-6-P ⇄(glutamine) glcNH$_2$-6-P ⇄(AcSCoA) glcNAc-6-P ⇄ glcNAc-1-P ⇄(UTP) UDPglcNAc

glcNAc-6-P → glcNAc → manNAc

UDPglcNAc → manNAc

UDPglcNAc ⇅ UDPgalNAc

manNAc —ATP→ manNAc-6-P —PEP→ neuNAc-9-P → neuNAc —+CTP→ CMPneuNAc

FIG. 4. Reactions involved in synthesis of the nucleotide sugar intermediates (22).

TABLE III

Per Cent Saturation of Precursor Pools[a]

	Isotope administered		
	$1\text{-}^{14}C$-glc	$1\text{-}^{14}c$-gal	$1\text{-}^{14}c\text{-glcNH}_2$
Precursor pool		Saturation, %	
N-acetyl neuraminic acid	3	10	100
N-acetyl galactosamine	5	36	92
galactose	5	25	-
glucose	6	24	-

[a]The per cent saturation was estimated by the procedure described in the text.

While a consideration of metabolic pathways will occur later, it will be useful to outline the pathways for these five isotopic precursors at this time. Figure 4 shows the interrelationships which exist for glucose, galactose, N-acetyl glucosamine, N-acetyl galactosamine and N-acetyl neuraminic acids (22). The incorporation of the carbohydrate units into the glycolipids is via the nucleotide sugars, i.e., UDP-gal, UDP-glc, UDP-galNAc, and CMP-neuNAc. The incorporation of radioactivity from $3\text{-}^{14}C$-serine is direct in the formation of sphingosine and indirect in forming acetic acid which will label the fatty acids. Incorporation from $4\text{-}^{14}C$-aspartic is totally nonspecific.

From the data presented in Table II and Figure 3 it is possible to estimate the extent of precursor pool saturation by the isotope labeled substance administered (8). The example which follows is for the incorporation of $1\text{-}^{14}C$-D-glucosamine; the hexoses can be analyzed in a similar manner.

The rate of incorporation of $1\text{-}^{14}C$-glucosamine radioactivity into gangliosides (a) and the increase in gangliosides (b) are:

(a) Ganglioside radioactivity, 65,600 (dpm/g ww brain/12 hr). (dpm, radioactive atoms decomposed per minute, ww, wet weight of tissue).

(b) Increase in total gangliosides, 0.018 (μmole/12 hr).

The specific radioactivity of the newly synthesized ganglioside can be obtained by dividing (a) by (b):

(c) New ganglioside, 3.6×10^6 (dpm/μmole).

The relative specific radioactivities of the galNAc and neuNAc isolated from the ganglioside fraction are:

(d) N-acetyl galactosamine, 4,250 (dpm/μmole).

(e) N-acetyl neuraminic acid, 4,660 (dpm/μmole).

The calculation of the actual specific radioactivity for galNAc is shown in (f); the brackets indicate *specific radioactivity, relative* (rel) or absolute (abs). The proportion of galNAc to neuNAc is given in Figure 8 for the dineuraminyl type of ganglioside.

(f) $$[\text{galNAc}]_{abs} = \frac{[\text{galNAc}]_{rel}}{[\text{galNAc}]_{rel} + 2\,[\text{neuNAc}]_{rel}} \times [\text{ganglioside}]$$

Therefore,

$$[\text{galNAc}]_{abs} = \frac{4250}{4250 + 2\,(4660)} \times 3.6 \times 10^6$$

$$= 1.1 \times 10^6 \text{ dpm/}\mu\text{mole}$$

Since the specific radioactivity of the $1\text{-}^{14}C$-glucosamine administered was 1.2×10^6 dpm/μmole, the per cent of saturation by the glcNAc of the amino sugar precursor pool is:

(g) $$\frac{[\text{galNAc}]_{abs}}{[\text{glcNH}_2]_{abs}} \times 100 = \frac{1.1 \times 10^6}{1.2 \times 10^6} \times 100 = 92\%$$

Similar calculations produce data which provide a relative description of the size of the precursor pools for the carbohydrate components of gangliosides (Table III).

It should be noted that the pool cannot be specifically defined from this data alone, since the pool may represent a dilution at any of the steps from the compound administered through the various intermediates to the product (Fig. 4).

Metabolic Activity of the Lipid

The concept advanced by Folin (28) in 1905 was that constituents of the body are either structural or energy producing compounds. The introduction of the modifying idea that body constituents exist in steady state equilibrium

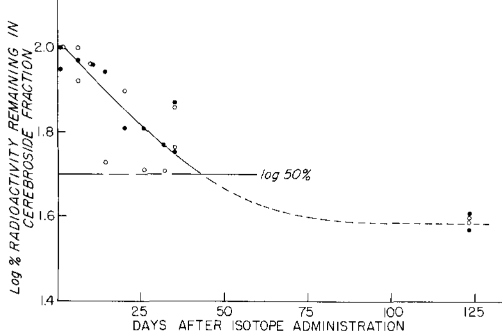

FIG. 5. Turnover of rat brain cerebrosides. 1-^{14}C-D-Glucose (open circles) and 1-^{14}C-D-galactose (closed circles) were administered ip to young rats, i.e., 13 and 14 days old. Fourteen days after the isotopic sugars were given, the initial groups of rats were killed and the cerebrosides isolated (Fig. 1). The radioactivity was determined and set equal to 100%. All subsequent radioactivity measurements were expressed as percentages of the initial radioactivity values.

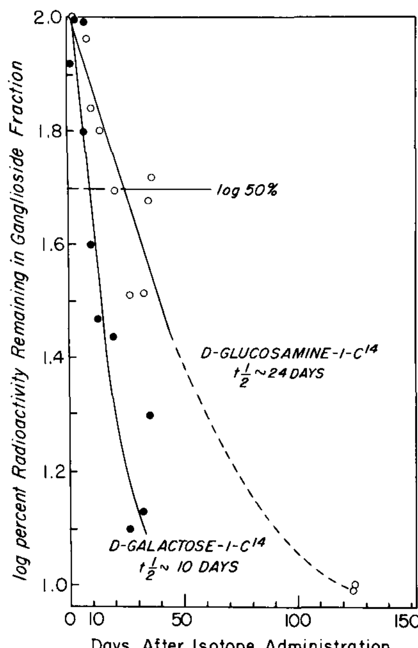

FIG. 7. Turnover of rat brain gangliosides (22). 1-^{14}C-D-Galactose (closed circles) and 1-^{14}C-D-glucosamine (open circles) were administered ip to young rats, i.e., 13 days old. Fourteen days after the isotopic substrates were given, the first group of rats were killed and their brain gangliosides isolated (Fig. 1). The radioactivity of this first group of rats was set equal to 100%. All subsequent measurements are expressed as percentages of the initial radioactivity values.

was due to the use of isotopes to measure these dynamic states (31). Subsequent work reviewed by Davison and Dobbing (31) indicates a continuum of turnover rates and a suggested functional classification of lipids (27,32). The functional classification divides the lipids into three classes based upon their turnover time, where $t_{1/2}$ is the time required to synthesize or catabolize one half of the total substance. The $t_{1/2}$ is often measured by the loss of radioactivity from the labeled compound.

Class 1. $t_{1/2} < 1$ week: Functions as fuel and energy reserves (triglycerides); in active synthesis as intermediates; in transport and related uses (e.g., phosphatidyl inositols in sympathetic neurones).

Class 2. 4 weeks $> t_{1/2} >$ 1 week: Functions as membrane components.

Class 3. $t_{1/2} >$ 4 weeks: Functions as structural components (e.g., myelin).

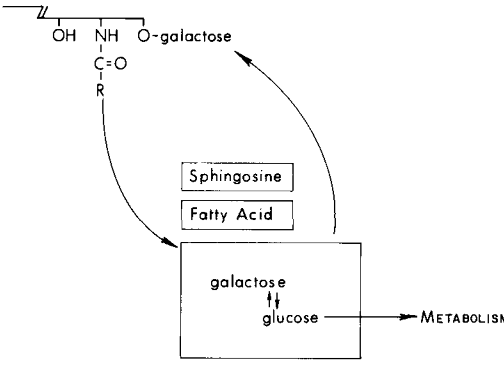

FIG. 6. Schematic pools of components involved in cerebrosides turnover (27).

It is apparent that the incorporation of precursor substances into structural components will be slower than incorporation into compounds undergoing rapid turnover. Two illustrations are cerebrosides, constituents of myelin, and gangliosides, components of neuronal membranes. The detailed description is given in the following section.

Metabolic Pathways

The Position of the Isotope Label in the Compound Synthesized. Cerebrosides are composed of sphingosine, a fatty acid in amide linkage, and a galactosyl residue attached to O^1 of sphingosine, i.e., gal($1 \rightarrow O^1$) ceramide. The fatty acid in the ceramide is long chain (centering around tetracosanoic and tetracosenoic acids) and over 50% are stable α-hydroxy fatty acids. The enzymatic pathway of synthesis requires the formation of UDP-gal (Fig. 4) and the transfer of the galactose to sphingosine followed by N-acylation (22,26,33,34). The intraperitoneal (ip) or intra-

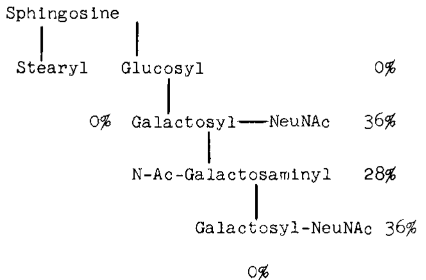

FIG. 8. Distribution of radioactivity incorporated into gangliosides from 1-^{14}C-D-glucosamine. The experimental details are as described in Figure 7 and the text. The isolated gangliosides were acid hydrolyzed step-wise and the hexoses and galactosamine separated by column and paper chromatography as previously reported (8).

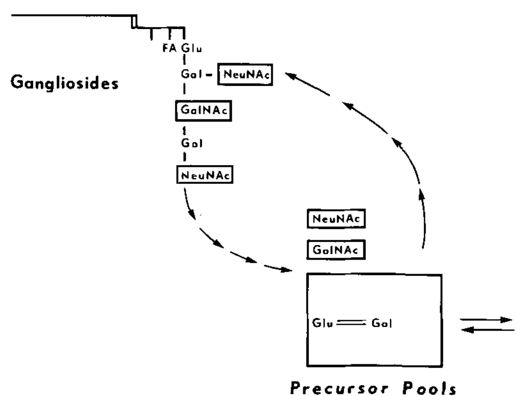

FIG. 9. Schematic pools of components involved in gangliosides turnover.

cerebral (ic) administration of 1-^{14}C-glucose or 1-^{14}C-galactose to young rats undergoing myelination results in the formation of radioactive cerebrosides. After the cerebrosides have been labeled, the subsequent loss of radioactivity can provide an estimation of the turnover of cerebrosides (i.e., the galactose moiety). Such an experiment is shown in Figure 5, where the $t_{1/2}$ (gal) is about 45-46 days. In addition, there appears to be a hard-core of radioactive cerebrosides with a very long $t_{1/2}$. In similar experiments, Davison et al. (35) and Smith and Ing (36) have evaluated the turnover of myelin cerebroside labeled in the fatty acid moiety by administering 1-^{14}C-sodium acetate. The fatty acid label indicated a $t_{1/2}$ of over one year. These studies can be interpreted in two ways, first, the structures embodying the cerebroside do so with the galactosyl residue exposed and able to be hydrolyzed and resynthesized, or second, the entire cerebroside molecule undergoes turnover with a $t_{1/2}$ of 45 days. In this later situation, the galactose would enter a large galactose-glucose pool which would dilute the radioactive carbohydrate, whereas the fatty acid and sphingosine enter small pools and the dilution of the ^{14}C-label is slight. The radioactive fatty acid and sphingosine would be reincorporated into the cerebroside. This is pictorially shown in Figure 6 and discussed in detail in Reference 27.

A similar study of gangliosides showed that both 1-^{14}C-D-galactose and 1-^{14}C-D-glucosamine can be readily incorporated. The subsequent loss of radioactivity indicated a $t_{1/2}$ (galNAc) = 24 days and a $t_{1/2}$ (gal) = 10 days [Fig. 7 and (22)]. Since the entire oligosaccharide is labeled by 1-^{14}C-galactose (8), the amino sugars must be removed from the oligosaccharide unit at least at the same rate as the hexoses. The best explanation for the apparent slower turnover of the amino sugar is that the ^{14}C introduced from the 1-^{14}C-glucosamine specifically labeled N-acetyl galactosamine and the N-acetylneuraminic acid as indicated in Figure 8 (8). Reference to Table III, indicates the saturation of the amino sugar pools by 1-^{14}C-glucosamine, only minimal saturation of the hexose pools by administered 1-^{14}C-glucose and about 25% saturation by 1-^{14}C-galactose. As illustrated by the schematic diagram in Figure 9, the entire oligosaccharide unit is hydrolyzed to the individual sugars and amino sugars. The 1-^{14}C-N-acetyl galactosamine and 1-^{14}C-N-acetyl neuraminic acid enter small pools and are reincorporated into gangliosides with only a small reduction of their specific radioactivities. Whereas the hexoses have labeled all components of the oligosaccharide unit, and with turnover, the 1-^{14}C-glucose and 1-^{14}C-galactose enter large pools and are quickly diluted. However, the amino sugars (labeled by the 1-^{14}C-hexose) enter small pools and are reincorporated. Thus, the $t_{1/2}$ (gal) of 10 days represents a value larger than the true turnover time since reincorporation of the amino sugars still occurs. While 1-^{14}C-acetate can label the fatty acid and sphingosine of the gangliosides, the rate is slow (compared to the carbohydrates) and different for the two lipid moieties (37). 3-^{14}C-Serine is rapidly metabolized and the isotope can be found in all parts of the gangliosides; howeever it is more selectively incorporated into the sphingosine moiety (8,22) as shown by Zabin and Mead (38), Sprinson and Coulon (39), and Brady et al. (42).

The Position of the Isotope Label Within the Precursor Compound Administered. The administration of 1-^{14}C-glucosamine results in

TABLE IV

N-Acetyl Glucosamine and Acetate Incorporation Into Rat Brain Lipids[a]

Route	Precursor	Neutral plus phospholipids mμmoles/g brain	Gangliosides μμmoles/g brain
ip	N-acetyl-^{3}H glucosamine	95	1
	sodium acetate-^{3}H	2,550	19
ic	N-acetyl-^{3}H glucosamine	1,610	358
	sodium acetate-^{3}H	12,400	73

[a]Data from Sena-Esteves and Burton, in preparation.

rather specific labeling of gangliosides (Fig. 8); only a small amount of radioactivity is found in the neutral and phospholipid fraction. However, when N-acetyl glucosamine, labeled in the acetyl moiety with tritium, is given ip to young rats, most of the incorporated radioactivity is found in the neutral plus phospholipid fraction of brain (Sena-Esteves and Burton, in preparation). Thus, the nonspecific incorporation of radioactivity must result from the removal of the ^{3}H-acetate from the parent compound and incorporation of the acetate itself. It should be noted that the control experiment in which ^{3}H-sodium acetate was given in the amount equivalent to the N-(^{3}H-acetyl)-glucosamine, resulted in a much greater incorporation into the neutral plus phospholipid fraction by factors of 10 to 250 times.

Route of Administration

The route of administration becomes important when the nature of the compound is such that a barrier exists between the compound and the organ being studied. In addition, the point of administration may route the compound to the organ of study before, for example, passing to the liver where detoxication or other reactions may occur (e.g., administration into the portal vein vs. the femoral artery).

It has been cited previously that glucose, galactose and glucosamine have free access to the brain since these are small molecular weight, neutral, water soluble compounds. However, the ip injection of N-(^{3}H-acetyl) glucosamine shows over 350-fold less incorporation into gangliosides than the ic route [Table IV; (Sena-Esteves and Burton, in preparation)]. This is most likely due to the liver deacylating the N-acetyl glucosamine. When the N-(^{3}H-acetyl) glucosamine is given by the intracerebral route, the radioactivity is found in the acetate moiety of the amino sugars of the gangliosides.

Nature of the Substrate

Molecular Size. The simple hexoses such as glucose and galactose have ready access to the central nervous system, however high molecular weight carbohydrates (e.g., inulin) cannot pass the blood brain barrier. Neutral lipids such as cerebrosides cannot enter the brain (41), however cerebrosides given ic can be metabolized (42).

Ionic or Nonionic Substrates. In general, ionic compounds cannot cross the blood brain barrier (Table IV, sodium acetate), whereas neutral compound can (shown by experiments with the ip and ic administration of glucose, galactose and glucosamine, cited earlier). Notable exceptions are compounds such as the amino acids, e.g., 3-^{14}C-serine, which are actively transported into the brain. 3-^{14}C-Serine is a direct precursor of sphingosine.

Water-Lipid Solubilities and Micelle Formation. In general, the more lipid-soluble and less water-soluble compounds can pass the blood brain barrier (e.g., diethyl ether vs. sodium acetate). This principle is certainly associated with the last subsection, which mentions ionic and nonionic compounds. The example cited in this subsection, for a neutral, lipid soluble, higher molecular weight substance not capable of crossing blood brain barrier, i.e., cerebrosides, is certainly an example of a compound which forms micelles and thereby assumes in effect a high molecular weight (24).

Influence of Hormones and Drugs

Little is known about the effects of hormones and drugs upon the glycolipids of the central nervous system. Unpublished data of Burton et al. have shown that high dosages of deoxyglucose and deoxygalactose will inhibit the incorporation of glucose and galactose into cerebrosides and gangliosides. It is not known if this is a true inhibition or if the deoxy sugars are competitively incorporated themselves.

Burton (unpublished) has not been able to inhibit the incorporation of galactose into cerebrosides by the prior administration of azauracil or other analogues of uridine compounds. It has been shown by Kanfer and Richards (43) that puromycin administered ic into the parietal region of the rat brain retards the formation of gangliosides as evidenced by the use of radioactive sugars. While the mechanism of puromycin action in this example is obscure, the authors suggest that ganglioside synthesis may require the concurrent synthesis of proteins.

However, other aspects of lipid metabolism are under the control of hormones and can be affected by drugs. For example, the plasma level of cholesterol can be reduced by thyroxin and estrogens (44,45) as well as by drugs such as Atromid-S, chlorophenoxyisobutyrate, clofibrate; Triparanol, 1-[(4-diethylaminoethoxy) phenyl]-1-(*p*-tolyl)-2-(*p*-chlorophenyl) ethanol; MER-29, etc. (46,47).

One of the more fascinating examples of hormones that affect lipid metabolism is the hormonal regulation of triglyceride lipolysis in adipose tissue. Hormones such as ACTH (adrenocorticotropic hormone), growth hormone, glucagon, etc., increase the rate of lipolysis (48). Of particular interest is the increased lipolysis due to norepinephrine released by stimulation of the sympathetic nerves innervating fat pads (49). Similarly, denervation, the use of ganglionic blocking drugs and drugs that interfere with norepinephrine actions all reduce the rate of triglyceride lipolysis (49,50).

Finally, experiments by Hokin et al. (51) and Hokin (52) have implicated the phospholipids in membrane activities by showing that neurohormones such as acetyl choline and norepinephrine will stimulate the incorporation of $^{32}PO_4^{3-}$ into phospholipids. A skillfully executed experiment by Larrabee (53) provides support for the studies of Hokin and has provided possible physiological relevance. In Larrabee's experiment the cervical sympathetic nerves were cut (preganglionic), one nerve was stimulated while the contralateral nerve was unstimulated and provided a control. The superior cervical ganglia from the stimulated side showed increase incorporation of $^{32}PO_4^{3-}$ into the phosphatidyl inositols.

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SHORT COMMUNICATIONS

Phospholipid Content of Human and Guinea Pig Muscle: Post-Mortem Changes and Variations With Muscle Composition

ABSTRACT

The phospholipid composition of human and guinea pig skeletal muscle was determined. Virtually no autolytic changes occurred in the first half hour post-mortem and after 12 hr only very small changes were detected. There were significant differences in the phospholipid composition of red and white muscle, especially in the diphosphatidyl glycerol (DPG) content, red muscle having over 50% more DPG than white muscle.

Red fibers of rat gastrocnemius have a higher total phospholipid content than white fibers (1). Some difference is to be expected since red muscle is richer in mitochondria than white muscle (2) and it has been demonstrated that diphosphatidyl glycerol is found predominantly in the mitochondria while sphingomyelin is absent from these structures (3).

It is essential to know what autolytic changes occur in the phospholipids of muscle since it is often necessary to use autopsy material when studying muscle function and metabolism.

Human rectus muscle was obtained as a biopsy specimen from three patients showing no muscle abnormalities. For the zero time control, 1 g of the material was frozen within 1 min of its removal and within 4 min of the original surface incision.

Young adult male guinea pigs were decapitated and the entire muscle from the hind limbs removed and freed of adipose tissue. In those experiments in which the red and white muscles were separated, the pink muscle which is a mixture of the two types of fibers was discarded. Muscle samples removed 30 min and 12 hr post-mortem were obtained from the left hind

TABLE I

Phospholipid Composition of Human Rectus Muscle: Autolysis Study[a]

Phospholipid[b]	Zero time	1 hr (25 C)	4 hr (25 C)	10 hr (25 C)	1.5 hr (25 C) 9 hr (4 C)
PC	55.7	55.0	56.7	51.7	55.1
PE	21.9	21.8	21.6	22.7	21.6
DPG	4.7	4.9	4.8	5.3	4.3
SPH	5.6	5.4	5.3	5.7	5.7
PS	4.2	4.4	4.2	4.9	4.8
PI	6.0	6.8	5.2	6.5	6.2
PA	0.3	0.3	0.4	0.5	0.5
LPE	0.0	0.1	0.2	0.4	0.1
LPC	0.5	0.4	0.7	1.2	0.6
Other	0.8	0.8	0.7	1.1	0.7
Origin	0.0	0.1	0.1	0.2	0.3
mg lipid/ g muscle	16.4	17.5	17.2	13.7	15.0
% PL of muscle	0.58	0.60	0.61	0.47	0.53

[a]Values expressed as per cent of total phospholipid. Heading denotes length of time at designated temperature for sample prior to freezing at -70 C. Maximum standard error for values over 10% ± 1.2, for values above 0.5% and below 10%, ± 0.5 and for values 0.5% or less, ± 0.1. Three samples were utilized and each samples was analyzed in quadruplicate.

[b]Abbreviations: PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; DPG, diphosphatidyl glycerol; SPH, sphingomyelin; PS, phosphatidyl serine; PI, phosphatidyl inositol; PA, phosphatidic acid; LPC, lysophosphatidyl choline; LPE, lysophosphatidyl ethanolamine; PL, phospholipid.

TABLE II

Phospholipid Composition of Guinea Pig Muscle[a]

Phospholipid	Per cent phospholipid			Milligram per gram muscle wet weight		
	0 hr (7)[b]	0.5 hr (5)	12 hr (5)	0 hr (7)	12 hr (5)	% Change
PC	50.0±1.7	50.4±0.5	48.3±0.8	5.15	4.54	-12
PE	27.7±1.0	26.6±0.1	27.0±0.7	2.85	2.54	-11
DPG	6.4±0.8	7.0±0.8	6.6±0.5	0.66	0.62	-6
SPH	4.2±0.6	4.0±0.2	4.5±0.2	0.43	0.42	-2
PS	3.6±0.4	3.6±0.2	3.4±0.2	0.37	0.32	-13
PI	7.9±0.6	8.0±0.5	6.9±0.3	0.81	0.65	-20
PA	0.2±0.1	0.2±0.1	0.3±0.1	0.02	0.03	+
LPC	0.3±0.0	0.4±0.1	1.6±0.3	0.03	0.15	+
LPE	0.0	Trace	0.3±0.3	0	0.03	+
Other	0.3±0.1	0.4±0.1	0.6±0.2	0.03	0.06	+
Orig.	0.0	Trace	0.7±0.1	---	---	+
mg lipid/g muscle	---	---	---	15.5±0.7	13.9±0.5	-10
mg PL/g muscle	---	---	---	10.5±0.3	9.4±0.3	-10
% Water	75.3	76.0	76.3			

[a]All values expressed ± standard error, see Table I for abbreviations. Autolysis allowed to preceed at 25 C for indicated period of time.

[b]Time post-mortem; number in parentheses is number of animals in sample.

limbs and control or zero time samples were obtained from the right hind limbs of the same animals. Water content was determined by drying tissue samples to constant weight. The samples were frozen in dry ice, pulverized, weighed and extracted with chloroform-methanol solvents according to the method of Rouser et al. (4). The phospholipids were separated by two-dimensional thin layer chromatography (chloroform-methanol-concentrated ammonium hydroxide, 65:25:4; chloroform-acetone-methanol-acetic acid-water, 5:2:1:1:0.5, charred, the spots aspirated and the phosphorous contents quantitated (5).

It is apparent from Table I that human muscle undergoes slow phospholipid autolysis. In the first 4 hr of autolytic action there is essentially no change in the phospholipid (PL)

TABLE III

Phospholipid Composition of Guinea Pig Red and White Muscle[a]

Phospholipid	Per cent phospholipid			Milligram per gram muscle wet weight		
	Red (5)[b]	White (5)[b]	P	Red (5)[b]	White (5)[b]	P
PC	47.1±0.9	52.0±0.5	>.05	5.98±0.17	5.15±0.07	<01
PE	29.9±0.9	26.9±0.3	<02	3.80±0.09	2.72±0.05	<01
DPG	7.3±0.2	4.5±0.4	<01	0.93±0.02	0.45±0.03	<01
SPH	4.4±0.4	4.7±0.2	>.05	0.56±0.05	0.47±0.03	<02
PS	3.7±0.2	3.9±0.4	>.05	0.47±0.02	0.39±0.01	<02
PI	6.9±0.1	7.4±0.2	>.05	0.88±0.03	0.74±0.08	>.05
PA	0.1	0.1	---	---	---	---
LPC	0.2	0.1	---	---	---	---
LPE	0.0	0.0	---	---	---	---
Other	0.2	0.2	---	---	---	---
Orig.	0.0	0.0	---	---	---	---
mg lipid/g muscle				22.4±1.9	19.6±1.4	>.05
mg PL/g muscle				12.7±0.4	10.1±0.5	<01
% Water	73.4	73.4				

[a]All values expressed ± standard error, see Table I for abbreviations.

[b]Number in parentheses is number of animals in sample.

ratios and no change in the absolute amounts of the PL. After 10 hr there is a change in both absolute and relative amounts of the PL. The largest decrease takes place in phosphatidyl choline (PC) with lysophosphatidyl ethanolamine (LPE), lysophosphatidyl choline (LPC) and others showing an increase. During this time there has been an increase in phosphorus-containing compounds that do not leave the origin. There appears to be a difference in the PL autolysis depending upon whether the specimen is kept at 25 C for 10 hr or at 25 C for 1.5 hr and then at 4 C for 9 hr. The slow autolysis of human skeletal muscle reported here is similar to the slow autolysis of aorta reported by Rouser and Solomon (6) and is in marked contrast to the high rate of lipid autolysis reported by Rouser et al. for liver (7).

The changes which occurred in PL content of guinea pig muscle during the two post-mortem periods are illustrated in Table II. No appreciable changes were found in the percent distribution of the individual PL when the samples were obtained 30 min after killing. When the post-mortem period was increased to 12 hr a definite decrease in the per cent of each of the PL except diphosphatidyl glycerol (DPG) and sphingomyelin was found. On the basis of milligram per gram wet weight of tissue, the content of the individual PL in the 12 hr samples ranged from 80-98% of the content in the zero time samples with the greatest decrease in phosphatidyl inositol. Both total lipid and PL contents decreased with a more pronounced decrease in the amount of total lipid signifying neutral lipid decrease is greater than PL decrease.

The per cent of DPG in white muscle is significantly lower than that in red (Table III). A lower per cent of PE is also suggested by the data but is not statistically significant. When the comparison is made on the basis of milligram per gram wet weight of tissue a lower content of PL, total lipid and of each of the individual PL in white muscle is evident and the differences are statistically significant for total PL, phosphatidyl choline and DPG.

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Chain Length Specificity of the Saturated Fatty Acyl Desaturase in Rat Liver Preparations

ABSTRACT

Chain length specificity of the saturated fatty acyl desaturase was studied in rat liver preparations using both de novo synthesized long chain fatty acids and equimolar mixtures of preformed fatty acids. Both experiments show that myristic acid is least desaturated and palmitic and stearic acids are desaturated in about equal amounts.

Aerobic desaturation, which produces a *cis* double bond at the 9,10-position of saturated fatty acids, is the predominant pathway for the biosynthesis of monoenoic acids in animal tissues. The most common monoenoic fatty acids in animal lipids are, in increasing order of prevalence: myristoleic, palmitoleic and oleic. Nakagawa and Uchiyama (1) have reported that palmitic acid is desaturated about twice as rapidly as stearic acid in mitochondria-free rat liver preparations. Very recently Johnson et al. (2) reported that in mitochondria-free hen liver

ratios and no change in the absolute amounts of the PL. After 10 hr there is a change in both absolute and relative amounts of the PL. The largest decrease takes place in phosphatidyl choline (PC) with lysophosphatidyl ethanolamine (LPE), lysophosphatidyl choline (LPC) and others showing an increase. During this time there has been an increase in phosphorus-containing compounds that do not leave the origin. There appears to be a difference in the PL autolysis depending upon whether the specimen is kept at 25 C for 10 hr or at 25 C for 1.5 hr and then at 4 C for 9 hr. The slow autolysis of human skeletal muscle reported here is similar to the slow autolysis of aorta reported by Rouser and Solomon (6) and is in marked contrast to the high rate of lipid autolysis reported by Rouser et al. for liver (7).

The changes which occurred in PL content of guinea pig muscle during the two post-mortem periods are illustrated in Table II. No appreciable changes were found in the percent distribution of the individual PL when the samples were obtained 30 min after killing. When the post-mortem period was increased to 12 hr a definite decrease in the per cent of each of the PL except diphosphatidyl glycerol (DPG) and sphingomyelin was found. On the basis of milligram per gram wet weight of tissue, the content of the individual PL in the 12 hr samples ranged from 80-98% of the content in the zero time samples with the greatest decrease in phosphatidyl inositol. Both total lipid and PL contents decreased with a more pronounced decrease in the amount of total lipid signifying neutral lipid decrease is greater than PL decrease.

The per cent of DPG in white muscle is significantly lower than that in red (Table III). A lower per cent of PE is also suggested by the data but is not statistically significant. When the comparison is made on the basis of milligram per gram wet weight of tissue a lower content of PL, total lipid and of each of the individual PL in white muscle is evident and the differences are statistically significant for total PL, phosphatidyl choline and DPG.

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Chain Length Specificity of the Saturated Fatty Acyl Desaturase in Rat Liver Preparations

ABSTRACT

Chain length specificity of the saturated fatty acyl desaturase was studied in rat liver preparations using both de novo synthesized long chain fatty acids and equimolar mixtures of preformed fatty acids. Both experiments show that myristic acid is least desaturated and palmitic and stearic acids are desaturated in about equal amounts.

Aerobic desaturation, which produces a *cis* double bond at the 9,10-position of saturated fatty acids, is the predominant pathway for the biosynthesis of monoenoic acids in animal tissues. The most common monoenoic fatty acids in animal lipids are, in increasing order of prevalence: myristoleic, palmitoleic and oleic. Nakagawa and Uchiyama (1) have reported that palmitic acid is desaturated about twice as rapidly as stearic acid in mitochondria-free rat liver preparations. Very recently Johnson et al. (2) reported that in mitochondria-free hen liver

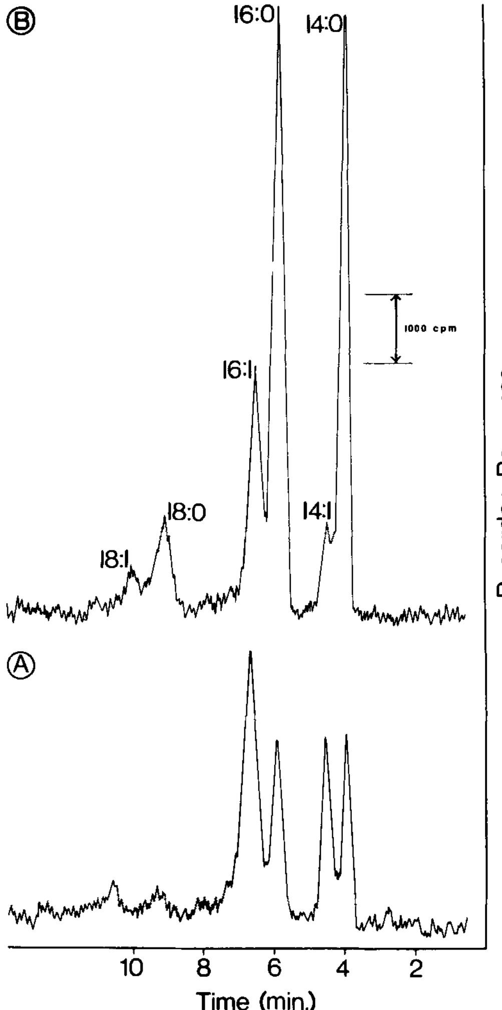

FIG. 1. (A) Radiochromatogram of the fatty acids synthesized from labeled acetate. Complete system contained 2 mg protein of rat liver high speed supernatant, 5 μmoles of ATP, 15 μmoles of reduced glutathione, 0.25 μmoles of CoA, 2.5 μmoles of $MnCl_2$, 10 μmoles of potassium bicarbonate, 10 μmoles of potassium citrate, 350 μmoles of potassium phosphate buffer pH 7.4 and 50 mμmoles of 1-^{14}C-acetate (1 x 10^6 cpm) in a total volume of 1.5 ml. Incubations were carried out in a Dubnoff metabolic shaker at 37 C for 30 min. One milligram of microsomal protein was added and the incubation continued for an additional 30 min. Incorporation of labeled acetate in long chain fatty acids was 10.2 mμmoles. Fatty acid methyl esters were analyzed by gas radiochromatography (column 9 ft x 1/4 in., packed with 15% diethyleneglycol succinate on Gaschrom HP and operated isothermally at 180 C with a flow of 40 ml/min of helium). (B) Radiochromatogram showing the desaturation of myristic, palmitic and stearic acids. Complete system contained mitochondria-free supernatant (5 mg microsomal protein), 10 μmoles of $MgCl_2$, 20 μmoles of ATP, 100 μmoles of phosphate buffer, 0.2 μmoles of CoA, 15 μmoles of reduced glutathione, 2 μmoles of NADPH and 750 mμmoles of K salts of 1-^{14}C-labeled fatty acids (250 mμmoles of 1-^{14}C-myristic, 250 mμmoles of 1-^{14}C-palmitic and 250 mμmoles of 1-^{14}C-stearic acid) in a total volume of 2 ml. Incubations were carried out at 37 C for 30 min. The methyl esters were analyzed by gas radiochromatography.

preparations, myristic acid is desaturated about 1.5 times as rapidly as palmitic or stearic acids. These in vitro studies do not correlate with the monoenoic acid concentrations in animal tissues. All the in vitro studies to date were carried out under conditions in which one fatty acid was tested at a time, and thus are not a measure of the possible competition between fatty acids of different chain lengths. In the studies reported here, long chain fatty acids were desaturated in competition with each other in rat liver preparations. In one experiment, fatty acids were synthesized de novo from 1-^{14}C-labeled acetate in rat liver high speed supernatant fraction and then desaturated by the addition of microsomes. In a second experiment, equimolar amounts of 1-^{14}C-myristic, 1-^{14}C-palmitic and 1-^{14}C-stearic acids were subjected to desaturation to their corresponding Δ^9 monoenes by a mitochondria-free rat liver preparation. It was found that myristic acid is the least desaturated and palmitic and stearic acids are desaturated in about equal amounts.

Adult, ca. 200 g, male rats, maintained at least a week on a fat-free diet, were used. The animals were killed by cervical fracture and the livers homogenized in 3 vol of 0.25 M sucrose. The homogenate was centrifuged at 1,000 x *g* for 15 min. The resultant supernatant was then centrifuged at 11,000 x *g* for 20 min. The mitochondria-free supernatant so obtained was used for the desaturase experiments. High speed supernatant was prepared by centrifugation of the mitochondria-free supernatant at 104,000 x *g* and the pellet so obtained was used as microsomes. The incubation conditions are described under Figure 1. Enzyme action was stopped by the addition of 5 ml of chloroform-methanol (2:1 v/v) and the lipids were extracted after acidification with dilute HCl according to the procedure of Folch et al. (3). The fatty acid methyl esters of the extracted lipids were prepared by refluxing with a solution of 5% boron trifluoride in methanol for 5 min. The methyl esters were analyzed by gas radiochromatography using an Aerograph A-90-P gas chromatograph connected to a Nuclear-Chicago Biospan proportional counter, and the signal fed to a 10 mv strip chart recorder.

Figure 1A shows the fatty acid activity

spectrum obtained when fatty acids were synthesized from labeled acetate for 30 min by the rat liver high speed supernatant fraction and then desaturated by the addition of microsomal protein. The extent of desaturation of myristic acid was less than that of palmitic acid, showing that of the two, palmitic acid is a preferred substrate. That the desaturase enzyme for myristic and palmitic acid is located in microsomes is evidenced from the observation that no myristoleic or palmitoleic acids were produced when the high speed supernatant was used alone.

Figure 1B shows the relative degrees of desaturation of myristic, palmitic, and stearic acids when all three were allowed to compete for the desaturase in equimolar proportions. In this case, when 250 mμmoles of each acid was used, myristic acid was also desaturated less than palmitic (39 vs. 70 mμmoles) whereas stearic acid was desaturated slightly more (75 mμmoles) than palmitic acid. Thus the desaturation of myristic and palmitic acids in rat liver are in reverse order to that of hen liver preparations, as reported by Johnson et al. (2). Also, unlike the observation of Nakagawa and Uchiyama (1), the amount of palmitic acid desaturated is no higher than that of stearic acid. Holloway et al. (4) have also found that slightly more stearoyl-CoA is desaturated than palmitoyl-CoA in rat liver microsomes. A significantly higher rate of desaturation of stearic acid than palmitic acid was observed by Elovson (5) in in vivo experiments with rats. Thus there are discrepancies in the reported desaturase activities for palmitic and stearic acids as determined in vitro and in vivo.

Under in vivo conditions, palmitic acid is utilized for at least three important biosynthetic processes: elongation to stearic acid, desaturation to palmitoleic acid, and esterification to glycerides. The rate of esterification of stearic acid by rat liver microsomes (6) is very low in comparison to that of palmitic acid. Further, the elongation of stearic acid is not a very active pathway in rat liver in comparison to the very active elongation of palmitic acid to stearic acid. These differences in the rates of utilization of the two saturated fatty acids could produce differences in their effective concentrations in vivo, which may be a reason for the apparent anomaly in the desaturase activities determined in vitro.

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Separation of the C_{20} Unsaturated Fatty Acids From Rapeseed Oil by Countercurrent Distribution

ABSTRACT

A C_{20} fatty acid mixture (5.9 g), which was obtained from fractional distillation of 95 g methyl esters of rapeseed oil, was subjected to countercurrent distribution. This procedure yielded 4.5 g eicosenoic acid (20:1), 0.4 g eicosadienoic acid (20:2), and 0.04 g eicosatrienoic acid (20:3), 4.7%, .42% and .04%, respectively, of the total methyl esters.

A number of procedures have been used for the isolation and purification of unsaturated fatty acids from seed oils, e.g., mercury or urea adduct formation of the unsaturated acids and successive column chromatography, low temperature crystallization, countercurrent distribution, thin layer and gas chromatography. In the present paper a procedure based on fractional distillation and countercurrent distribution is described for the isolation of the unsaturated C_{20} fatty acids of rapeseed oil. The

spectrum obtained when fatty acids were synthesized from labeled acetate for 30 min by the rat liver high speed supernatant fraction and then desaturated by the addition of microsomal protein. The extent of desaturation of myristic acid was less than that of palmitic acid, showing that of the two, palmitic acid is a preferred substrate. That the desaturase enzyme for myristic and palmitic acid is located in microsomes is evidenced from the observation that no myristoleic or palmitoleic acids were produced when the high speed supernatant was used alone.

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TABLE I

Fatty Acid Composition of the Segments Under the Countercurrent Distribution Curve

	20		20:1		20:2		20:3		C_{18} acids		Impurities
Segment	g	%	g	%	g	%	g	%	g	%	%
I	0.18	30	0.3[a]	50							20
II			4.14[a]	99					0.02	0.5	
III					0.4	76			0.13	22	
IV			0.012	8	0.01	6	0.04	25	0.09	60	

[a]These two values and the corresponding areas under the distribution curve cannot be compaired, since most of the 20:1 acid found in segment I stems probably from tubes 95-110, a part of the main 20:1 peak.

obtained acids, 20:1, 20:2 and 20:3, of which the last one was present only in very small amounts, are known to occur in various Cruciferae seed oils (1-5; also Haeffner), and their structures have also been determined (1,3,6).

Rapeseed oil, which was purchased from the Hospital-and Wohlfahrts-apotheke in Cologne, Germany, was used in these experiments. The standard values of the oil, its long chain fatty acid and lipid composition have been determined (6). The distillation procedure has been described elsewhere (6).

An automatic H.O. Post apparatus from the Scientific Instruments Company, Inc., N.Y., was used, consisting of 500 elements with volumes of 10 ml for each lower and upper phase. The solvent system *n*-heptane-methanol-acetic acid-acetonitrile (3:1:1:1), as described by Ahrens and Craig (7) was used for the fatty acid separation. The distribution was started by adding upper phase to each element in a stepwise manner, mixing both phases by shaking (20 times), allowing the phases to separate for 2 min, and then transferring the upper phase to the next element. After all 500 elements were filled with upper phase, the distribution process was continued by decanting the upper phases into a fraction collector; two transfers were combined per tube. One milliliter of every fourth tube was taken for bromine value determinations (8). The fatty acid distribution pattern was obtained by plotting the consumption of 1/10 N bromine solution versus the tube number.

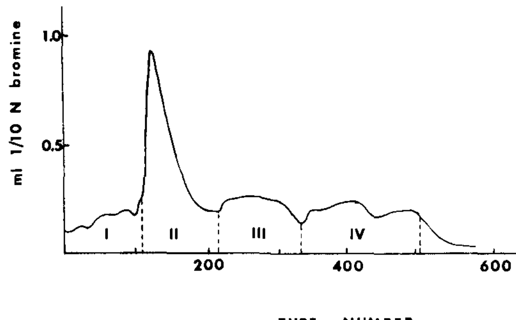

FIG. 1. Countercurrent distribution profile of the C_{20} fatty acids of rapeseed oil. An automatic H.O. Post apparatus with 500 elements of each 10 ml lower and upper phase was used. Solvent system: *n*-heptane-methanol-acetic acid-acetonitrile (3:1:1:1). The distribution of the fatty acids was followed by measuring the consumption of 1/10 N bromine of an aliquot of every fourth tube.

An amount of 95 g methyl esters of rapeseed oil was subjected in two batches to a fractional distillation (No. 1) to yield 19.1 g material enriched in C_{20} acids. This mixture was again distilled (No. 2) to give 4.4 g of more than 90% pure C_{20} acids (by gas liquid chromatography analysis). Some of the side fractions (9.4 g) of distillation No. 2 were subjected to a further distillation (No. 3) to yield 1.9 g of almost pure (GLC) C_{20} acids. The combined methyl esters of distillation No. 2 (4.4 g) and No. 3 (1.9 g) were saponified to give 5.9 g of free fatty acids, which were then separated by countercurrent distribution according to their degree of unsaturation. After dissolving the acids in 30 ml of each lower and upper phase of the solvent system, the solution was filled into the elements 4 to 6, while all other elements were filled with lower phase (10 ml of each). The elements 16 to 30 were filled with additional 10 ml of lower and upper phase in an alternative way for the purpose of further equilibration of the two phases during the distribution process. The result of the countercurrent distribution, operated at a constant temperature of 20 C, is shown in Figure 1. The distribution curve was divided into four segments according to its profile. The tubes of each segment were combined, the solvent system evaporated (traces were removed by putting the samples into a dessicator under high vacuum), and the amounts of fatty acids and their composition were determined by weighing and gas chromatographic analysis (Table I).

As can be seen from Table I, the segments II, III and IV contained the desired unsaturated C_{20} fatty acids. The total yield after the countercurrent distribution was 5.5 g (94%), of which 4.9 g (88%) were C_{20} acids. The 20:3 amounts to only 0.04% of the rapeseed oil. The C_{20} fatty acids can be further purified by silver nitrate thin layer chromatography.

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[Received September 3, 1969]

Graphic Presentation of Computer-Derived Schlieren Lipoprotein Data[1]

ABSTRACT

Computer produced graphs of lipoprotein spectra visually present a large amount of information and greatly facilitate error detection. Data derived from schlieren patterns in the analytical ultracentrifuge are fully corrected to standard conditions allowing valid comparison of serum lipoprotein concentrations and profiles. The computer program is flexible enough to provide appropriate representations for many types of spectra and several possibilities for comparison of data.

The computer analysis of schlieren photographs of serum lipoprotein distributions has been described in detail by Ewing et al. (1). This computer program yields numeric output fully corrected to standard conditions in terms of lipoprotein concentration of the original sample, allowing valid comparisons to be made from sample to sample. In addition, an analytic ultracentrifuge data acquisition system (2) provides the accumulated value of $k\int\omega^2(t)dt$ for the mean time of each schlieren photograph. These data permit more accurate schlieren analysis of very low-density lipoproteins, where the precise equivalent up-to-speed centrifugation during the acceleration phase of the run is required. In order to extend and improve the computer analysis of lipoprotein distributions, a program has been written to present these data in graphic form, allowing rapid visual evaluation, comparison of samples and error detection.

The computer input data consist of lipoprotein concentrations for a series of standard flotation rate (S_f or F rate) intervals in the card format produced by the schlieren analysis program (1). Although the low-density analysis is presented here in terms of S_f values, the program is similarly used for high-density graphic analysis or for single frame analysis at any time, including the acceleration frame. The results from any frame may be plotted at any desired up-to-speed time value for ease of comparing data. The plotting program converts these S_f rates into appropriate linear dimensions and lipoprotein concentrations into areas equivalent to those of the corrected schlieren patterns, routinely presented at three times the concentration of serum. It then calculates frequent points along a best fit curve through the resulting histogram, from which the graph is subsequently plotted on a physical device. At the same time the program sums lipoprotein concentrations of the total pattern and specified subfractions (S_f° 0-12, S_f° 12-20, S_f° 20-100 and S_f° 100-400 in the standard low-density run), as well as determining the S_f rate where

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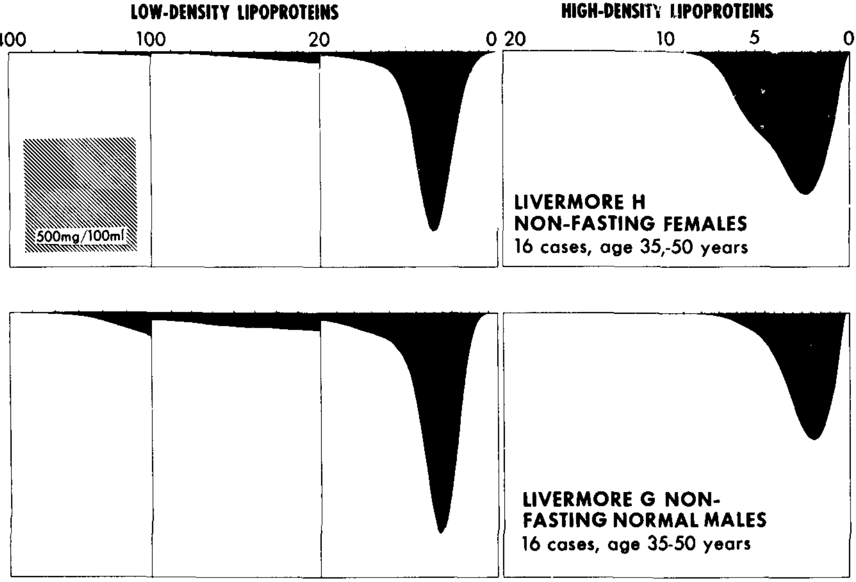

FIG. 1. Typical low- and high-density lipoprotein spectra obtained from a Cal-Comp plotter. The areas under the curves were shaded to provide profiles for visual comparison.

the maximum concentration occurs. The final steps are to draw a rectangle around each frame, with tick marks and frame boundaries which normally correspond to the template upon which the schlieren pattern was initially traced, label the plot and print standard interval lipoprotein concentration values. It is possible to plot several patterns on one frame, or to plot the mean or standard deviation, or both, of a group of cases.

At the present time we normally use an S_f rate scale identical to that of the 0′, 6′ and 30′ up-to-speed (52,640 rpm) frame template used for tracing the enlarged schlieren patterns (Fig. 1). However, the montage of three frames (taken at different times required to include all the low-density lipoproteins) results in a discontinuous curve (Fig. 1 and 2a) representing what is essentially a continuous spectrum. In Figure 2b, a logarithmic scale has been chosen to achieve continuity across the entire S_f 0-400 lipoprotein spectra, avoiding discontinuities at the frame boundaries of S_f 20 and S_f 100. To accomplish this a variable k log (S_f + 5) is used to avoid negative values while preserving the relative widths represented by the individual schlieren frames. The usefulness of a similar logarithmic scale, which has been applied to β lipoprotein fractions (S_f 0-12), has been discussed earlier (3). This detailed theoretical analysis of distribution functions also includes corrections for both diffusion and concentration dependence. Another potentially useful scale using the square root of S_f rate would yield a scale nearly linear in particle diameter.

Although patterns may be compared visually or by plotting them on a single graph, small but significant differences still may be hard to detect. A modified version of the program subtracts the first pattern of a series from each subsequent one and plots the difference at specified magnification (Fig. 2d).

Figure 1 was drawn using a Cal-Comp plotter, which moves a pen about a segment of chart paper under computer control. To present a visually effective profile, the pattern has been shaded, producing a high quality figure. Since the Cal-Comp plotter is accurate to 1/100 in., it is therefore also useful in drawing master templates for tracing schlieren patterns from the film. However, the main disadvantage of the Cal-Comp plotter is that it is a relatively slow process requiring extensive operator intervention; thus, plotting on it, especially in any quantity, is expensive and subject to delays.

In contrast, the plots in Figure 2 were traced electronically on a cathode ray tube (CRT) and photographed on 35 mm film. This CRT system lacks the resolution and absolute dimensions given by the Cal-Comp, but it is rapid and requires only occasional removal of film. Rough 8 1/2 x 11 in. prints in any quantity usually are available on an overnight basis.

Logically there is one plotting program with an extra input routine to handle population means. It is written in Fortran IV for the

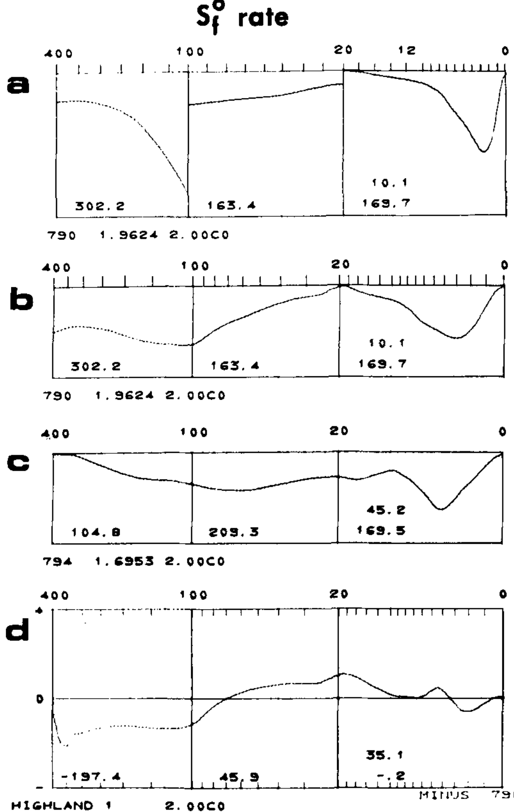

FIG. 2. Cathode ray tube plots of low-density lipoprotein spectra. Traditional discontinuous plot (a) and a continuous log plot (b) of a fasting subject and the same subject 24 hr after a fat tolerance test (c); d shows b subtracted from c. Note significantly different up-to-speed equivalent (1.9624 and 1.6953 min) of the S_f 100-400 time frame for run 790 and 794, respectively.

CDC-6600 computer using system plotting routines of the Lawrence Radiation Laboratory Berkeley computer center. [Copies of the source program listings, card decks and documentation for the schlieren analysis program (1) and for our plotting program are available on request.] The present program calls a package of local subroutines, which in turn create a plot file only interpretable by our own local system programs. Thus, conversion to another computer system, with different local subroutines and plotting facilities, would necessarily involve appropriately re-writing the program. For reasons of technical efficiency, separate but similar versions for Cal-Comp and CRT plotting are used. The program itself is quite flexible. After selecting the proper version, one specifies by optional control cards such requirements as input intervals, number of curves to plot on one frame, template dimensions, magnification factors, positions of S_f rate labels, frame boundaries and concentration sub-regions. Standard values or procedures are assumed unless overridden by special options.

This program was conceived as a means for preparing data, such as Figure 1, for publication in place of the tediously hand-drawn graphs previously required (1). Soon it became obvious that one had such a clear and immediate visual presentation that it would be valuable to have for all schlieren data. Emphasis was then given to ease of use and conversion to the CRT medium. The program now runs following computer analysis of the schlieren tracing as part of the same job, i.e., without human intervention, so that plots are routinely obtained.

Many errors are now caught which before graphic presentation undoubtedly went undetected. Our data reduction from the schlieren film to punch-card numbers involves two technicians and two keypunch operators. To illustrate a typical error, suppose some interval on the tracing is 1.36 cm but is read by the computer as 1.86 cm. This error does not stand out at all in a column of figures, however, on the plot it immediately is detected as a bump on an otherwise smooth curve.

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Two Dimensional Thin Layer Chromatographic Separation of Polar Lipids and Determination of Phospholipids by Phosphorus Analysis of Spots

ABSTRACT

Separation of polar lipids by two-dimensional thin layer chromatography providing resolution of all the lipid classes commonly encountered in animal cells and a sensitive, rapid, reproducible procedure for determination of phospholipids by phosphorus analysis of spots are described. Values obtained for brain and mitochondrial inner membrane phospholipids are presented.

Two-dimensional thin layer chromatography (TLC) has been shown to provide good resolution of polar lipids and determination of phosphorus in spots by aspiration and color development without prior elution from TLC adsorbent was found to be a rapid and accurate procedure for determination of molar amounts of phospholipids (1). This report presents a modified TLC procedure providing better resolution of polar lipids, a more accurate and sensitive procedure for determination of phosphorus in TLC spots, and typical analytical data for phospholipids of adult human brain and beef kidney mitochondrial inner membrane.

Silica Gel H (Merck) as a slurry of 20 g in 65 ml of water containing either 0.50 or 1.50 g of magnesium acetate was spread with a 0.25 mm fixed distance spreader and the plates air-dried. Just before use, the plates were placed in a TLC chamber equipped with a cardboard top through which nitrogen of 50% humidity was passed for 20 min. The plates were then transferred for sample application to another humidity controlled chamber constructed of clear plastic supported by a metal rod frame. The desired humidity was obtained by mixing the required amount of dry nitrogen with nitrogen saturated with water by bubbling through a water tower heated electrically at the base.

Immediately after spotting, plates were transferred to TLC chambers (11 3/4 in. long by 2 3/4 in wide by 10 1/2 in. high) lined on all sides with paper saturated with the chromatographic solvent (about 200 ml added per chamber) by tilting the chamber first to one side and then the other about 30 min before use. Plates spread with 1.50 g of magnesium acetate were developed in the first dimension with chloroform-methanol-28% aqueous ammonia 65:25:5, chromatograms were dried for about 10 min in a TLC chamber flushed with nitrogen and then developed in the second dimension with chloroform-acetone-methanol-acetic acid-water 3:4:1:1:0.5. Plates spread with 0.50 g magnesium acetate were developed with the same solvent mixtures in the proportions 65:35:5 and 5:2:1:1:0.5. Plates were air-dried for a few minutes, sprayed with a char reagent composed of 3 vol of 37% formaldehyde and 97 vol of 98% sulfuric acid and heated at 180 C for 30 min. Spots were circled and numbered and each chromatogram photographed.

Spots were aspirated as described previously (1) except that ignition tubes (16 mm o.d. x 125 mm) were substituted for Kjeldahl flasks, the paper filters were discarded in favor of small circles of glass fiber paper (No. 994, H. Reeve Angel Co., Clifton, N.J.) first washed with freshly distilled constant-boiling hydro-

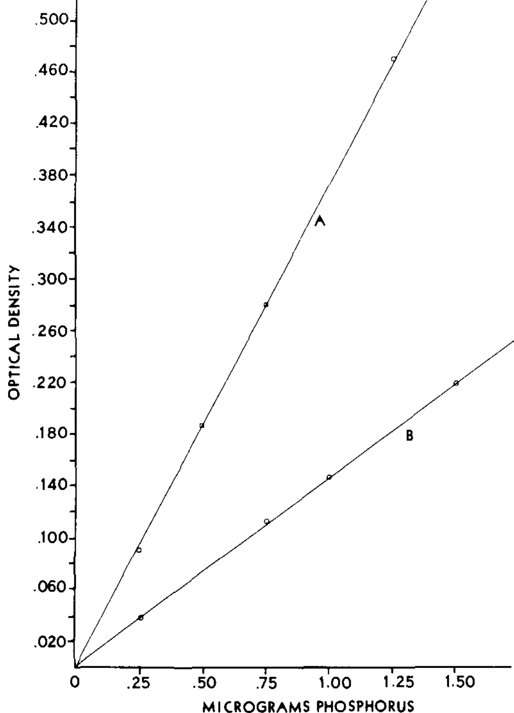

FIG. 1. Typical lines passing through the origin obtained with inorganic phosphate standards by the procedure described in the text for the smaller (A) and larger (B) reagent volumes.

TABLE I

Values Obtained in Quadruplicate Determinations of Different Amounts of Phosphorus

	O.D.[a]	Blank[b]	Corrected O.D.	Ave. μg P[c]	Per cent total P	Std. Dev.[d]
Phosphatidyl ethanolamine	0.588	0.006	0.582	3.751	31.26	±0.47
	0.581	0.006	0.575			
	0.568	0.006	0.562			
	0.572	0.006	0.568			
Phosphatidyl inositol	0.151	0.013	0.138	0.325	2.08	±0.13
	0.141	0.013	0.128			
	0.143	0.013	0.130			
	0.136	0.013	0.123			
Lysophosphatidyl choline	0.035	0.013	0.022	0.044	0.34	±0.05
	0.033	0.013	0.020			
	0.030	0.013	0.017			
	0.031	0.013	0.018			

[a]Optical density values for three brain lipids.

[b]The total blank including TLC adsorbent. Reagent blanks (excluding adsorbent) are very low or zero with carefully acid-washed glassware and the purest reagents. Glass fiber paper washed with hydrochloric acid and the formaldehyde-sulfuric acid spray do not contribute to blank values.

[c]The lower limit for positive detection of phosphorus is about 0.003 μg.

[d]Maximum reproducibility is obtained when: (a) the amount of TLC adsorbent in each tube is kept constant, the desired total amount being obtained by aspiration of additional adsorbent from a blank plate for spots moving close together; (b) variable loss of perchloric acid is prevented by neutralization with hydrochloric acid of silicate in the adsorbent and avoidance of loss of fumes during digestion; (c) before aspiration of small spots the aspirator is carefully cleaned by aspiration from a blank plate of adsorbent which is then discarded; (d) spurious color production after addition of ascorbic acid is prevented by immediate and thorough mixing; (e) accidental inclusion of sedimented TLC adsorbent is prevented by centrifugation in relatively small bore tubes; and (f) OD readings are reproduced to ± 0.001 units.

chloric acid and then water, dried and cut to uniform size with a paper punch. Water (0.5 ml) was added to each tube to serve as a liquid trap for the adsorbent. The glass fiber filter was held in place by suction during aspiration and allowed to fall into the test tube by release of vacuum after aspiration of a spot was complete. Large spots (total area 4.5 cm^2) were aspirated first and the aspirator carefully cleaned by aspirating adsorbent from a blank plate before aspiration of small spots (total area 2.25 cm^2). After aspiration, 0.5 ml of concentrated hydrochloric acid was added to each tube for neutralization of silicate, and water and excess acid removed by placing the tubes for 10 min in an electrically heated metal block (heating base No. 2127-A, Model 100-300 C; tube blocks No. 2127-B-2; Hallikainen Instruments, Richmond, Calif.) maintained at 180 C.

The procedure for large spots (4.5 cm^2) was then as follows. Perchloric acid (0.65 ml, 70% triple distilled into Vycor, G. Frederick Smith Chemical Co., Columbus, Ohio) was added and the lipid digested by heating for 20 min at 180 C in the heated metal block with the upper one half of each tube extending outside of the block to prevent loss of perchloric acid fumes. After cooling, reagents added in order were: water (3.30 ml), 2.5% ammonium molybdate (0.50 ml), and 10% ascorbic acid solution (0.50 ml). The additions were made rapidly and accurately with RePipettes (Lab Industries, Berkeley, Calif.) and the tube contents were mixed after each addition with a vibrator mixer (De Luxe Mixer, S8220, Scientific Products, Evanston, Ill.). Small spots (2.25 cm^2) were treated similarly except that 0.26 ml of perchloric acid was used for digestion and 0.92 ml of water, 0.40 ml of 1.25% ammonium molybdate, and 0.40 ml of 5% ascorbic acid solution were added for color development.

Color was developed by heating for 5 min in a boiling water bath (Renwall electric water bath, No. 3025, Scientific Products, Evanston, Ill.). Adsorbent was sedimented by brief centrifugation, the solutions from the smaller reagent volumes first being transferred to 3 ml centrifuge tubes. After centrifugation, the solutions were transferred to cuvettes with Pasteur pipettes with care not to disturb the sedimented adsorbent, and the color intensity was determined at 797 mμ in a Gilford Model 240 spectrophotometer equipped with a digital readout. Both reagent blanks and reagent-TLC adsorbent blanks were carried through the procedure. The optical density values were read against a water blank and the reagent-TLC adsorbent blank was subtracted to give the cor-

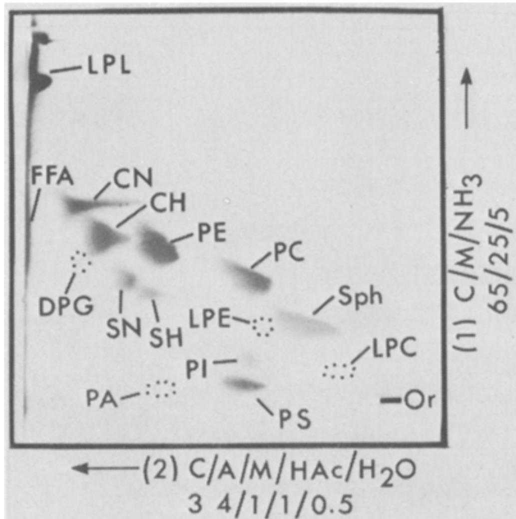

FIG. 2. Two-dimensional TLC of normal human (23 year old male) whole brain lipids. Lipid (800 μg) was applied at the lower right (see text for further details). Abbreviations and percentage of the total phosphorus ± standard deviation: LPL, less polar lipid (cholesterol, triglyceride, etc.); CN and CH, cerebroside with normal and hydroxy fatty acids; SN and SH, sulfatide with normal and hydroxy fatty acids; PE, phosphatidyl ethanolamine (31.3 ± 0.5); PC, phosphatidyl choline (29.2 ± 0.5); PS, phosphatidyl serine (15.4 ± 0.5); PI, phosphatidyl inositol (2.1 ± 0.1); LPE, lysophosphatidyl ethanolamine (1.14 ± 0.06); PA, phosphatidic acid (0.50 ± 0.02); Sph, sphingomyelin (13.2 ± 0.1); DPG, diphosphatidyl glycerol (0.9 ± 0.07). Total phosphorus recovery 97.5% (including areas devoid of spots but containing phosphorus).

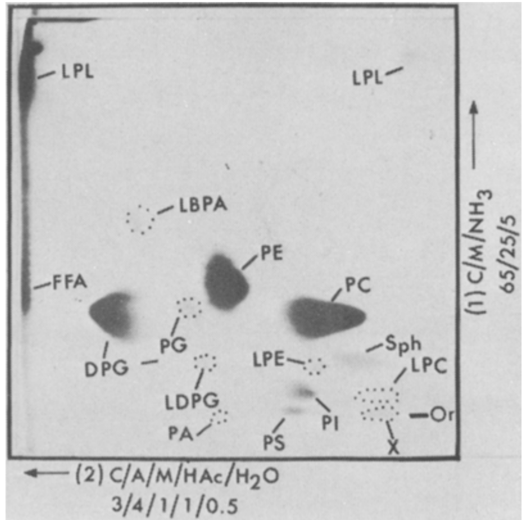

FIG. 3. Beef kidney mitochondrial inner membrane lipid class separation as described in the text. Abbreviations (as for Fig. 2 except as noted) and percentage of the total phosphorus ± standard deviation: PE (35.7 ± 0.3); PC (35.0 ± 0.4); PS (1.36 ± 0.3); PI (3.40 ± 0.07); LPE (0.98 ± 0.12); PA (0.22 ± 0.04); Sph (2.43 ± 0.02); DPG (15.8 ± 0.06); LPC, lysophosphatidyl choline (0.8 ± 0.07); PG, phosphatidyl glycerol (1.17 ± 0.04); LDPG, lysodiphosphatidyl glycerol (0.71 ± 0.05); LBPA, lysobisphosphatidic acid (0.58 ± 0.02). Total phosphorus recovery 99.4%. Note that some of the less polar lipid (LPL) is insoluble in the solvent mixture used in the second dimension and does not migrate.

rected optical densities. The latter were converted to micrograms of phosphorus by multiplication by a factor which was determined from known amounts of disodium hydrogen phosphate spotted onto TLC plates and, except for chromatography, treated in the same manner as the sample spots. Phosphorus recovery was determined by analysis of eight separate 100-200 μg applications of the lipid mixture to a TLC plate that was not developed with solvent but which was otherwise treated in the same manner as the phosphate standards and TLC spots.

Typical results with inorganic phosphate standards are shown in Figure 1 and typical values obtained for various amounts of phosphorus are shown in Table I. The improved TLC separation of polar lipids and the reproducibility of quadruplicate determinations of phospholipids are shown for brain (Fig. 2) and a beef kidney mitochondrial inner membrane preparation (Fig. 3). In both cases, complete resolution of all detectable components was obtained. This was judged by TLC of diethylaminoethyl cellulose column fractions which also disclosed the presence of trace components below the level of detectability by two-dimensional TLC of the total lipid mixture.

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Pancreatic Lipolysis of Some Brominated Vegetable Oils

ABSTRACT

Pancreatic lipolysis of several commercially used brominated vegetable oils has shown that although hydrolysis proceeds more slowly, these oils are degraded in a similar way to the common vegetable oils.

A recent report (1) on the possible toxicity of brominated vegetable oils has prompted us to investigate their degradation by pancreatic lipase. As these oils are extensively used in the soft drink industry and consequently may be consumed in quantity, it was considered important to obtain this information.

Brominated olive, sesame and cottonseed oils were obtained from Abbott Laboratories, and brominated corn oil was prepared in the laboratory (2). Pancreatic lipase (27.5 units/mg) was purchased from the Worthington Biochemical Corporation.

Lipolyses were carried out using the semimicro technique of Luddy et al. (3) and the hydrolysis products separated by thin layer chromatography. Triglycerides, triglycerides recovered from hydrolysates, and monoglycerides were analyzed by gas liquid chromatography (GLC) after reaction with sodium methoxide-methanol as described by Conacher et al. (4). Free fatty acids were analyzed in a similar fashion after esterification with sulfuric acid-methanol and were quantitated by the addition of methyl pentadecanoate as internal standard. GLC separations were carried out on a Varian 1740-10 instrument equipped with dual columns and twin flame detectors. Columns, 3 ft x 1/8 in. stainless steel, were packed with 3% JXR on Anakrom ABS 80-90 mesh, and programmed from 150 to 275 C at 8 C/min. with a helium flow of 35 ml/min. The injection point was held at 250 C and the detector at 280 C. Weight response factors (relative to methyl pentadecanoate) for the 9,10-dibromo- and 9,10; 12,13-tetrabromostearates were 1.45 and 1.92 respectively; correction was made for 10% of the tetrabromoester derivatives eluting with the dibromo derivative peak. Peak areas were measured with a disc integrator.

The data in Table I are in good agreement with those previously reported for the lipolysis of nonbrominated olive, sesame, corn and cottonseed oils (5) and indicate there is no lessening in specificity of pancreatic lipase for hydrolysis of the primary ester linkages in brominated oils. A further indication that lipolysis proceeds satisfactorily with no preferential hydrolysis is shown by the recovery of triglyceride from the hydrolysate with a similar composition to that of the original oil (Table I).

A decrease in the activity of the enzyme

TABLE I

Lipolysis of Brominated Vegetable Oils

	Fatty acid composition							
	16:0		18:0		Di Br		Tetra Br	
Oil	Area %	Mole %	Area %	Mole %	Area %	Mole %	Area %	Mole %
Br olive	8.6	13.8	5.0	7.2	74.6	70.7	11.8	8.3
2-MG	2.4	4.0	6.3[a]	1.1[b]	74.6	82.6	16.7	12.3
Br sesame	5.9	10.5	5.0	8.1	42.7	45.0	46.4	36.4
2-MG	1.1	2.1	3.3[c]	1.7[d]	43.7	49.4	51.9	46.8
Br corn	6.3	12.2	1.6	2.8	21.7	24.9	70.4	60.1
Br corn[e]	6.4	12.5	1.6	2.8	20.5	23.6	71.5	61.1
2-MG	1.6	3.3	0.5	0.9	20.6	25.3	77.3	70.5
Br cottonseed	14.5	25.9	1.9	3.1	19.4	20.5	64.2	50.5
Br cottonseed[e]	14.0	25.1	2.5	4.1	18.5	19.6	65.0	51.2
2-MG	2.5	5.1	0.8	1.5	21.5	26.0	75.2	67.4

[a]18:0, 0.8%; 18:1, 5.5%.

[b]18:1, 8.5% combined with Di Br.

[c]18:0, 1.0%; 18:1, 0.3%; 18:2, 2.0%.

[d]18:1, 0.5% combined with Di Br; 18:2, 3.5% combined with Tetra Br.

[e]Triglyceride recovered from hydrolysate.

with these oils was however apparent. Under conditions which gave 50% hydrolysis of corn oil (3 min reaction time, 10 mg corn oil, 3 mg lipase, as measured by free fatty acids), approximately 20% hydrolysis was obtained with brominated olive oil, 16% hydrolysis with brominated sesame oil, and 12% hydrolysis with brominated corn and cottonseed oils. It appears that as the content of tetrabromostearate is increased, resulting in a higher melting point of the substrate, the activity of the lipase decreases. A similar decrease in enzyme activity has been observed previously with tristearin and tripalmitin substrates (6).

These results indicate that except for a reduction in hydrolysis rate, these brominated oils are degraded enzymatically in the same way as common vegetable oils; also, they may be absorbed and deposited in a similar fashion, since 9,10-dibromo- and 9,10; 12,13-tetrabromostearates were detected in the livers and hearts of rats after administration of brominated cottonseed oil (Conacher and Hand, unpublished observations).

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[Received December 17, 1969]

Lipolysis, Esterification and Glucose Oxidation by Human Omental Isolated Adipose Cells: The Effects of pH, Buffer and Epinephrine

ABSTRACT

The rates of glycerol productions, glucose oxidation and glucose conversion to glyceride-glycerol by human omental fat cells were compared in vitro at pH's from 7.0 to 8.4, with and without epinephrine, and in both Krebs-Ringer-bicarbonate and -phosphate buffers. Basal glycerol production was not significantly altered by changes in pH, and was similar in each buffer. Epinephrine markedly increased glycerol production at each pH in both buffers, but significantly more was produced at pH 8.4 than at pH 7.4. Basal glucose metabolism was not affected by pH or by the type of buffer. Epinephrine caused a small increase in glucose oxidation at each pH in bicarbonate buffer but only at pH 7.8 in phosphate buffer, and had no effect on glucose conversion to glyceride-glycerol.

Epinephrine-stimulated lipolysis in rat adipose tissue is pH dependent, reaching a maximum at pH 8.5 (1). No comparable studies of human adipose tissue, which is less sensitive to epinephrine (2) have been reported. In this report, data for human omental adipose tissue are presented.

The Krebs-Ringer-bicarbonate and -phosphate buffers (3) contained half the recommended amounts of calcium (2.5 mEq/1), bovine serum albumin fraction 5 (Armour, 40 mg/ml), glucose (1 mg/ml), and epinephrine (10 μg/ml) where indicated. The pH of the phosphate buffer was adjusted with 1 N hydrochloric acid or sodium hydroxide while the pH of the bicarbonate buffer was altered by substituting sodium bicarbonate for sodium chloride.

Omentum removed at cholecystectomy or hysterectomy was promptly used to prepare isolated adipose cells (4,5) which were washed thrice in the buffer appropriate to the study and then dispensed into siliconed flasks. The ratio of packed cells (containing approximately 600 μmoles of triglyceride per 1 ml) to buffer was constant at 1:30 in all studies.

There were two groups of studies, each comprising six identical experiments. The first group measured lipolysis by means of the glycerol produced at 37 C during 2 hr incu-

with these oils was however apparent. Under conditions which gave 50% hydrolysis of corn oil (3 min reaction time, 10 mg corn oil, 3 mg lipase, as measured by free fatty acids), approximately 20% hydrolysis was obtained with brominated olive oil, 16% hydrolysis with brominated sesame oil, and 12% hydrolysis with brominated corn and cottonseed oils. It appears that as the content of tetrabromostearate is increased, resulting in a higher melting point of the substrate, the activity of the lipase decreases. A similar decrease in enzyme activity has been observed previously with tristearin and tripalmitin substrates (6).

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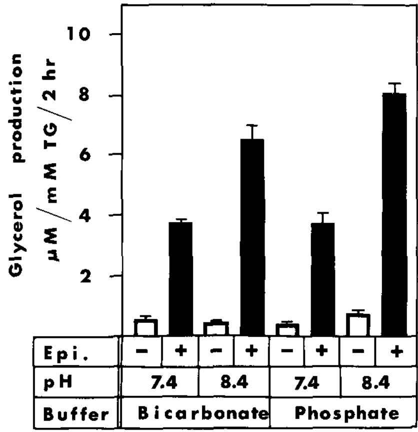

FIG. 1. A comparison of lipolysis occurring in bicarbonate- or phosphate-buffered medium at pH 7.4 and 8.4, with and without epinephrine 10 μg/ml. Means + 1 S.E.M. of six experiments.

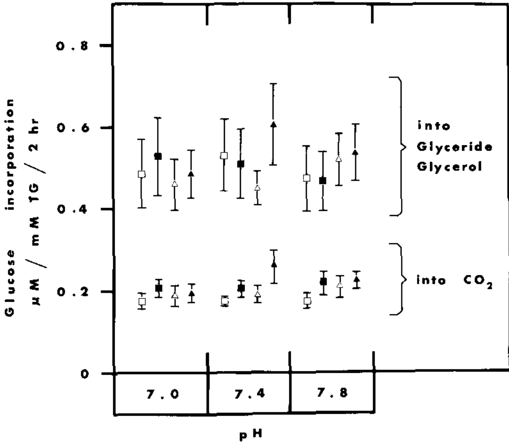

FIG. 2. Glucose incorporation into glyceride-glycerol and oxidation to carbon dioxide compared in bicarbonate and phosphate buffers at pH 7.0, 7.4 and 7.8, in the presence and absence of epinephrine (10 μg/ml). □ Krebs-Ringer bicarbonate; ■ Krebs-Ringer bicarbonate + epinephrine; △ Krebs-Ringer phosphate; ▲ Krebs-Ringer phosphate + epinephrine. Means ± 1 S.E.M. of six experiments.

bations at pH 7.4 and 8.4, with and without epinephrine, and in both buffers. Glycerol was measured enzymatically (6) on duplicate samples, and was found to accumulate at a linear rate at either pH.

The second group of experiments measured the conversion over a 2 hr period of U-^{14}C-D-glucose (Radiochemical Centre, Amersham) from the medium to carbon dioxide and glyceride-glycerol at pH 7.0, 7.4 and 7.8, in each buffer, with and without epinephrine. The methods have been described (5). The triglyceride content of the incubation flasks was estimated as carboxyl ester (7). Results were assessed by the analysis of variance, the chosen level of significance being p = < .05.

There was a decrease in pH of the media during incubation. The change was less than 0.1 unit for buffers of initial pH 7.0 and 7.4, less than 0.2 units for the pH 7.8 buffer and did not exceed 0.5 unit with the pH 8.4 buffer.

The lipolysis results are illustrated in Figure 1. Basal lipolysis was not significantly affected by the type of buffer or by a change in pH. Epinephrine markedly increased lipolysis in each buffer and at each pH but the increase was significantly greater at pH 8.4 than at pH 7.4. Preliminary experiments established that maximal lipolysis with epinephrine was achieved at pH 8.4 in each buffer. Thus, human adipose cells exhibit a pH dependance to epinephrine stimulation similar to that observed for rat adipose tissue (1) where the influence of pH has been ascribed to alterations in adenyl cyclase activity (8).

Esterification was measured as the incorporation of glucose into glyceride-glycerol and is shown in the upper section of Figure 2. There was a wide scatter of individual values and no significant influence of pH, buffer or epinephrine could be demonstrated. The lack of effect of epinephrine on esterification despite a several-fold increase in lipolysis contrasts markedly with results from rat adipose tissue (9) and a possible explanation is diminished responsiveness of the glucose transport mechanisms, as had been reported for insulin (5). Glucose incorporation into glyceride fatty acids did not occur, confirming our previous observations that fatty acids are not synthesized in such dilute cell suspensions (10).

Glucose oxidation, shown in the lower portion of Figure 2, was not significantly affected by pH, epinephrine or buffer unless the results were expressed as means of the individual changes from basal levels, when it

TABLE I

Percentage Increases in Glucose Oxidation Above Control Values in the Presence of Epinephrine (10 μg/ml)[a]

	pH 7.0	pH 7.4	pH 7.8
Bicarbonate	19.8±5.3[b]	16.5± 6.2[b]	24.9±10.6[b]
Phosphate	6.2±7.0	40.9±16.6	13.8± 5.2[b]

[a]Means ± 1 S.E.M. of six experiments.
[b]p <.05.

became apparent (Table I) that epinephrine accelerated glucose oxidation at each pH in bicarbonate buffer and at pH 7.8 in phosphate buffer. It has been reported that epinephrine does not influence glucose oxidation by human adipose tissue (11) whereas its marked effect on rat adipose tissue is well known (9).

Our results are also relevant to the conduct and interpretation of in vitro experiments where a lipolytic response may be inhibited by an associated decrease in pH. This is likely to occur when lipolysis is very active, and especially when the volume of incubation medium is small in comparison to the tissue volume.

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Isolation of Human Serum Low-Density Lipoproteins With the Aid of an Immune-Specific Adsorber

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ABSTRACT

Human serum lipoproteins containing B-protein have been isolated using an immunoadsorber. Bromoacetyl cellulose was combined with pure antibodies to low density lipoprotein (LDL) and an immunoadsorber of high capacity was obtained. With 1 g of this immunoadsorber all LDL and very low density lipoprotein (VLDL) from 30 ml pooled human serum were adsorbed and then eluted with glycine-HCl buffer pH 3.2 at 0 C. The isolated lipoproteins were investigated by electrophoresis, immunodiffusion and ultracentrifugation, and found to be identical to LDL + VLDL isolated by ultracentrifugation.

INTRODUCTION

Lipoprotein fractions homogeneous with respect to density, regardless of their protein portion, have mostly been isolated from blood serum by repeated flotation in the ultracentrifuge (1). The lipoproteins prepared by other methods are inhomogeneous either with regard to their protein components or with regard to their hydrated density and have to be further fractionated in the preparative ultracentrifuge. Such methods are: fractionation according to Cohn (2); column chromatography on Sephadex G-200 (3), agarose gel (4) or glass powder (5); precipitation with polysaccharide sulfate (6) and different special methods (7-9).

For a long time there was confusion concerning the protein components of low density lipoproteins (LDL) as well as very low density lipoproteins (VLDL). Many authors assumed complete antigen identity for LDL and VLDL (10) as well as the presence of a homogeneous protein portion, which is supposed to be constructed from diverse monomers. Others assumed, with or without consideration of the genetically determined polymorphism, a heterogeneity of the apoprotein of LDL and VLDL (11,12). According to contemporary knowledge, there seem to be at least three distinct apoproteins in human serum: the A, B and C proteins (13). High density lipoproteins (HDL) contains the A-protein, LDL the B-protein and VLDL the A, B and at least two other proteins (14).

Because lipoproteins are easily irreversibly changed, a quick isolation out of a fresh serum is desirable. In order to produce lipoproteins homogeneous with regard to their protein portion, the use of specific antibodies is especially suited. Recently antibodies to β-lipoproteins (B-protein) have been isolated in high purity and quantity (15) and hence immunoadsorbers with sufficient capacity could be prepared.

MATERIALS AND METHODS

Isolation of Antibodies to β-Lipoprotein

The immunization of animals and isolation of pure antibodies were performed according to Kostner and Holasek (15). After precipitation of the antibodies from a monospecific horse antiserum with pooled human serum β-lipoprotein the immune precipitate was dissolved in a 0.2 M glycine-HCl buffer at pH 3.2 and the antibodies were separated from antigen by centrifugation in a preparative ultracentrifuge at a density of 1.07 and 4 C. The antibody fraction was dialyzed against 0.15 M NaCl and employed for the preparation of the immunoadsorber without further purification. Bromoacetyl cellulose (16) was used to bind the antibody; 1.5 g of this adsorber binds approximately 100 mg of pure antibody.

Adsorption and Removal of Lipoproteins

Before use the immunoadsorber was first washed with 0.2 M glycine-HCl buffer of pH 3.2 until no substance adsorbing at 280 nm could be eluted, and then with 0.15 M NaCl of pH 7.4. This immunoadsorber was incubated with pooled human serum of 4-6 fasted males and females for 1 hr at 36 C and overnight at 4 C. Before use of the serum the chylomicrons were removed by centrifugation for 10 min at 9500 x *g*. The charged immunoadsorber was washed free from unbound protein with 0.15 M NaCl and then with distilled water. The adsorbed lipoproteins were eluted with a small amount of 0.2 M glycine-HCl buffer at pH 3.2 and 0 C. The eluted lipoproteins were brought up to a concentration of about 1% by pressure dialysis against 0.15 M NaCl and kept at 4 C. All the buffers used as well as the washing solutions contained 0.05% EDTA.

Protein was determined by the method of

TABLE I

Chemical Analysis of Three Different Lipoprotein Samples Isolated Via Immunoadsorber

Sample	Protein % by wt.	Total lipid % by wt.	Per cent of total number of lipids: Total cholesterol	Phospholipid	Triglycerides	Free fatty acids	LDL:VLDL ratio[a]: Lipoprotein isolated at a density of 1,063 in the ultracentrifuge	Lipoprotein isolated via immunoadsorber
1	18.5	80.4	43.9	26.1	28.8	0.9	ND[b]	ND
2	19.8	79.1	45.1	26.8	26.2	1.1	2.4	2.5
3	21.0	77.2	46.5	25.7	25.5	1.3	2.7	2.7

[a]Determined gravimetrically after separation of VLDL at a density of 1.006 g/ml.

[b]ND, not determined.

Lowry et al. (17) and by measuring the extinction at 280 nm. The standard absorption curve for human LDL was prepared by the use of highly purified lyophilized material. The total lipid was determined by gravimetry after extraction with chloroform-methanol (2:1) according to Folch et al. (18), cholesterol according to Zak (19) and the neutral fat according to Eggstein and Kreutz (20). Phospholipid content of the lipoproteins was determined by a phosphorus assay (21). The estimation of free fatty acids was performed by the method of Duncombe (22). The analytical ultracentrifuge was a Beckman Spinco Model E; and the preparative ultracentrifuge a Beckman Model L 4 equipped with fixed-angle rotors.

Electrophoresis and staining of lipoproteins in starch gel were performed according to Cohen and Djordjevich (23).

The polyvalent antiserum to human serum protein and the monovalent antiserum to α_1-lipoprotein for immunoelectrophoresis were purchased from Behring-Werke AG., Marburg a.d.L.

RESULTS

With 1 g of the described immunoadsorber the total LDL plus VLDL could be adsorbed from 30 ml pooled human serum. After removal and dialysis, the yield of lipoproteins amounted to about 110-160 mg (weights from three samples). The yield of VLDL + LDL isolated from the same serum with a preparative ultracentrifuge at a density of 1.063 was 105-150 mg after dialysis. After removal of the lipoproteins, the immunoadsorber was extracted with chloroform-methanol (18) and an analysis of cholesterol was carried out. It was found that more than 99.5% of the adsorbed lipoproteins had been removed by the buffer. The serum used for the preparation of lipoproteins was examined by starch gel and immunoelectrophoresis before and after treatment with immunoadsorber. After adsorption, no LDL and VLDL could be detected by either method. Analytical ultracentrifugation revealed the complete absence of particles floating at a density of 1.063.

The density of adsorbed serum was brought to 1.21 with KBr and the remaining lipoproteins separated in the ultracentrifuge. These lipoproteins were characterized as α-lipoproteins by starch gel and immunoelectrophoresis.

The lipoproteins extracted via immunoadsorber were examined by immunoelectrophoresis, electrophoresis in starch gel and on acetate foil strips. In all cases they behaved as a mixture of pre-β- and β-lipoprotein (Fig. 1 and 2). Examination of these lipoproteins in the

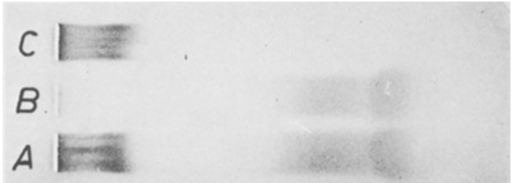

FIG. 1. Starch gel electrophoresis pattern of (A) complete human serum and (B) human serum from which lipoproteins had been removed by immunoadsorber, and (C) lipoproteins isolated via immunoadsorber. Buffer: tris-borate-versene, pH 8.5. Time of run: 18^h at 150 V. Staining: oil red O.

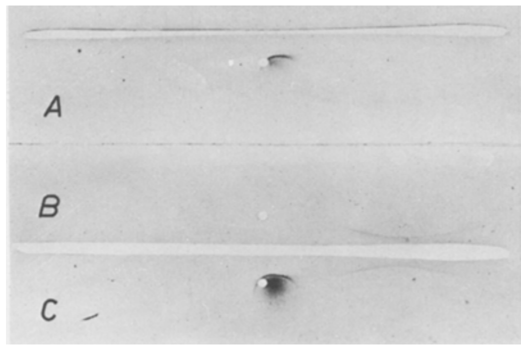

FIG. 2. Immunoelectrophoresis pattern of (A) lipoproteins isolated via immunospecific adsorber, (B) human serum treated with immunoadsorber and (C) complete human serum. The slits were filled with antiserum to complete human serum. Staining: sudan black.

analytical ultracentrifuge demonstrates that the main peak floated with an S_f of 5-8. Faster floating lipoproteins (VLDL) were also visible in low quantities. The ultracentrifuge pattern resembles that of lipoproteins of human serum, which had been isolated in the ultracentrifuge at a density of 1.063 (Fig. 3). No selective loss of VLDL or LDL occurred during absorption since the ratio of LDL-VLDL in the lipoproteins isolated via immunoadsorber was almost the same as the ratio in the lipoproteins isolated in the preparative ultracentrifuge at a density of 1.063 from the same serum (Table I). No sedimenting particles were visible at this density. In different sera the ratio was found to be different. In immunodiffusion tests the lipoproteins extracted via immunoadsorber showed a strong precipitation curve with anti-β-lipoprotein and a weak one with anti-α-lipoprotein (Fig. 4). The chemical analysis of the isolated lipoproteins is shown in Table I.

The immunoadsorber could be reused frequently. After using it 10 times a loss of capacity of less than 10% was found. After six months of storage at -20 C the activity of the immunoadsorber amounted to more than 90%.

DISCUSSION

The classical method for the preparation of lipoproteins, namely the flotation in the ultracentrifuge, has been shown to be successful for fractionation of lipoproteins according to their density. However, since the protein portions of lipoproteins, the A, B and C proteins, are newly taken into account for their characterization (13), it appeared to be necessary to develop a method for the isolation of lipoproteins with definable apoprotein. With the described immunoadsorber all the lipoproteins with B specificity could be adsorbed from pooled human serum (VLDL + LDL). The VLDL also showed a reaction with anti α-lipoprotein because they possess, besides the B protein, an A protein portion (24). Although the concentration of VLDL in the sample shown in Figure 3 was very small, in one case of hyperlipoproteinemia, Type 4, all VLDL could be removed and recovered using the immunoadsorber technique.

Different carriers such as *p*-amino benzyl cellulose (25) and cyanogen bromide with polysaccharides (26) were used for the binding of the antibodies. Bromoacetyl cellulose however proved to be the most suitable one. It could be applied more than 10 times without considerable loss of capacity and was stable for several months. The most delicate step, the removal of the bound lipoprotein from the immunoadsorber, was best achieved by the use of 0.2 M glycine-HCl buffer, pH 3.2, in the absence of neutral salts. The investigation of the isolated lipoproteins showed that they migrate as LDL and VLDL in starch gel, cellulose acetate foil strips and immunoelectrophoresis.

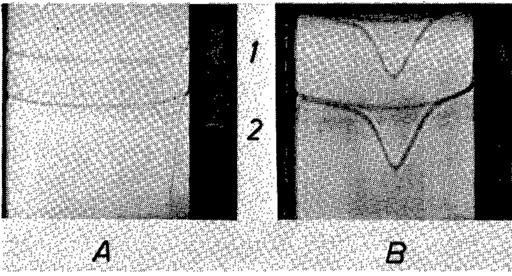

FIG. 3. Ultracentrifugal analytical runs at a density 1.0630 and 48.000 rpm. Concentration of samples: 1%. Curve 1, LDL isolated via immunoadsorber. Curve 2, complete LDL + VLDL from human serum isolated at a density of 1.0630. A: 28 min after start. B: 98 min after start. S_f values of peak in both samples: 6.1 Svedberg units.

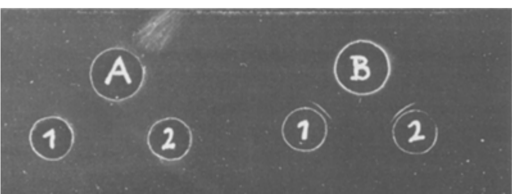

FIG. 4. Immunodiffusion test of 1, lipoproteins isolated via immunoadsorber; 2, lipoproteins from human serum isolated at a density of 1.063. A: Anti α_1-lipoprotein (Behringwerke). B: Anti-LDL.

It is well known that LDL are heterogeneous and show an antigen polymorphism (27,28). In spite of this fact, all LDL and VLDL were adsorbed out of the pooled human serum by the described immunoadsorber, because polymorphic proteins also possess identical antigen determinants.

Most representative methods for lipoprotein isolation such as column chromatography and specific precipitation procedures deliver either no pure products or cause changes in their physicochemical properties. In order to obtain immunologically pure products many sequential flotations in the preparative ultracentrifuge have to be carried out. Furthermore, alteration of proteins can occur with increasing salt concentration (29). Immunoadsorbers with high capacity permit the fast isolation of pure lipoproteins in large amounts. As was shown by analytical ultracentrifugation, lipoproteins were not noticeably changed during the preparation.

The method described in this article for the preparation of VLDL and LDL should be applicable to the isolation of lipoproteins with A protein specifity as well. With the aid of an immunoadsorber it might also be possible to prepare lipid-free apoproteins which were found in serum in small amounts (30).

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NMR Spectra of Mercury Adducts of Unsaturated Fatty Acids: Quantitative Determination of *Cis-Trans* Ratio

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ABSTRACT

By stereospecific adduct formation between mercuric acetate and unsaturated fatty acids, derivatives are obtained suitable for NMR determination of *cis-trans* ratio. The preparation and optimal experimental conditions are determined. Experimental data are found to be within a few per cent of the true values.

INTRODUCTION

For several decades it has been known (1) that mercury salts can form adducts by reaction with unsaturated compounds. The method has been applied in preparative separation of fatty acids (2-4) as well as analytically in paper and thin layer chromatography (5).

The reaction of an unsaturated aliphatic compound with mercury acetate can formally be expressed as

$$M_1HC = CHM_2 + Hg(OAc)_2 + ROH \rightarrow$$

$$(M_1CHCHM_2; Hg\ OAc; RO) + HOAc$$

Here nothing has been said about the details in the reaction mechanism. Investigations by Wright (8) and Spengler (9) indicate a reaction of the type I → II followed by alchoholysis II → III whereby OAc is replaced by OR. Jantzen and Andreas (2,3) found the Reaction I → III to be irreversible. In most cases the reaction proceeded until more than 95% of the unsaturated material was converted into adduct within a period of 12 hr.

$$\underset{\text{I}}{M_1HC = CHM_2} + Hg(OAc)_2 \rightarrow \underset{\text{II}}{M_1\,\overset{H}{\underset{OAc}{C}}-\overset{H}{\underset{HgOAc}{C}}-M_2}$$

$$\underset{\text{II}}{M_1\,\overset{H}{\underset{OAc}{C}}-\overset{H}{\underset{HgOAc}{C}}-M_2} + ROH \rightarrow \underset{\text{III}}{M_1-\overset{H}{\underset{OR}{C}}-\overset{H}{\underset{HgOAc}{C}}-M_2} + HOAc$$

A problem of major importance is the specificity of the reaction. A number of authors (9-12) all found that the reaction is exclusively a *trans* addition. In a few ring compounds with highly unfavorable steric configurations a *cis* addition was found, but the degree of reaction and the reaction rate were then drastically reduced. Jantzen and Andreas investigated the specificity by forming the adduct of a pure *cis* monoene. After acid decomposition of the adduct they isolated the original monoene having a *trans* content of less than 1%. This results leads to the conclusion that in unsaturated aliphatic compounds the reaction can be regarded as a pure *trans* addition.

Molecules symmetric about the double bond ($M_1 = M_2$) form only one adduct. When $M_1 \neq M_2$, two position isomers can be formed differing in the relative position of the OR and HgOAc groups. In the case R = CH_3 evidence (6) has been found that OCH_3 will be located at the same carbon as the most bulky groups.

In the remaining part of the article the discussion will be restricted to the symmetric case ($M_1 = M_2$).

Starting from a pair of *cis-trans* isomers two different adducts are formed (IV and V), each consisting of a pair of mirror images (Fig. 1). The NMR spectra of these types of compounds will be discussed in the following sections.

Brownstein (6) has reported NMR spectra of mercury adducts, but they were all derived from compounds of the type $CH_2 = C^X_Y$, X,Y being small alkyl groups. Addition to these compounds resulted in only one asymmetric carbon atom and the problems discussed here therefore did not arise.

To classify the NMR spectra of the adducts it is important to know whether the rotation about the carbon-carbon bonds can be considered as fast. Even when a bulky substituent, as HgOAc, is present the barrier is expected to be below 10 Kcal/mole. This is sufficiently low for the rotation to be classified as fast.

The chemical shifts observed in the spectra will then correspond to proper weighted average values over the three different rotamers. Compounds which are mirror images as Va and Vb yield the same average while IV and V result in different average signals.

APPLICATIONS

Analysis of mixtures of similar aliphatic molecules is normally an almost impossible task

FIG. 1. Geometric arrangement in the initial addition products formed by addition of $Hg(OAc)_2$ to a *cis*-monoene (IV) or a *trans*-monoene (V). The OAc group is replaced by OR in the following alcoholysis.

in NMR spectroscopy. In the present paper the attention is focused on mixtures of *cis-trans* isomers represented by oleic and elaidic acid methyl esters. In Figure 2 the characteristic part of each spectrum arising from the double bond protons is the pure ester is given followed by the spectrum of the same region in a 50-50% mixture. Since the rest of the spectra of the two compounds are identical the only source of information concerning the *cis-trans* ratio is the overlapping bands of Figure 2. The spectrum of each species consists of a large number of lines, and they overlap to such an extent (Fig. 2c) that direct intensity measurements cannot be used. In the adducts separate sharp signals are found for each isomer suitable for accurate intensity measurements.

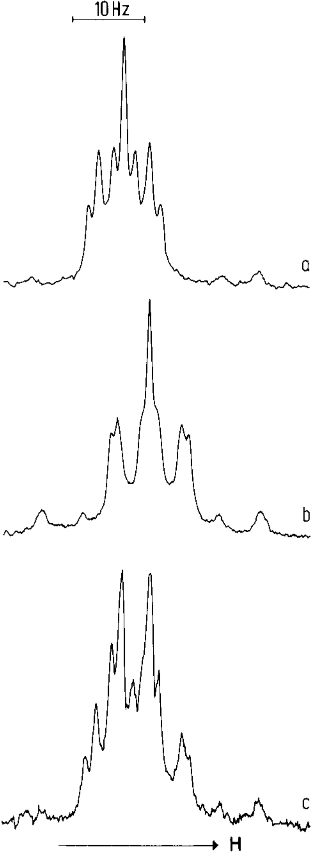

FIG. 2. Spectra of the double bond protons in (a) pure *trans*-monoene elaidic acid, (b) pure *cis*-monoene oleic acid, (c) approximately 50-50% mixture of (a) and (b). Recorded at 100 MHz.

In the actual case the double bond can be regarded as being in symmetric surroundings (13) due to the large number of methylene groups attached on both sides of the double bond. According to the discussion above, two distinct spectra arise from the adducts of oleic and elaidic acid. Decisive for the practical applicability of the adducts in determination of *cis-trans* mixtures is the chemical shift differences between similar signals in the two compounds. When the difference is sufficient, measurements of the line intensities will yield the proportions of the two compounds.

In Figure 3 the spectrum of an approximate 50-50% adduct is reproduced. In principle it would be most favorable to study the signals arising from the protons located at the asymmetric carbon atoms. Unfortunately these signals are obscured by the large methylene signal. Alternatively the OCH_3 signal which occurs as a doublet can be studied. Since the line shape for the OCH_3 signals can be expected to be very nearly the same, peak height measurements can be used to determine the *cis-trans* ratio.

TABLE I

Chemical Shift of New Signals Produced by Adduct Formation Between Mercuric Acetate and Fatty Acid Methyl Esters

Fatty acid methyl esters	Chemical shift ppm[a]	
	OCH_3[b]	HgOAc
Oleic	3.22_4	1.98_3
Erucic	3.22_4	1.99_2
Elaidic	3.18_5	1.98_7

[a]Measured relative to internal TMS at 100 MHz.

[b]Due to the increased overlap the peak separation in the 60 MHz spectra is only 0.025 ppm.

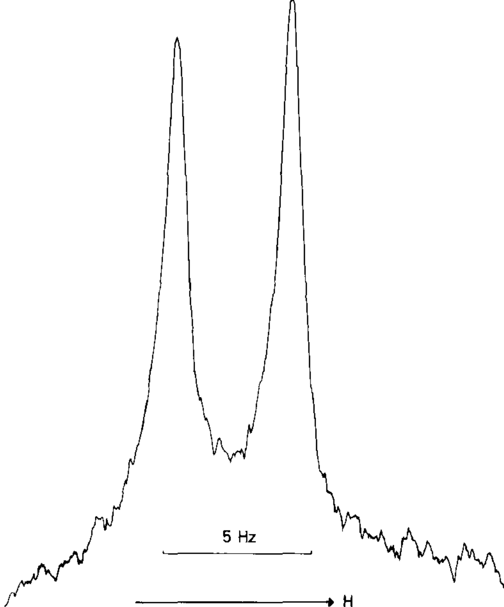

FIG. 3. Spectrum showing the OCH_3 signals in the adduct of an approximate 50-50% mixture of *cis*-and *trans*-isomers at 100 MHz.

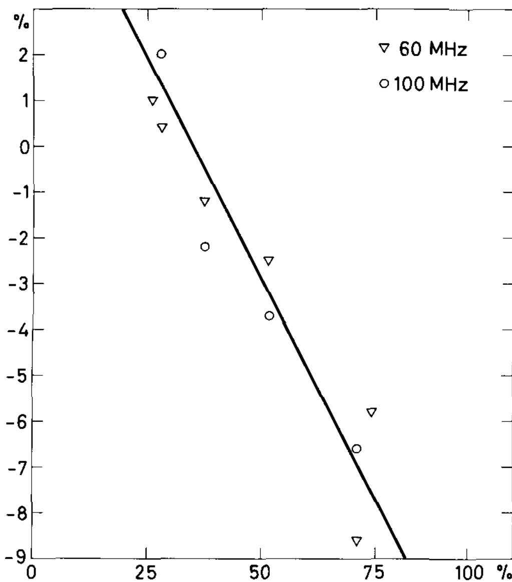

FIG. 4. Correlation between *cis-trans* ratios determined by NMR and gravimetrically. Abscissa: true content of oleic acid methyl ester. Ordinate: Δ the deviation between NMR data and true content of oleic acid methyl ester.

RESULTS

Table I summarizes the chenical shifts for the methoxy groups and the acetate groups in the methyl esters of the two acids measured relative to internal tetramethylsilane (TMS). In Table II calculated data for the molar ratio obtained by line intensity measurements are given as well as the true values.

EXPERIMENTAL PROCEDURES

Spectra were obtained using Varian A 60 and Varian HA-100 spectrometers operating in frequency sweep mode; sample temperature, 32 C. Line positions are measured relative to internal TMS. The oleic and elaidic acid methyl esters were commercial samples purified by gas chromatography. The mixtures were prepared gravimetrically and dissolved in analytical grade solvents at a concentration of approximately 10% w/v.

The adducts were synthesized according to Jantzen and Andreas (2,3). The adducts were dissolved in benzene and the solution extracted with water until a clear benzene phase was obtained. The benzene solution was distilled in

TABLE II

Comparison of *Cis-Trans* Ratio in Mixtures of Oleic and Elaidic Acid Methyl Esters[a]

Method	NMR		Gravimetric		
	Oleic %	Elaidic %	Oleic %	Elaidic %	Δ[b]
100 MHz	28.1	71.9	27.9	72.1	+0.2
	35.1	64.9	37.3	62.7	- 2.2
	47.9	52.1	51.6	48.4	- 3.7
	64.3	35.7	70.9	29.1	- 6.6
60 MHz	26.8	73.2	25.8	74.2	+1.0
	28.5	71.5	27.9	72.1	+0.6
	36.1	63.9	37.3	62.7	- 1.2
	48.9	51.1	51.4	48.6	- 2.5
	62.3	37.7	70.9	29.1	- 8.6
	68.5	31.5	74.3	25.7	- 5.8

[a]As determined by NMR or gravimetrically in the preparation of samples.
[b]Δ Represents the deviation between results obtained by the two methods.

vacuo until the pressure was below 10 μ. The remaining yellow oil decomposes slowly at room temperature under influence of light. At 4 C in darkness the adducts are stable for periods of months.

DISCUSSION

The observed chemical shifts of the OCH_3 group in *cis* and *trans* adducts are time average values over a large number of possible configurations of the molecule. This prevents a detailed understanding of the average values. The difference in chemical shift reflects primarily short range phenomena. The aliphatic chains extending to both sides from the OCH_3 position are identical in a pair of *cis-trans* isomers and can be expected to yield comparable contributions to the average chemical shift.

Accordingly a model was chosen on the basis of which the problem is discussed in terms of the population of the rotamers about the carbon-carbon bond. This population can be influenced by variation of temperature and solvent. Experiments show CCl_4 and $CDCl_3$ to give a similar separation of the OCH_3 signals studied, while dimethylsulfoxide resulted in a decreased separation. Temperature studies in CCl_4 resulted in an increasing line separation with increasing temperature. Since the thermal stability of the compounds is poor, optimum results are obtained between 30 and 40 C in CCl_4 solution. This result is highly convenient since such a temperature range corresponds closely to the standard operational conditions for the spectrometer. As the populations of the various rotamers depend only little on groups far from the asymmetric carbon atoms it is to be expected that various fatty acids in *cis* conformation yield almost identical adducts. This is confirmed by inspection of Table I where data for the line positions for erucic acid are given, being nearly identical to the one obtained for oleic acid. When 100 MHz and 60 MHz spectra are compared it is observed that the apparent separation of metoxygroup signals increases with increasing field strength. Thus the peak separation is 0.039 ppm at 100 MHz and 0.025 ppm at 60 MHz. The variation is due to the partial overlap of the signal at 60 MHz resulting in a reduction in the peak maximum separation. From this it follows that a much better separation is observed at 100 MHz improving the accuracy of the data.

The proposed method lends itself to a comparison with the long known IR spectroscopic determination. Three features favor the present NMR method. First, it is possible to observe directly the signal representing both the *cis* and the *trans* compound; secondly, signal intensities are linearly related to the concentrations in solution. Finally, the method is not restricted to cases where two protons are located at the double bond. Admittedly the present method involves preparation of a derivative, but the reaction is simple and the compound can be recovered in its original steric configuration after the determination.

In the present paper the emphasis has been placed on demonstrating the possibility of applying the mercury adducts rather than obtaining the highest possible accuracy in the results. By plotting Δ of Table II against the true content of oleic acid one obtains a linear relationship from which a calibration curve for the obtained data may be deducted (Fig. 4). Both data obtained at 60 and 100 MHz fit the curve within 0.5%. Application of this or other correction methods improves the accuracy of the results considerably.

ACKNOWLEDGMENTS

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Aliphatic Hydroxylamines as Lipid Antioxidants[1]

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ABSTRACT

It was previously noted that N,N-dioctylhydroxylamine is formed from the oxidation of trioctylamine in oxidizing fats and that N,N-dioctyl- and N,N-diethylhydroxylamine preparations have antioxidant activity. In this paper we summarize some additional observations with these and some other N-substituted hydroxylamines. ESR spectra indicate that nitroxides are present in oxidized preparations of hydroxylamines. Since some stable nitroxides have been shown to be exceptionally effective antioxidants, we hypothesize that the antioxidant effectiveness of the hydroxylamines is accounted for by their conversion to nitroxides during the initial stages of oxidation.

INTRODUCTION

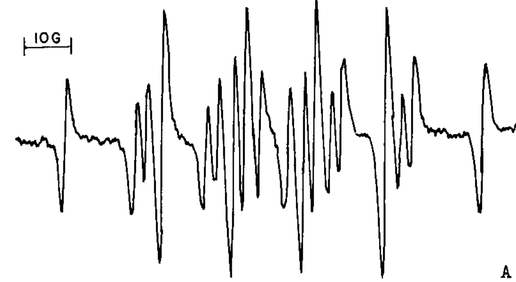
Squalene containing added trioctylamine is more stable at 60 C than at 50 C (1). The concept that the effective antioxidant was an intermediate of the oxidation of the original amine followed from the demonstration that N,N-dioctylhydroxylamine could be isolated from an oxidizing lipid containing trioctylamine and that the isolated compound had antioxidant activity. In a parallel case, Barnard et al. (2) had shown that the effective oxidized intermediates accounting for the antioxidant effect of thio- and dithio-compounds in rubber were sulfoxides and thiosulfinates.

In this paper we give results obtained in further studies with aliphatic hydroxylamines and suggest that their oxidized products, the nitroxide radicals, may be the effective antioxidants. The very effective antioxidant activity of some stable nitroxides in squalene at room temperature was recently described (3).

METHODS

With the exception of N,N-diethylhydroxylamine (DEHA), the hydroxylamines were synthesized by the method of Schopf et al. (4). The parent amines (Pennsalt and Aldrich) were oxidized with hydrogen peroxide. Sodium carbonate was added and the hydroxylamines were extracted with diethyl ether. The ether solutions were dried over anhydrous sodium sulfate, evaporated under nitrogen, and distilled under reduced pressure. The hydroxylamines were then further purified by recrystallization as the oxalate derivatives. The oxalate was removed by treatment with liquid ammonia (dry ice-acetone bath) and the free hydroxylamines were redistilled under reduced pressure. DEHA preparations (85% and 100%, Pennsalt) were purified directly from the oxalate derivative as described earlier (5), and distilled under reduced pressure.

Homogeneity was checked by thin layer



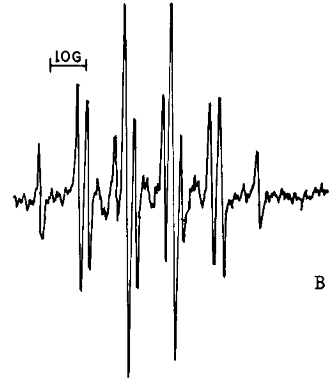


FIG. 1. A, Spectrum of the nitroxide radical formed in oxidized proline. (See text for experimental details). B, Spectrum of the nitroxide radical formed in oxidized N-hydroxyglycine. (See text for experimental details).

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TABLE I

Properties of Hydroxylamines and Their Oxalates[a]

Hydroxylamine	Hydroxylamine Found	Hydroxylamine Literature	Oxalate derivative Found mp	Oxalate derivative Literature mp
N,N-diethylhydroxylamine	bp_{30}57-62 C	bp_{30}60 C (20)	138 C	136-137 C (17)
N-hydroxypiperidine	mp38.5-39.5 C	mp39-40 C (3)	112 C	112 C (18)
N-hydroxypyrrolidine	bp_{24}75 C	bp_{25}74-76 C (21)	123-124 C	123-124 C (19)
N-hydroxymorpholine	$bp_4$72-75 C	bp76-79 C (21)	--	--

[a]Figures in parentheses are references.

chromatography (6). The melting and boiling points reported in Table I corresponded to those in the literature. Samples of purified hydroxylamines were kept at dry ice temperature in sealed vials until just prior to usage.

All solvents were distilled through a 15 plate Oldershaw column. Antioxidant activity in purified squalene (7) was measured by a weight gain method (8) as follows: covered beakers containing 200 mg of substrate with and without additions were held in a constant draft oven for 50 C runs and in a laboratory cabinet for those at room temperature. Once or twice daily they were tested for rancidity by odor, cooled to room temperature and weighed. The end of the induction period was indicated by a sharp gain in weight which coincided with the development of the odor of rancidity. An increase in weight of 1 mg. (0.5%) was arbitrarily chosen as the end-point but in most cases the break was sharp.

ESR spectra were obtained with aqueous solutions in a micro flat-cell at a temperature range of +1.0 C to -10 C using a Varian Model E-3 X-band spectrophotometer. Infrared spectra were obtained with a Perkin Elmer Model 137 Infracord Spectrophotometer, and ultraviolet spectra, with a Cary Model 15 spectrophotometer.

RESULTS AND DISCUSSION

The antioxidant activities of purified hydroxylamines and of their oxalate derivatives in squalene are shown in Tables II and III. The oxalate salts were in the hydrogen form with the exception of DEHA which was in the neutral form. Concentrations of DEHA below 0.1 μm/200 mg squalene had little antioxidant activity at 24 C (Table II). This result may be due to its loss by volatilization under the conditions of the test. The oxalate salts of the hydroxylamines were effective at 50 C, but the free hydroxylamines were not, possibly also because of their volatility. Volatilization is assumed because of the low boiling points of these compounds and also because the characteristic odors were readily discernible in the oven when beakers containing samples of squalene containing the free hydroxylamines were present. Different results might be expected if determinations were run in closed systems. At room temperature (24 C) the oxalate salts and the free hydroxylamines had about the same antioxidant activity. Oxalic acid itself was inactive under these conditions.

Fresh samples of hydroxylamines showed little or no ESR signals, no IR bands at 6.1-6.3 μ attributable to nitrones (9,10), and no UV bands at 240 mμ typical of nitrone or nitroxide

TABLE II

Antioxidant Activity of Hydroxylamines in Squalene

Temperature	Amount (μmoles/200 mg)	Induction period (days)[a] N-hydroxy pyrrolidine	N-hydroxy morpholine	N-hydroxy piperidine	N,N-Diethyl hydroxylamine	Ethoxyquin[b]
24 C[c]	0.01	9	9	19	--	44
	0.02	11	16	28	--	100
	0.05	28	32	42	5	--
	0.1	52	47	54	10	--

[a]Averages of six runs; controls, four days.

[b]6-Ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline.

[c]Room temperature.

TABLE III

Antioxidant Activity of Hydroxylamine Oxalates in Squalene

Temperature	Amount (μmoles/200 mg)	Induction period (days)[a] N-hydroxypyrrolidine acid oxalate	N-hydroxypiperidine acid oxalate	N,N-diethyl hydroxylamine oxalate
24 C[b]	0.01	8	8	5
	0.02	11	12	5
	0.05	27	34	11
	0.1	42	46	---
50 C	0.02	4	2	1
	0.5	10	14	4
	1.0	16	22	5
	2.0	30	43	11
	4.0	68	108	26

[a]Averages of three runs; controls with no additive or with oxalic acid only at all levels had induction periods of three days at 24 C and one day at 50 C.

[b]Room temperature.

(10). However, when these were oxidized with hydrogen peroxide or with *t*-butyl-hydroperoxide or irradiated with UV light, there was an immediate development of ESR signals, typical of nitroxides (5), and also of UV bands at 240 mμ and weak absorption bands at 6.1-6.3 μ in the IR. These conditions thus result in the formation of nitroxide radicals which then decompose to nitrones as previously proposed by De LaMare and Coppinger (9).

According to Brownlie and Ingold (11) the labile hydrogen of hydroxylamines can be abstracted by a peroxy radical in the rate determining step of autoxidation as follows:

$$R_2NOH + ROO^\bullet \rightarrow ROOH + R_2NO^\bullet \quad [1]$$

The hydroperoxide so formed may react rapidly with additional hydroxylamine and then act as oxidation initiator as follows:

$$R_2NOH + ROOH \rightarrow RO^\bullet + R_2NO^\bullet + H_2O \quad [2]$$

Both reaction [1] and [2] include nitroxide radical formation. The nitroxide radical is reported to react with the alkyl radical as follows (11,12):

$$R_2NO^\bullet + R'^\bullet \rightarrow R_2NOR' \quad [3]$$

and to compete favorably with O_2 for $R^\bullet$ (12). Alkoxy radicals [2] have been reported to react with hydrocarbons to give further alkyl radicals (13):

$$RO^\bullet + R'H \rightarrow ROH + R'^\bullet \quad [4]$$

Thus if nitroxide radicals were present in large enough amounts, they would react with alkyl radicals before there was an opportunity for the latter to react with oxygen. These reactions lend credence to the possibility that after initial hydroperoxide formation and its subsequent reaction with substituted hydroxylamine, the unsaturated substrate may be protected from further oxidation by the nitroxide that is formed.

Proline is known to have antioxidant activity (16). The above observations suggest that an hydroxylamine intermediate may be formed during its oxidation in an autoxidizing lipid system. Alkaline L-proline was oxidized with 30% hydrogen peroxide with sodium tungstate catalysis. The sample was immediately frozen and the ESR spectra observed at -10 C. The 18 line spectrum is shown in Figure 1A. From the coupling constants (Table IV) the spectrum was interpreted to show two equivalent protons

TABLE IV

ESR Coupling Constants of Oxidized L-Proline and Oxidized N-Hydroxyglycine

Compound	Coupling constants (Gauss) AH	AH_1	AH_2	No. of lines
L-proline	15.5	17.7	21.1	18
N-hydroxyglycine	13.6	13.6	11.6	12

[a]See text for experimental details.

having the same coupling constant and third proton with a smaller coupling constant due to the proton alpha to the carboxyl group. These results are consistent with the nitroxide which could occur via an N-hydroxylamine intermediate (22). The nitroxide radical could account for the antioxidant properties previously described (16). Attempts to synthesize or isolate the N-hydroxyproline have to date been unsuccessful.

Several N-hydroxyamino acids have recently been found to occur naturally (14). A sample of N-hydroxyglycine was found to have measurable antioxidant activity (15) despite its apparent insolubility in the substrate. The oxidation of N-hydroxyglycine with 30% hydrogen peroxide resulted in a 12 line spectra (Fig. 1B). The coupling constant (Table IV) for the nitrogen proton was the same as for the nitrogen. The methyl protons had coupling constants of 11.06 G. This is consistent with the structure of a nitroxide radical formed by the abstraction of the hydroxyl proton. These observations suggest that naturally occurring N-hydroxyamino acids or amino acids that can be oxidized to N-hydroxyamino acids in vivo, may play an intermediate antioxidant role in animal or plant tissues.

Such a mechanism would be favorable for survival in biological systems. The nitroxide radical formed from the N-hydroxyamines would be an effective scavenger for alkyl radicals. However since all of our antioxidant observations were made in the anhydrous state, it remains to be determined whether similar results can be demonstrated in aqueous or tissue systems.

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Retarded Hydrolysis by Pancreatic Lipase of Seed Oils With *Trans*-3 Unsaturation

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ABSTRACT

Triglycerides containing acids with *trans*-3 unsaturation ($16{:}1^3$, $18{:}1^3$ and $18{:}3^{3,9,12}$) showed a marked retardation of reaction rate with pancreatic lipase. An inverse relationship was found between content of *trans*-3 unsaturated acids in seed oils and lipolysis rate. The *trans*-3 unsaturated acids were concentrated in the diglyceride and residual triglyceride fractions of the lipolysates, and only small amounts were present in the free acid and monoglyceride fractions.

INTRODUCTION

Although pancreatic lipase (E.C. 3.1.1.3) is used in establishing the position of acyl groups in triglycerides of certain oils, it is recognized that there are differences in the rates of hydrolysis of some triglycerides (1,2) which can lead to ambiguities in the structures derived from the data. Triglyceride lipolysis rates can vary because the structure of the acyl group may affect the reaction at the ester linkage (3,4) or the reaction of the entire triglyceride molecule (5,6).

Studies with triglycerides containing acids with functional groups close to the carboxyl group have previously shown reduced rates of hydrolysis with pancreatic lipase. This reduction has been found with triglycerides containing 5,6-double bonds (namely, whale oil) (3), *cis*-6-octadecenoic acid (4), or 2,2-dimethyl stearic acid (7). Our studies demonstrate marked retardation of hydrolysis of triglycerides containing acids having *trans*-3 unsaturation.

MATERIALS AND METHODS

A range in concentration of triglycerides with *trans*-3 unsaturation was obtained by using seed oils of *Grindelia oxylepis* (8), *Aster hayatae*, *Calea urticaefolia* (9) and *Stenachaenium macrocephalum*, all members of the family Compositae (Table I). Typical crambe (*C. abyssinica*) and soybean oils were used as reference materials.

Stenachaenium oil (0.37 g) was partially randomized by refluxing the oil in benzene for 22 hr with *p*-toluenesulfonic acid (8.7 mg) as the catalyst. Attempts to use sodium methoxide as the randomization catalyst produced a complex mixture of products.

Oils were hydrolyzed by pancreatic lipase by the method of Mattson and Volpenhein (11). Samples weighing 250 mg were hydrolyzed with 80 mg of commercial Steapsin in 4.5 ml 1 M tris(hydroxymethyl)aminomethane buffer at pH 8.0, 0.5 ml 22% $CaCl_2$, and 1.0 ml 0.1% sodium cholate solution. The mixture was emulsified at 40 C in a 50 ml round-bottom flask by rapid stirring with a 1/2 in. magnetic bar. Samples weighing 50 mg were emulsified in a 1 x 2 3/4 in. screw-cap vial containing 1 ml 1 M tris(hydroxymethyl)aminomethane buffer solution at pH 8.0, 0.1 ml 22% $CaCl_2$, 0.25 ml 0.1% sodium cholate solution and 10 mg lipase. The mixture was stirred rapidly with a magnetic stirrer or a mixer giving vortex action.

Lipolysis was terminated by acidification with HCl. The reaction mixture was then immediately extracted three times with diethyl ether. The ether solution was washed with water and dried over sodium sulfate. Solvent was removed under nitrogen on a steam bath.

A portion (10-25 mg) of the recovered lipid material was titrated in neutral ethanol with 0.01 N NaOH to determine the amount of free acid present. Calculation of the percentage of free fatty acids in the mixture (i.e., per cent hydrolysis of the oil) was based on the mean molecular weight of these acids determined by gas liquid chromatography (GLC) of their methyl esters.

Lipolysis products were separated by preparative thin layer chromatography (TLC) on 20 x 20 cm plates spread with a 1 mm layer of Silica Gel G. The slurry was prepared with a saturated solution of boric acid. The plates were developed with hexane-diethyl ether (70:30 v/v). Bands were detected under ultraviolet light with Rhodamine 6G or dichlorofluorescein as the fluorescent indicator. In subsequent workup, total monoglycerides and total diglycerides were treated as single fractions. No attempt was made to isolate individual partial glycerides in either fraction.

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TABLE I

Lipolysis Data[a]

Acid[b]	Per cent in oil	Per cent in monoglyceride	Per cent in diglyceride	Apparent extent of hydrolysis from 1,3-positions, %
Grindelia oxylepis				
16:0	9.6	1.6	1.4	92
$16{:}1^{3t}$	15.1	5.3	47.7	9
18:0	2.9	0	0.5	123
18:1	14.6	17.3	12.4	88
$18{:}1^{3t}$	1.9	0	3.7	0
18:2	54.0	75.8	30.7	110
Aster hayatae				
16:0	5.6	2.6	1.2	110
$16{:}1^{3t}$	10.4	4.2	21.4	13
18:0	1.7	0.2	--	127
18:1	6.9	11.1	4.7	98
$18{:}1^{3t}$	2.0	0	4.0	0
18:2	62.7	76.7	45.0	68
$18{:}3^{3t,9c,12c}$	10.8	5.2	23.6	16
Calea urticaefolia				
16:0	9.0	1.9	0.4	90
18:0	3.9	1.2	0.3	91
18:1	5.3	8.6	4.0	104
18:2	46.3	75.5	47.9	54
$18{:}3^{3t,9c,12c}$	35.5	12.2	47.4	5
Stenachaenium macrocephalum				
16:0	3.6	1.5	0.5	120
18:0	2.5	0.4	--	127
18:1	6.3	9.0	4.9	48
18:2	38.0	66.4	45.3	41
$18{:}3^{3t,9c,12c}$	48.6	21.6	49.4	8
Randomized *Stenachaenium macrocephalum*				
16:0	3.6	2.4	0.4	116
18:0	2.5	2.2	1.5	143
18:1	6.3	8.8	5.4	41
18:2	38.0	47.5	41.8	30
$18{:}3^{3t,9c,12c}$	48.6	39.2	50.9	8

[a]Hydrolysis at 40 C for 20 min.

[b]Small amounts of presumed $18{:}2^{3t,9c}$ (10) were observed in the ester from *Grindelia, Aster* and *Stenachaenium* oils by $AgNO_3$-TLC.

Methyl esters of lipolysate fractions were prepared by scraping individual areas from the TLC plate into a 100 ml round-bottom flask, covering the Silica Gel G with anhydrous ether, adding 10 ml of 5% HCl-methanol, and refluxing the mixture for 3 hr (12). The mixture was then transferred to a 125 ml separatory funnel and extracted with ether; the ether solution was washed with water. Benzene was added to remove any water azeotropically and esters were recovered by evaporating the solution to dryness under nitrogen on a steam bath.

Methyl esters were analyzed with an F&M Model 810 gas chromatograph equipped with a flame ionization detector and a 10 ft x 1/8 in. column packed with 20% LAC-2-R 446 held at 200 C.

A 260 mg sample of *G. oxylepis* oil was hydrogenated with 10% palladium on charcoal as the catalyst and ethanol as the solvent. Lipolysis of the hydrogenated oil (solid) with pancreatic lipase was performed as described above, but at 40 C for 120 min and at 55-60 C for an additional 120 min.

Rates of hydrolysis were determined on 100 mg samples by measuring the alkali consumption at constant pH (13).

Soybean and *Grindelia* oils (125 mg) were hydrolyzed with 10 mg of castor bean lipase (E.C. 3.1.1.3) at room temperature for 4 hr (14). The lipolysates resulting were handled in the same manner as those from the reaction with pancreatic lipase.

To measure the relative resistance of individual acids to lipolysis with pancreatic lipase, the amount of a given acid released was calculated as a percentage of the apparent total of

TABLE II

Rate of Hydrolysis of Individual Oils
(Initial Slope of Curve From Radiometer pH-Stat)

Oil	Free acids released/min, meq	Δ3 Acids in oil, wt %
Crambe abyssinica	1.05	0
Soybean	0.95	0
Grindelia oxylepis	0.77	17.0
Aster hayatae	0.67	23.2
Calea urticaefolia	0.59	35.5
Stenachaenium macrocephalum	0.36	48.6

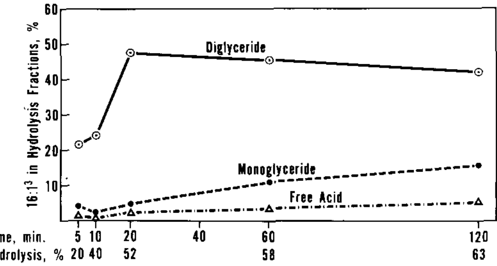

FIG. 1. Proportion of $16{:}1^3$ in lipolysis fractions of *G. oxylepis* oil with increasing time.

this acid in the 1,3-positions. The following equation was used in making this calculation:

Per cent of a particular acid in 1,3-positions released =

$$100 \times \left[\frac{\text{Per cent of the acid found in free acid fraction} \times \text{per cent hydrolysis of the oil}}{\text{Apparent proportion of acid in 1,3-positions} \times \text{per cent in original oil}}\right]$$

where the apparent proportion of acid in 1,3-positions is the percentage of the total of the acid in the oil that appears to be in these positions as determined by difference from the composition of the oil and of the monoglycerides obtained after lipolysis for 20 min.

RESULTS AND DISCUSSION

The acid compositions of *Grindelia oxylepis* (8), *Aster hayatae, Calea urticaefolia* (9) and *Stenachaenium macrocephalum* oils are listed in Table I. Identifications of the *trans*-3 unsaturated methyl esters from *A. hayatae* and *S. macrocephalum* were made by comparison of the mobility of these esters on GLC and TLC ($AgNO_3$ impregnated) with the mobility of known compounds from *G. oxylepis* ($18{:}1^3$ and $16{:}1^3$) (8) and *C. urticaefolia* ($18{:}3^{3,9,12}$) (9). In addition, nuclear magnetic resonance of the *A. hayatae* and *S. macrocephalum* oils showed a doublet at τ 7.0, indicative of the presence of 3,4-unsaturation (8). Infrared, with a strong band at 10.36 μ, disclosed the presence of isolated *trans* unsaturation. Interrupted ozonolysis of the 18:3 from *S. macrocephalum* proved this ester to be $18{:}3^{3,9,12}$ (15). The presence of *trans*-3 unsaturated acids in seed oil of *A. hayatae* is in agreement with the recent demonstration of them in several other species of *Aster* (10).

The retarded hydrolysis by pancreatic lipase of oils containing *trans*-3 unsaturation became apparent during an investigation of *G. oxylepis* oil in which 15% of the acyl groups is *trans*-3-hexadecenoic acid and 2% is *trans*-3-octadecenoic acid (8). In one reaction of this *Grindelia* oil with pancreatic lipase, only 44% of the lipolysate mixture was composed of free acids; whereas with crambe oil, run under identical conditions, 59% free acids was produced. Thirty per cent of the *Grindelia* oil lipolysate was made up of diglycerides and 11% residual triglycerides was left vs. 10% diglycerides and 3% triglycerides from the crambe oil. Analysis of the *Grindelia* oil lipolysate fractions gave further evidence of an abnormal reaction with pancreatic lipase in that both the monoglyceride and free acid fractions were low in $16{:}1^3$, whereas the diglyceride and triglyceride fractions were enriched in this same acid. *G. oxylepis* oil contained 2% $18{:}1^3$, which was also hydrolyzed at a slow rate. Only trace amounts were ever found in either monoglyceride or free acid fractions, but increased amounts were found in both the diglyceride and residual triglyceride fractions.

To test the hypothesis that *trans*-3 unsaturation is indeed the cause of retardation of lipolysis, four oils with a range of *trans*-3 unsaturated acid concentration were treated with pancreatic lipase under constant pH in a recording pH-stat to determine initial rates of hydrolysis (13). Results from these analyses (Table II) show an inverse relationship between the concentration of *trans*-3 unsaturated acids and rate of lipolysis; the *Grindelia* oil has the least amount of such acids (17%) and the fastest rate (0.77 meq free acid per minute); the *Stenachaenium* oil, the most *trans*-3 unsaturated acids (49%) and the slowest rate (0.36 meq free acid per minute). As with *Grindelia* oil, *trans*-3 unsaturated acids were concentrated in the residual triglyceride and the diglyceride fractions from the other three oils. Amounts of these acids found in the diglycerides after a 20 min hydrolysis were all about 50% (Table I).

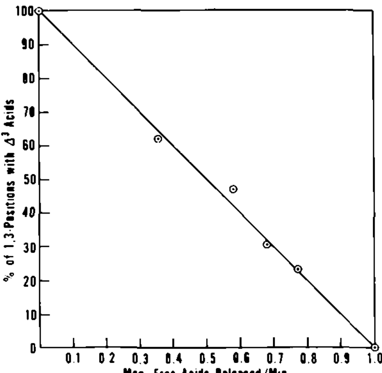

FIG. 2. Rate of lipolysis vs. apparent percentage of *trans*-3 acids esterified at the 1,3-positions of glycerol.

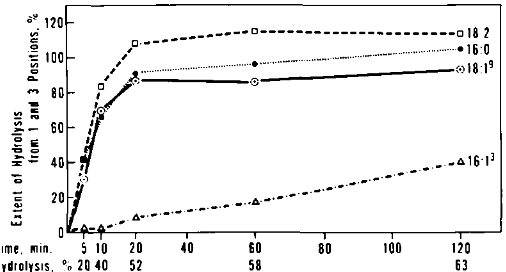

FIG. 3. Apparent extent of hydrolysis by pancreatic lipase of individual acids from 1,3-positions of *G. oxylepis* oil with increasing time. See text for calculations.

The increase to this level indicates that the proportion of *trans*-3 unsaturated acids removed by lipolysis was much less than for the other acids.

Figure 1 shows the contrast in *trans*-3 unsaturated acid content of lipolysate fractions from *G. oxylepis* oil as a function of time. When 50 mg samples of this oil were lipolyzed to different extents, the proportion of $16{:}1^3$ in the monoglycerides increases little from 5 to 20 min, or from 20% to 52% hydrolysis (the decrease at 10 min is unexplained), but from that point, the percentage of $16{:}1^3$ increases, and at the end of 120 min the concentration of this acid in the monoglyceride fraction is greater than in the original oil. The percentage of $16{:}1^3$ in the diglyceride fraction increases to 48% after 20 min and decreases to 42% after 120 min hydrolysis. If migration of acyl groups occurred in the mono and diglycerides, and if the usual acids were hydrolyzed much more rapidly than $16{:}1^3$, the $16{:}1^3$ would be enriched in the monoglyceride fraction, but the fraction would include both 1- and 2-monoglycerides. This migration would more likely be significant after an extended reaction time and, presumably, is the reason for the change in composition in the monoglycerides of *G. oxylepis* after 20 min.

Brockerhoff (5) reported two types of retardation of lipolysis of triglycerides with pancreatic lipase. He demonstrated that the structure of the fat droplets affected the rate of hydrolysis of human depot fat. During this lipolysis, stearic acid was concentrated in the resulting diglyceride, an indication that stearic acid was hydrolyzed at a slower rate than other acids. Inclusion of hexane in the hydrolysis reaction mixture abolished this discrimination against stearic acid. This response suggests that the decreased rate of lipolysis of human depot fat is a physical phenomenon. Brockerhoff (5) also found that 20:5 and 22:6 acids (both with double bonds in the 5,6-position, i.e., relatively close to the carboxyl group) from marine oils were also concentrated in the diglycerides upon pancreatic lipolysis. Inclusion of hexane, however, did not prevent this accumulation. The effect, presumably, is not the result of the physical factors restricting the lipolysis of human depot fat. Bottino et al. (3) showed that there was a similar resistance to hydrolysis of synthetic 1,2-dioctadecenoyl-3-eicosapentaenoyl glycerol when treated with pancreatic lipase. They found little monoglyceride formed during lipolysis and only 17% 20:5 among the liberated acids.

To ascertain if increased emulsification would change the hydrolysis rate of oils containing *trans*-3 unsaturated fatty acids, we treated soybean and *C. urticaefolia* oils with pancreatic lipase while they were mixed vigorously with a mixer generating vortex action. Under these conditions soybean oil was 45% hydrolyzed in 6 min at room temperature, whereas with the magnetic stirrer initially employed 20 min at 40 C was required to obtain 55% hydrolysis. Similarly, the *Calea* oil was 23% hydrolyzed in 6 min of vigorous mixing and only 31% after 20 min with magnetic stirring at 40 C. Although a quantitative comparison cannot be made because of the different conditions required, it is apparent that the increased emulsification hastened the hydrolysis of both oils but did not eliminate the difference between them. This result suggests that the retardation of lipolysis for the *Calea* oil is more analogous to that for triglycerides containing 20:5 and 22:6 acids than to that for human depot fat.

Sampugna et al. (6) reported an interesting contrast in the behavior of synthetic glycerides containing butyric acid. Butyrate triglycerides hydrolyzed more rapidly than triolein, whereas

lipolysis of diglycerides with butyryl groups was retarded. The slow initial rates given in Table II show that the inhibitory influence of *trans*-3 unsaturation is operative at the triglyceride stage. The accumulation of diglycerides containing *trans*-3 unsaturated acids indicates that retardation is also operative at the diglyceride stage.

An intermolecular effect on lipolysis due to the presence of *trans*-3 unsaturated acids and arising via some mechanism other than the ones responsible for the behavior of either human depot fat or butyrate glycerides cannot be ruled out. However, additional evidence presented below supports the alternative view that we are dealing with a specific inhibition of enzymatic hydrolysis of the ester linkages by which *trans*-3 unsaturated acyl groups are attached to glycerol. The presumption is that this inhibition is caused by the presence of this functional group near the site on which the enzyme must act. In view of the positional specificity of pancreatic lipase (16), such an explanation implies that a substantial portion of the *trans*-3 unsaturated acids are in the 1,3-positions.

That a short exposure to lipolysis produced monoglycerides representative of the 2-position is suggested by the results obtained by lipolysis of hydrogenated *G. oxylepis* oil. Since these solid triglycerides (mp 55-56 C) were difficult to hydrolyze, they were reacted for 2 hr at 40 C and 2 hr at 55-60 C. Even with this long a reaction time only 30% hydrolysis took place. Eliminating unsaturation should nearly equalize the rates at which the acids were hydrolyzed and, therefore, a monoglyceride fraction approximately representative of the 2-position should result. The composition of these monoglycerides showed 6% 16:0, a percentage which agreed well with the total C_{16} acids found in the monoglycerides formed from the unsaturated oil after hydrolysis for 20 min (2% 16:0 + 5% $16{:}1^{3}$). Thus, 20 min hydrolysis of *G. oxylepis* oil produced monoglycerides apparently representative of the acids esterified at the 2-position.

The apparent percentage of *trans*-3 unsaturated acids in the 1,3-positions of the individual oils can be calculated by difference from the mono- and triglyceride compositions given in Table I. When data so calculated are plotted against the rates of lipolysis of the oils (Table II), the points fall near a straight line, as shown in Figure 2. The two extreme points represent two assumptions: At 100% *trans*-3 unsaturated fatty acids in the 1,3-positions, the rate would be zero; at 0% of such acids in these positions, the rate would be approximately 1.0 meq of acids released per minute (from the rates for crambe and soybean oils given in Table II). In view of the method of derivation of the intermediate points, their distribution along the straight line connecting the extremes lends further credibility to the belief that monoglycerides from the 20 min lipolyses were representative of 2-position acids in the original oils.

Assuming the 20 min monoglyceride composition is representative of the 2-position of the triglycerides of *Grindelia* oil, we can calculate the extent of hydrolysis of individual acids from the 1-3 positions of the molecule as explained under Materials and Methods. We show this hydrolysis for four acids as a function of time in Figure 3, which is a graphic display of the relative resistance of individual acids to release during lipolysis of *G. oxylepis* oil. The amount of the $16{:}1^{3}$ acid released is much less than that of the usual acids at any given time. Hydrolysis of more than 100% of the 18:2 from the 1,3-positions suggests migration from the 2 position and subsequent hydrolysis. In the other three oils, percentage hydrolysis of 18:2 from the 1,3-positions decreases as the amount of *trans*-3 unsaturated acids increases. The reason for this decrease is unknown, but one factor may be inhibition of migration of 18:2 from the 2-position as the amount of *trans*-3 unsaturated acid increases and available 1,3-positions decrease. Besides 18:2, other examples of more than 100% hydrolysis in Table I involve minor components (especially 18:0) and are probably related to errors in the chromatographic method.

Complete randomization of *Stenachaenium* oil was not attained. However, the apparent percentage of palmitic acid in the 2-position increased from 1.5 to 2.4 and the $18{:}3^{3,9,12}$ from 22 to 39. The extent of hydrolysis of individual acids from the 1,3-positions was similar to that for the natural *Stenachaenium* oil. In both, only 8% of the $18{:}3^{3,9,12}$ in the 1,3-positions was hydrolyzed.

Castor Lipase Hydrolysis

Oils from *G. oxylepis* and soybean were hydrolyzed with castor bean lipase (14). Ninety-two per cent of the soybean oil was hydrolyzed in contrast to 71% of *G. oxylepis* oil. Analysis of the castor lipase hydrolysis fractions from *G. oxylepis* shows an increase in amount of $16{:}1^{3}$ acid in both the residual triglyceride (25%) and diglyceride fractions (31%) and a reduction in the free acid fraction (9%). The resistance to hydrolysis of $16{:}1^{3}$ with castor lipase suggests that the same factors which limit the hydrolysis of *trans*-3 acids with pancreatic lipase are in effect when castor lipase is used.

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Variation in Lipase Activities During Germination (in Dark) of γ-Irradiated Castor-Seeds

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ABSTRACT

A special variety of *R. communis* selected for the present investigation, indicated two types of lipases. One of these, is active at pH 4.5 and another at pH 6.8. The acid lipase was maximum in dry seeds. The control and the irradiated seeds when germinated in dark showed a decrease in acid lipase. The decrease in acid lipase was dependent on the period of germination and the applied irradiation dose. The presence of a de novo synthesis of another activity measured at pH 6.8 was found after 24 hr of germination of the control as well as the irradiated seeds. The new activity termed as a neutral lipase showed a gradual increase with the period of germination. Irradiation played a characteristic role in the production of this activity.

INTRODUCTION

In oleaginous seeds lipase activity is manifested upon germination, but in castor bean there is also an active lipase in the resting seeds. Green (1) was the first to establish the existence of highly active lipase described as Ricinus lipase in the germinating castor seeds.

Ramakrishnan et al. (2,3) made a comparative study of lipases from several oil seed cakes and found that the castor and peanut cake lipases are the most active. The acetate buffer system was the most effective at the optimum pH.

Yamada (4) found blastolipase (reacting at pH 6.8) in the embryo during the first 48 hr of germination of castor beans. In addition to the neutral lipase, an acid lipase is also reported in the endosperm of the dry seeds. Angelo and Altschul (5) failed to note the development of a neutral lipase in germinating castor seeds.

Previous work (6) on the behavior of lipase activity of γ-irradiated peanut indicated that different dosage levels have different effects on the liberation of the lipase activity. The increase in activity has been explained on the basis of other metabolic products.

A little information is available on the effect of ionizing radiation on the lipase activity on germination of castor seeds. There are also contradictory views regarding the production of the acid and the neutral lipases. The present work has been undertaken to study the effect of γ-irradiation on the lipase activity and also to correlate the metabolism of castor seeds during germination.

EXPERIMENTAL PROCEDURES

Irradiation and Germination of Castor (Ricinus communis, L) Seeds

A special variety of castor seeds S-20 obtained from the Institute of Agriculture, Anand, (Gujaret State, India) was used in the present work. The seeds were irradiated with different dosage levels of γ-rays from a ^{60}Co-source (1000 k.curies) located at the Bhabha Atomic Research Center (BARC), Trombay, India. The following dosage levels were selected for the work: 10, 30, 50, 70, 90 and 120 kiloroentgen (kr). Control and irradiated seeds were weighed individually and then sown in chemically purified and sterilized sand after piercing a hole in each seed. This enabled uniform germination. Germination was carried out in darkness in a double walled chamber maintained at constant temperature of 30 ± 1 C. No other nutrients except distilled water was given daily in a measured quantity to the germinating seedlings. The periods of germination selected were: 0, 1, 2, 3, 5, 7 and 9 days. At the end of each period four sets each consisting of 10 seedlings from each treatment were removed from the sand, cleaned with cold distilled water and used for the extraction of the enzyme source.

Preparation of Enzyme

The method adopted for the preparation of the lipase enzyme is essentially the same as described by Patel et al. (6).

After the removal of testa, 10 cleaned seedlings were crushed with cold acetone in a mortor and pestle for about 10 min. The homogenates were filtered in the cold and washed with cold acetone until free of oil. The residuals, finely crushed plant materials were dried in a vacuum desiccator, weighed and stored cold until ready for assay. Ungerminated castor seed cakes were prepared by the same method.

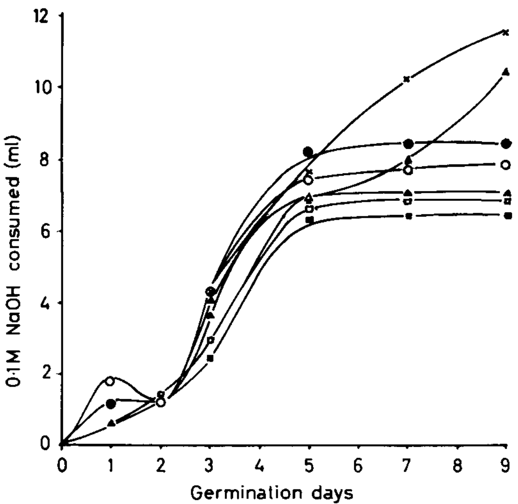

FIG. 1. Activity of acid lipase during germination. Radiation dose: ○ Control, ● 10 kr, x 30 kr, △ 50 kr, ▲ 70 kr, □ 90 kr, ■ 120 kr.

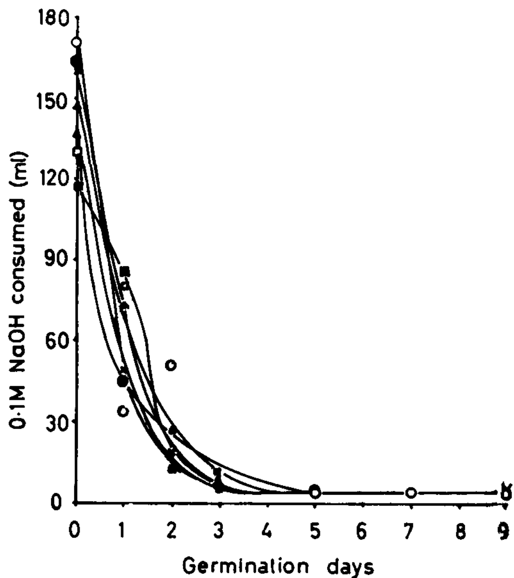

FIG. 2. Activity of neutral lipase during germination. Radiation dose: ○ Control, ● 10 kr, x 30 kr, △ 50 kr, ▲ 70 kr, □ 90 kr, ■ 120 kr.

Lipase Assay

As a source of glyceride freshly extracted peanut oil was used. One gram of oil was taken in a glass-stoppered Erlenmeyer flask and, to that, 5.0 ml of 0.1 M tris-acetic acid buffer (pH = 4.5) were added. The contents were stirred for 5 min to have through mixing. Then 50.0 mg of the test material (enzyme) were added with vigorous shaking. Hydrolysis was carried out at room temperature (28-30 C) for 24 hr. During hydrolysis the contents of the flask were shaken continuously on a rotary shaker. At the end of the hydrolysis period, 10 ml of neutral 1:1 (v/v) ethanol-ether mixture were added to the flask. The liberated fatty acids were titrated against 0.10 M NaOH. The determination of blank (7) was also carried out in a similar manner without addition of the test materials from the beginning. The test material was added to the glyceride-buffer mixture after 24 hr. This was immediately followed by 10 ml neutral ethanol-ether mixture. Lipase activity has been expressed in terms of ml of 0.10 M NaOH after subtraction of the blank. From the weight of the total test material the activity per gram of the original seed was calculated. The variation in the activity of the neutral lipase was determined by the same procedure, except that the buffer used was kept at 6.8 pH. The results are presented in Fig. 1 and 2.

RESULTS

The results on lipase assay of two replications and among the four sets of each dosage levels showed relative errors of 5% in the activity of acid lipase and 0.5% in the activity of neutral lipase respectively.

Preliminary experiments pointed out that in resting seeds maximum lipase activity could be obtained at optimum pH = 4.5 in tris-acetic acid buffer. Fazio and Lado (8) also reported maximum activity in acetate buffer at pH = 4.7. In comparison to acetate, phosphate and other buffers, greatest activity was observed in tris-acetic acid buffer at pH 4.5 Hence this buffer was used to determine the activity of the acid lipase. It has been also experienced that there was no appreciable difference in the rate of hydrolysis (9) in the temperature range 25-40 C. Therefore in the present work the hydrolysis was carried out at room temperature (28-30 C).

Results on the activity of the acid lipase determined at pH 4.5 are shown graphically in Figure 1, which indicates the change in the activity with the period of germination. The activity of control as well as irradiated seeds decreased considerably on the first day of germination. The rate of decrease in the acid lipase on the first day of germination was different for each treatment. There was a sudden decrease in the activity of the control seeds, whereas the rate of decrease was slow with the increase in the dosage levels. The lipase of seeds irradiated with 120 kr units was highest on the first day of germination.

On the second day of germination the acid lipase of the control seeds was high and that of the irradiated seeds decreased proportionately. A little activity was obtained on the third and the subsequent days of germination. In general the acid lipase of all seedlings was found to decrease with an increase in the period of germination.

Results on the determination of the activity of neutral lipase (pH = 6.8) are shown graphically in Figure 2, which indicates the variation in the activity with the period of germination. The neutral lipase was absent in the resting seed and the lipase activity is newly formed during germination. On the first day of germination the neutral lipase of the control seeds was high in comparison to other treated seeds. The activity diminished in the control seeds, while it was found to increase in the irradiated seeds on the second day of germination. Thereafter the neutral lipase was found to increase gradually in all the seedlings. There was no significant increase in the activity of the control seeds and the seeds irradiated with 10, 70, 90 and 120 kr of γ-rays after the fifth day of germination. Whereas the activity of seeds irradiated with 30 and 50 kr dosage levels, showed a gradual increase up to the end of the observation periods.

DISCUSSION

The present study reveals the existence of acid lipase in the resting seeds. The activity referred to 1 g of seed at pH 4.5 was highest in the control seeds and was found to decrease with the increase in radiation dose. These observations are in agreement with those of Chmyr (10) who noted that irradiation of corn grains lowered the lipase activity. According to Meisel (11) this might be ascribed to the fact that radiation interferes with the function of mitochondria.

The acid lipase of all the seedlings was considerably decreased during the first 24 hr of germination. This reduction in activity was about 1/5 in the control and less than 1/2 of its original values in the irradiated seeds. Fazio and Lado (8) also experienced a similar decrease of lipase activity in castor seeds on germination.

The maximum activity of the acid lipase detected in the dry seeds may be explained as follows. Calabrese (12) reported that there was a continuous decrease in the amount of reducing sugars with concomitant increase in the fat content when the castor seeds were entering the last stages of maturation. A rapid increase of lipase activity was also noted during the same stage. Ricinus lipase has been shown to give noteworthy synthetic action (12). In the present study reducing sugars were not detected in dry seeds (13). In order to have high synthetic action of triglycerides, highly active lipase is needed. Hence the activity of acid lipase is maximum in dry seeds.

Even though fat is not utilized up to the third day of germination the acid lipase decreased considerably. This is because in the initial stage of germination, cell division does not occur and therefore the number of mitochondria may remain the same. The enzymes are related proteins and hence other chemical processes may lead to the interconversion of the enzymes into the forms suitable to enhance the process of germination. In the case of irradiated seeds this process might be affected to a great extent as the dose rate increases. The effect of irradiation is found to increase in the following manner: 10 kr seeds $<$ 30 kr seeds $<$ 50 kr seeds $<$ 70 kr seeds $\simeq$ 90 kr seeds $\simeq$ 120 kr seeds. These observations once again confirm the view of Miesel (11) regarding the interfering effect of irradiation on mitochondrial system. The decrease in the acid lipase is reflected in the increase of carbohydrates (13) and amino acids (14) which are found to increase in considerable amounts after the fifth day of germination. This view is in agreement with that of Padoa and Spada (15) who experienced a lowering of activity of the acid lipase by the action of amino acids.

Another lipase, called neutral lipase, has been detected at pH 6.8 in germinating seedlings. Resting seeds either the control or the irradiated ones have no neutral lipase activity. The neutral lipase first appeared after 24 hr of germination of the control and the irradiated seeds. In most of the cases this lipase increased gradually up to the fifth day of germination. The increase of the neutral lipase is nearly fourfold in control seedlings. It is nearly sevenfold in plants of seeds irradiated with 10 kr; whereas for seeds irradiated with higher dosage levels of γ-rays, it is more than 10-fold for the same period of germination. Fazio and Lado (8) also noted that the lipolytic activity at pH 6.8 increase during seven days of germination of castor seeds. Padoa and Spada (15) investigated that the lipase activity at pH 6.8 was strongest after seven days and disappeared after nine days. They also showed that amino acids lowered the activity at pH 4.6 and caused an increase in the activity at pH 4.8. The increase in the neutral lipase up to five days of germination can be ascribed to the increase in the amount of amino acids (14) during this period.

It should be noted (Fig. 2) that maximum activity of the neutral lipase found on the fifth day of germination has the following trend: 10

kr seeds > control seeds > 70 kr seeds > 90 kr seeds > 120 kr seeds. In case of 30 and 50 kr treated seeds, the neutral lipase increased up to the end of the observation period. The dosage level of 10 kr of γ-rays has stimulatory effect on the activity of the neutral lipase.

The rate of increase in the activity to attain maximum value is different for the irradiated seeds (Fig. 2). This difference may be explained by assuming that in irradiated seeds, the active centers (6) whatever number initially present are damaged in proportion to the applied dose. This results in decreased rate of production of the neutral lipase. Continuous increase in neutral lipase of seeds irradiated with 30 and 50 kr dosage levels may be explained as follows. It is assumed that the radiation damage of the active centers in these seeds is slight and that as germination proceeded the interfering effects of irradiation on mitochondrial activity become less pronounced. This could explain the increase in the activity of these seeds up to the ninth day of germination. In the case of 10 kr level the lipase activity follow the trend that of the control seedlings. The activity of 10 kr seedlings do not follow the trend of 30 and 50 kr seedlings. It was observed that the growth of seedlings of 10 kr dosage level was similar to that of the control seedlings; whereas in 30 and 50 kr dosage levels, delayed growth was noted. In the initial stage of germination certain amount of activity may be required by the seedlings for normal growth. If this is not available the growth may be inhibited. Since lipase is affected by irradiation, the growth of 30 and 50 kr irradiated seeds is poor. As the germination period is increased other metabolic factors play a role in producing the lipase activity. Thus the lipase activity of 30 and 50 kr seedlings has been found to increase, up to the end of the observation period of nine days. It is noted that 10 kr dosage level induces little effect of irradiation in castor seedlings. Hence all metabolic products follow the trend of control seeds.

The present work confirms the presence of neutral lipase, in contradiction to the observation made by Angelo and Altschul (5), who stated that the recorded observation of neutral lipase (4) might be due to the adventitious growth of molds, sometimes not visible to the eye. The FFA content is lowered by raising the pH and adding *p*-chloromercuribenzoate (*p*-CMB) by these investigators. In the present study, the germination was carried out in sterilized medium and sufficient care was taken to keep the laboratory also free of bacteria; no neutrients except glass distilled water were added to the germinating seedlings; therefore molds had no chance to grow. This was also further visualized by the fact that FFA never accumulated during the period of germination. It is likely that the activity of the neutral lipase might have been inhibited by the presence of *p*-CMB and hence the previous investigators may have failed to detect the neutral lipase.

Irradiated seeds showed a comparatively poor rate of germination with an increase in the irradiation dose as compared to the control seeds. Even in that case lipase activity increased. In the initial stage of germination a certain amount of activity may be needed by the seedling for normal growth and other metabolic features. If this is not available the growth may be inhibited. Since lipase is affected by irradiation, as mentioned previously, the growth of irradiated seeds may be poor. Further, with the increase in the period of germination, other metabolic factors, such as carbohydrates, free amino acids and ascorbic acid (AA), which play a part in producing lipase activity (6), are found to increase (14,16). Hence this increase in lipase at the later stages is ineffective for promoting growth. Thus it is assumed that growth might not be the only criteria for the increase of lipase and vice versa. Various metabolic products have their mutual effect on each other and are responsible for the observed increase in the activity. These observations confirm the view stated by previous workers (6).

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Corticoid Release and Gluconeogenesis Following Triglyceride Ingestion in the Rat

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ABSTRACT

Following a 5.0 ml triglyceride (cottonseed oil) gavage, there was a 60% elevation in the blood glucose concentration by the seventh hour in naturally occurring hypertriglyceridemic rats of the Long Evans strain. Glycogenolysis from liver or gastrocnemius muscle glycogen did not seem to be the source of this glucose. A significant elevation in the plasma total amino acid concentration concomitant with a peak in the blood urea nitrogen concentration by the fifth hour after gavage implicated gluconeogenesis. A prolonged plasma total corticoid elevation from hours 1 1/2 through 3 post-ingestion, which did not occur in non-fed or mineral oil-fed cohorts, supported gluconeogenesis. The serum total protein concentration rose significantly and progressively to a peak by the eighth hour due to an elevation in the albumin concentration. These elevations in serum proteins appeared to support glucocorticoid mediated gluconeogenesis. Adrenalectomy appeared to negate triglyceride-induced elevation in serum free amino acids, urea nitrogen, total protein and albumin.

INTRODUCTION

Previous studies revealed that triglyceride ingestion resulted in a 60% elevation in blood glucose by the seventh hour after such ingestion by naturally occurring hypertriglyceridemic rats. This elevation occurred concomitantly with a 317% rise in serum total free fatty acid. Adrenalectomy negated the elevation in blood glucose and significantly decreased the magnitude of the free fatty acid rise (1,2). In view of these findings it seemed desirable to determine the source of the glucose, and study some aspects of the control of the glucose synthesis.

MATERIALS AND METHODS

Male Long Evans rats from a light- and temperature-controlled inbred hypertriglyceridemic colony were maintained on Purina laboratory chow from weaning until they reached 200-250 g body weight (2-2 1/2 months of age). Initially, 200 rats were divided into two groups. One group was adrenalectomized by the method of Ingle and Griffith (3), ensuring that the gland was removed encapsulated, and the other group served as intact controls. Adrenalectomized rats were given no supportive therapy. Following an 18-hr fast (and five days postoperatively in the adrenalectomized rats), half of the rats in each group received 5.0 ml of cottonseed oil by gavage at 9 AM. At hourly intervals from 0 time through 9 hr, five rats from each of the four subgroups were exsanguinated by decapitation. The serum was analyzed immediately for total protein, albumin and total free amino acids by the biuret method of Weichselbaum (4), HABA dye method of Rutstein et al. (5), and the trinitrobenzene sulfonic acid method of Palmer and Peters (6), respectively. The standard errors for these methods were 0.05%, 0.05% and 0.11 mM/1 respectively. Total liver weights were recorded and a weighed aliquot digested

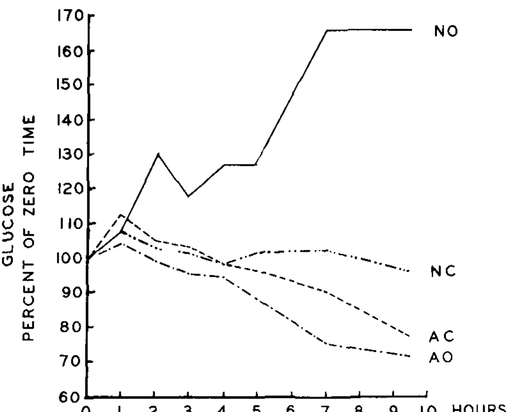

FIG. 1. The effect of triglyceride ingestion upon plasma glucose in intact and adrenalectomized male rats. NO, normal rats gavaged with 5.0 ml cottonseed oil; NC, normal rats without cottonseed oil; AC, adrenalectomized rats without cottonseed oil; AO, adrenalectomized rats gavaged with 5.0 ml cottonseed oil.

TABLE I

Effect of Triglyceride Ingestion on Liver and Gastrocnemius Muscle Glycogen in Intact and Adrenalectomized Rats[a]

	Intact		Adrenalectomized	
Hour	Oil	No oil	Oil	No oil
Liver glycogen, %				
0	0.091 ± 0.023[b]	0.091 ± 0.023	0.045 ± 0.002	0.045 ± 0.002
1	0.079 ± 0.041	0.065 ± 0.007	0.048 ± 0.011	0.048 ± 0.001
2	0.060 ± 0.052	0.063 ± 0.010	0.046 ± 0.010	0.054 ± 0.003
3	0.091 ± 0.041	0.050 ± 0.005	0.042 ± 0.001	0.044 ± 0.002
4	0.076 ± 0.028	0.085 ± 0.018	0.049 ± 0.011	0.051 ± 0.007
5	0.143 ± 0.028	0.051 ± 0.003	0.045 ± 0.005	0.045 ± 0.005
6	0.091 ± 0.043	0.099 ± 0.020	0.061 ± 0.018	0.043 ± 0.003
7	0.057 ± 0.015	0.087 ± 0.024	0.055 ± 0.002	0.040 ± 0.004
8	0.041 ± 0.011	0.102 ± 0.037	0.054 ± 0.006	0.043 ± 0.002
9	0.038 ± 0.005	0.050 ± 0.006	0.056 ± 0.011	0.044 ± 0.004
Muscle glycogen, %				
0	0.908 ± 0.070	0.908 ± 0.070	0.826 ± 0.040	0.826 ± 0.040
1	0.986 ± 0.070	0.809 ± 0.070	0.994 ± 0.058	0.867 ± 0.054
2	0.982 ± 0.038	0.959 ± 0.097	0.871 ± 0.097	0.900 ± 0.045
3	1.008 ± 0.067	0.823 ± 0.028	0.917 ± 0.018	0.790 ± 0.065
4	1.052 ± 0.137	0.931 ± 0.052	0.888 ± 0.036	0.930 ± 0.058
5	1.127 ± 0.099	0.798 ± 0.058	0.830 ± 0.025	0.800 ± 0.055
6	1.116 ± 0.132	0.644 ± 0.152	0.886 ± 0.008	0.780 ± 0.057
7	1.061 ± 0.040	0.877 ± 0.085	0.810 ± 0.106	0.800 ± 0.044
8	1.040 ± 0.074	0.937 ± 0.069	0.942 ± 0.040	0.750 ± 0.031
9	1.125 ± 0.063	0.873 ± 0.052	0.952 ± 0.106	0.808 ± 0.078

[a]Five rats per group in each time period.

[b]Mean ± S.D.

immediately in 30% potassium hydroxide for glycogen determination by the anthrone method of Seifter (7).

An additional group of 30 rats was used for the blood true glucose and urea nitrogen study. Ten intact rats received 5.0 ml of cottonseed oil (at 9 AM and after an 18 hr fast) one week, and served as their own controls the following week. Twenty rats were adrenalectomized and after five days ending with an 18 hr fast, 10 received 5.0 ml cottonseed oil and the remaining 10 served as controls. At hourly intervals for 9 hr approximately 100 μl of blood was taken via the tail in heparinized capillary tubes and the plasma analyzed immediately after separation for true glucose by an ultramicro modification of the glucose oxidase-peroxidase method (8-11) and for urea nitrogen by an ultramicro modification of the carbamidodiacetyl method of Marsh et al. (12), standard error 2.2 and 0.8 mg/100 ml, respectively.

A 10 rat cohort was used to study the effect of mineral oil (nonabsorbable and noncalorigenic), and cottonseed oil (absorbable and calorigenic) on the plasma total corticoid level. Each treatment was given one week apart at 9 AM following an 18 hr fast: the treatments were control (gavage needle only inserted into stomach); 5.0 ml cottonseed oil by gavage; and 5.0 ml mineral oil by gavage. The tails were clipped once at 9 AM, after which 50 μl blood samples were obtained in heparinized capillary tubes at 0, 1/2, 1, 1 1/2, 2, 3, 4, 5, 6, 7, 8 and 9 hr. The plasma was analyzed for total corticoids by the competitive protein binding method of Murphy (13), standard error 3.8 μg/100 ml.

RESULTS

In Figure 1, the plasma glucose response to triglyceride loading is depicted. For each response variable and for each animal, normalized values were obtained by computing the ratio of each value to the value at zero time (100%). This treatment materially stabilized the between animal variance observed at each measurement interval. A 29% rise in glucose was observed during the first 2 hr, $P < 0.01$, in the control rats. This was followed by a 10% drop, which was not significant, and a subsequent rise which at 7 hr was 64% above the value at zero time, $P < 0.05$. Plasma glucose remained at this level to at least 9.5 hr. In the absence of a fat load, there was an 8% rise at 1 hr, $P < 0.01$, after which the plasma glucose

TABLE II

Effect of Triglyceride Ingestion on Serum Total Free Amino Acids and Urea Nitrogen in Intact and Adrenalectomized Rats[a]

	Intact		Adrenalectomized	
Hour	Oil	No oil	Oil	No oil
Free amino acids (mM/liter)				
0	5.83 ± 0.25[b]	5.83 ± 0.25	6.46 ± 0.48	6.46 ± 0.48
1	5.93 ± 0.44	5.76 ± 0.33	6.12 ± 0.52	6.87 ± 0.51
2	6.06 ± 0.26	6.27 ± 0.19	6.31 ± 0.08	6.79 ± 0.43
3	6.04 ± 0.40	5.86 ± 0.30	6.41 ± 0.07	6.87 ± 0.58
4	6.26 ± 0.26	5.71 ± 0.19	5.99 ± 0.35	6.83 ± 0.40
5	6.81 ± 0.32[c,e]	6.06 ± 0.22	6.16 ± 0.48	7.57 ± 0.20
6	6.21 ± 0.27	5.66 ± 0.18	5.96 ± 0.43	6.95 ± 0.57
7	6.21 ± 0.20	5.85 ± 0.22	6.13 ± 0.40	6.87 ± 0.36
8	5.89 ± 0.27	5.82 ± 0.16	5.76 ± 0.39	7.12 ± 0.50
9	4.89 ± 0.20[d]	5.54 ± 0.26	6.05 ± 0.22	6.90 ± 0.49
Urea nitrogen (mg/100 ml)				
0	20.0 ± 1.0	20.0 ± 1.0	40.1 ± 2.1	40.1 ± 2.1
1	22.8 ± 0.9	22.3 ± 1.1	35.0 ± 1.7	42.1 ± 0.9
2	21.5 ± 0.9	16.1 ± 0.5	39.8 ± 1.2	39.6 ± 1.6
3	22.6 ± 0.5[c]	16.5 ± 0.8	35.9 ± 2.5	36.3 ± 2.7
4	23.6 ± 1.5[c]	15.5 ± 1.0	43.0 ± 2.7	37.6 ± 4.9
5	24.8 ± 1.4[c]	17.8 ± 1.2	41.2 ± 4.7	51.4 ± 4.1[c]
6	24.5 ± 1.5[c]	18.1 ± 1.2	58.5 ± 5.2	57.7 ± 2.2[d]
7	25.3 ± 1.5[c]	17.3 ± 1.4	43.0 ± 7.0	60.7 ± 2.0[d]
8	24.9 ± 1.0[d]	17.0 ± 1.5	63.9 ± 4.1[d]	65.7 ± 1.8
9	22.7 ± 1.0[c]	18.2 ± 1.6		77.5 ± 1.0[d]

[a]Five rats per group in each time period.
[b]Mean ± S.D.
[c]$P < 0.05$.
[d]$P < 0.01$.
[e]All statistical comparisons made to zero hour.

returned to and essentially remained at the zero time level. Adrenalectomy completely negated the glucose rise in response to the fat challenge and actually resulted in a significant drop in plasma glucose of 29% from the fasting level by 9.5 hr, $P < 0.01$. In the absence of a fat load, there was an apparent rise of 13% at 1 hr, which was not significant, followed by a significant drop of 23%, $P < 0.05$, by 9.5 hr. The response noted in the fat loaded and non-loaded adrenalectomized animals appeared similar. It would appear that adrenalectomy reverses the glucose response to fat load.

To determine whether the observed blood glucose elevation following triglyceride ingestion was the result of glycogen catabolism, liver and gastrocnemius muscle glycogen levels were determined at hourly intervals for 9 hr following a triglyceride meal (Table I). Liver glycogen levels were 0.091 ± 0.023% at zero time and did not vary significantly in either triglyceride-fed or control rats over the experimental period. Gastrocnemius muscle glycogen levels also remained at the zero time level of 0.908 ± 0.070% in both triglyceride-fed and control rats for the duration of the study period. Although liver glycogen appears to be lower in the adrenalectomized rats than in the intact rats, this difference was not statistically significant. Adrenalectomy did not appear to influence gastrocnemius muscle glycogen, nor did it alter the response of liver and gastrocnemius muscle glycogen to a triglyceride gavage. These data seemed to indicate that the rise in blood glucose following triglyceride ingestion was not due to a breakdown of liver or gastrocnemius muscle glycogen.

It has been reported previously that adrenalectomy prevented the glucose rise following triglyceride ingestion (1). Since glycogen was not the source of the glucose elevation, the adrenal catecholamines would not seem to have been involved, but the possibility existed that the additional glucose was synthesized from amino acids via gluconeogenesis due to the intervention of glucocorticoids. To test this possibility, serum free amino acid levels were determined (Table II). In the intact triglyceride-fed

TABLE III

Effect of Triglyceride Ingestion on Serum Total Protein and Albumin in Intact and Adrenalectomized Rats[a]

Hour	Intact		Adrenalectomized	
	Oil	No oil	Oil	No oil
Serum total protein (g/100 ml)				
0	6.21 ± 0.17[b]	6.21 ± 0.17	6.42 ± 0.15	6.42 ± 0.15
1	6.46 ± 0.25	6.38 ± 0.21	6.55 ± 0.18	6.24 ± 0.12
2	6.80 ± 0.10[d,e]	6.21 ± 0.24	6.97 ± 0.25	6.57 ± 0.17
3	7.03 ± 0.26[c]	6.27 ± 0.24	6.83 ± 0.27	6.66 ± 0.18
4	7.19 ± 0.26[d]	6.48 ± 0.19	6.74 ± 0.15	6.50 ± 0.09
5	6.94 ± 0.09[d]	6.39 ± 0.14	6.97 ± 0.11	6.64 ± 0.15
6	7.32 ± 0.38[c]	6.63 ± 0.21	7.04 ± 0.12	6.68 ± 0.11
7	7.30 ± 0.42[c]	6.40 ± 0.36	7.12 ± 0.31	6.68 ± 0.12
8	7.36 ± 0.47[c]	6.49 ± 0.24	6.92 ± 0.33	6.62 ± 0.08
9	7.14 ± 0.37[c]	6.37 ± 0.17	6.80 ± 0.20	6.73 ± 0.16
Serum albumin (g/100 ml)				
0	4.05 ± 0.19	4.05 ± 0.19	3.76 ± 0.17	3.76 ± 0.17
1	4.77 ± 0.43	4.18 ± 0.40	4.11 ± 0.38	3.50 ± 0.33
2	4.97 ± 0.37[c]	4.58 ± 0.36	4.51 ± 0.23	3.92 ± 0.30
3	5.29 ± 0.40[c]	3.79 ± 0.30	4.09 ± 0.21	3.89 ± 0.16
4	5.37 ± 0.61	4.11 ± 0.42	4.31 ± 0.23	3.76 ± 0.44
5	5.48 ± 0.45[c]	4.05 ± 0.38	4.22 ± 0.26	3.62 ± 0.29
6	5.15 ± 0.45[c]	3.77 ± 0.38	4.21 ± 0.12	3.72 ± 0.33
7	5.33 ± 0.42[c]	4.12 ± 0.20	4.26 ± 0.17	3.91 ± 0.28
8	5.60 ± 0.66[c]	4.05 ± 0.22	4.26 ± 0.17	3.36 ± 0.18
9	5.05 ± 0.20[d]	4.05 ± 0.40	3.83 ± 0.17	3.62 ± 0.11

[a]Five rats per group in all time periods.
[b]Mean ± S.D.
[c]$P < 0.05$.
[d]$P < 0.01$.
[e]All statistical comparisons made to zero hour.

rats, serum free amino acid levels rose from 5.83 ± 0.25 mM/l at zero time to 6.81 ± 0.32 mM/l ($P < 0.05$) by the fifth hour, and then significantly declined to 4.89 ± 0.20 mM/l ($P < 0.01$) by the ninth hour. Serum free amino acid levels in the controls did not significantly vary from the zero time level throughout the experimental period. The peak plasma free amino acid concentration at 5 hr was 0.75 mM/l greater in the triglyceride-fed rats than in the intact control rats ($P < 0.05$). At 9 hr, the free amino acid concentration in the triglyceride-fed rats was 0.65 mM/l lower than in the intact control rats ($P < 0.05$). Following adrenalectomy, no significant variation was noted from the zero time level in serum free amino acid levels in either the triglyceride-fed rats or the control rats over the 9 hr study period. Although the adrenalectomized control rats appeared to have a higher serum free amino acid concentration than their triglyceride-fed partners throughout the experimental period, this difference was not statistically significant due to the larger between-animal variability.

Since gluconeogenesis from amino acids would involve deamination of the amino acids with the concomitant formation of urea, the serum urea nitrogen concentration was examined (Table II). In the intact triglyceride-fed rats, urea nitrogen levels were significantly elevated over the zero time level of 20.0 ± 1.0 mg/100 ml for all time periods after 3 hr, $P < 0.05$; this elevation was also apparent when the triglyceride-fed animals were compared with the control animals at hourly intervals. Blood urea nitrogen was significantly higher in the adrenalectomized rats than in the intact rats at zero time, $P < 0.01$. In both the triglyceride-fed and control adrenalectomized rats there was a progressive increase in urea nitrogen concentration from the zero time level of 40.1 ± 2.1 mg/100 ml with time as death approached. All of the triglyceride-fed adrenalectomized rats died after the eighth hour of the experiment

TABLE IV

Effect of Absorbable and Non-absorbable Oils on Plasma Total Corticoid Levels, μg/100 ml[a]

Hour	Control	Mineral oil	Cottonseed oil, 5 ml
0	3.5 ± 4.8	2.4 ± 2.6	2.8 ± 2.8
½	35.8 ± 9.6[b,d]	46.3 ± 12.6[d]	36.9 ± 2.8[d]
1	21.2 ± 1.5[d]	30.8 ± 17.8[d]	46.8 ± 11.5[d]
1½	6.4 ± 1.5	12.0 ± 6.0[d]	32.8 ± 3.2[d]
2	5.6 ± 5.2	5.8 ± 6.1	40.1 ± 12.5[d]
3	3.3 ± 3.3	4.0 ± 6.3	43.0 ± 11.1[d]
4	10.9 ± 13.1	6.5 ± 4.9	23.5 ± 10.0[d]
5	21.3 ± 11.1[c]	3.7 ± 3.0	20.1 ± 7.4[d]
6	21.6 ± 10.1[d]	11.6 ± 9.6	28.0 ± 8.2[d]
7	21.1 ± 12.3[c]	23.9 ± 12.1[d]	33.3 ± 5.7[d]
8	21.3 ± 8.3[d]	38.5 ± 10.7[d]	33.9 ± 9.8[d]
9	29.6 ± 9.7[d]	32.1 ± 21.8[c]	27.9 ± 7.5[d]

[a]Five rats per group in all time periods.
[b]All statistical comparisons made to zero hour.
[c]$P < 0.05$.
[d]$P < 0.01$.

and all of the adrenalectomized controls died within 24 hr after completion of the experiment. The pattern of variability noted in serum free amino acids and urea nitrogen in the intact triglyceride-fed rats would seem to be indicative of amino acid metabolism and gluconeogenesis.

Glucocorticoids have been shown to elevate both the serum total protein and albumin concentrations (14,15). Thus, the effect of oil ingestion upon serum total protein and albumin was studied (Table III). In the intact triglyceride loaded rat, serum total protein and albumin were significantly elevated at all time periods after hour 2, $P < 0.05$. No significant variability was noted from the zero time levels in the intact control rats. In the adrenalectomized rats, no significant variability was noted in either the triglyceride-fed or the control rats.

The variation in plasma total corticoid levels was examined to confirm the possibility of corticoid intervention (Table IV). In the control, the total corticoid level rose from 3.5 ± 4.8 to a peak of 35.8 ± 9.6 μg/100 ml ($P < 0.01$) by 0.5 hr, returned to the zero time level by 1.5 hr, remained at this level to the fourth hour, rose to 21.3 ± 11.1 μg/100 ml by the fifth hour (2 PM) and remained at this level to the end of the experimental period. Following triglyceride gavage, the total corticoid level rose from 2.8 ± 2.8 μg/100 ml to 36.9 ± 2.8 μg/100 ml by 0.5 hr ($P < 0.01$), then further increased to 46.8 ± 11.5 μg/100 ml at 1 hr and remained elevated through hour 3, after which it declined to 23.5 ± 10.0 μg/100 ml by the fourth hour, and remained at this level to the end of the experimental period. The initial rise in total corticoids at 0.5 hr occurred in both groups and would seem to be due to the initial stress of handling. The total corticoid level was significantly higher in the triglyceride-fed rats than in the controls from hours 1 through 3 ($P < 0.01$), after which there was no significant difference for the remainder of the experimental period.

In order to determine whether the effect of a triglyceride gavage was a stress effect due to the distension of the gut, the same rats were gavaged with an equal volume of mineral oil one week after the triglyceride gavage (Table IV). Mineral oil did not cause the prolonged total corticoid elevation that was observed following triglyceride ingestion, indicating that the corticoid elevation noted after triglyceride ingestion was not simply an effect of stress caused by gut distension. The total corticoid levels following mineral oil gavage were almost identical to those of the controls.

DISCUSSION

It was noted that the 60% elevation in blood glucose found 7 hr after triglyceride ingestion could not be attributed to either liver or muscle glycogenolysis (Table I). Thus the glucose could have arisen either from the glycerol moiety of the triglyceride, from the fatty acid oxidation product, acetyl CoA, or from gluconeogenesis from protein. The prevention of the glucose rise by adrenalectomy (1) would appear to mitigate against either glycerol or acetyl CoA as the prime precursors since fat absorption and its metabolism have been noted to occur in the adrenalectomized rat (2).

Jacoby and LaDu (16) noted a marked increase in hepatic transaminase activity

following glucocorticoid administration. Feigelson and Greengard (17) reported a five-fold increase in hepatic tryptophan pyrrolase activity in rats approximately 4 hr after cortisone treatment. A similar increase in hepatic tyrosine-ketoglutarate transaminase activity was noted by Kenney (18,19). As Table IV indicates, maximum total corticoid levels were observed 1 hr after triglyceride gavage and maximal free amino acid levels occurred at 5 hr after the triglyceride load. Thus maximum serum free amino acid levels were noted 4 hr after the maximum corticoid levels. This time sequence is in agreement with that reported by Feigelson and Greengard and by Kenney. The triglyceride-induced plasma total corticoid elevation occurred well before the reported diurnal elevation: Guillemin et al. (20) and McCarthy et al. (21) showed that a diurnal increase in plasma corticoids occurs between 4 PM and 12 midnight in the rat. In the studies reported here, the triglyceride-induced plasma corticoid increase occurred between 9 AM and 12 noon. A diurnal elevation in agreement with the results of Guillemin et al. was noted in all rats tested, including mineral oil-fed and non-fed control rats.

The initial step in the conversion of a glucogenic amino acid to glucose would involve transamination followed by irreversible deamination (22) with subsequent formation of urea. A significant elevation in serum urea nitrogen was noted (Table II) in the triglyceride-fed rats. The highest concentration occurred at the fifth hour, concomitant with the peak concentration in free amino acids. Thus, the time sequence noted would appear to indicate that the rise in blood glucose (2 hr later) could be attributed to the deamination of amino acids and their conversion to glucose via gluconeogenesis.

In addition to the catabolic effects of corticoids, Tremolieres et al. (23) have shown that these hormones exert a marked protein anabolic effect in the liver. Feigelson et al. (24) reported maximum incorporation of 2-^{14}C-glycine into rat liver protein occurring approximately 4 hr after cortisone administration. Aschkenasy (15) and Hoch-Ligeti and Irvine (14) reported rises in serum albumin following glucocorticoid administration. White et al. (22) indicated that plasma albumin synthesis appears to be limited to the liver. The data in Table III indicate that a rise in total serum protein begins by the second hour following triglyceride ingestion and reaches a peak between the sixth and eighth hours. This rise in total protein can be almost entirely attributed to a rise in albumin. The rise in serum albumin occurs concomitant with a significant

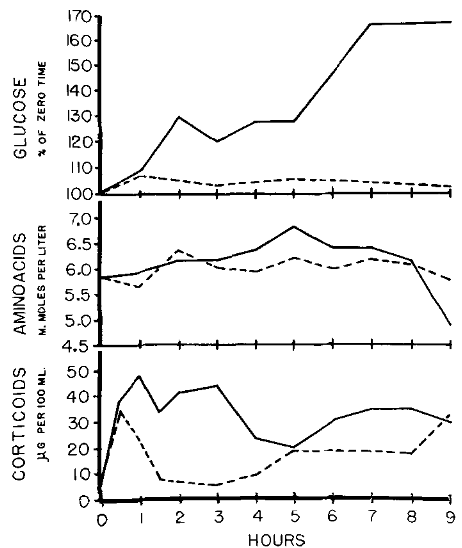

FIG. 2. The effect of triglyceride ingestion upon plasma glucose, amino acids and total corticoid concentrations in intact male rats. ——— intact rats gavaged with 5.0 ml cottonseed oil; - - - - - - intact rats without cottonseed oil.

precipitous rise in plasma free fatty acid (2) and could possibly be attributed to a requirement for a transport agent for the free fatty acid. In the adrenalectomized rat, there is a very significantly smaller rise in free fatty acid (2) and the albumin rise is quite small and not statistically significant.

In order to establish whether the corticoid release in response to triglyceride ingestion was simply a stress reaction due to distension of the stomach with the 5 ml of cottonseed oil, the rats were given mineral oil, a nonabsorbable oil. The effect in the presence of mineral oil was identical with that found in the control (Table IV). The prolonged elevation in plasma corticoid levels noted after the triglyceride gavage appeared to be in response to the triglyceride rather than to a simple stress reaction.

Thus the following major changes indicative of gluconeogenesis occurred following triglyceride ingestion (Fig. 2). The total plasma corticoid concentrations were elevated in the triglyceride-fed intact rats compared to intact controls from hours 1 through 3. At 5 hr the serum free amino acid concentration reached a peak in the triglyceride-fed rats, and then declined to a concentration below the zero time level by hour 9, indicating that the amino acids were being utilized at a faster rate than they were formed. By hour 7 the plasma true glucose concentration reached a peak value and remained

at this significantly elevated level, when compared to the zero time concentration as well as the concentrations in the intact controls at corresponding hours, until the end of the experimental period.

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The Relationship Between Eosinophilic Leukocytes and Phospholipase B Activity in Some Rat Tissues

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ABSTRACT

Spleen, peritoneal fluid, uterus and bone marrow of the rat have been studied for their content in phospholipase B (E.C. 3.1.1.5) and the presence of eosinophilic leukocytes. A close correlation has been found between cell counts performed on fixed and stained histological sections or cell suspensions and enzymatic activity of the same preparations. Equivalent values of phospholipase B activity per unit cell are found in spleen, uterus and peritoneal fluid. A lower average value is indicated for the bone marrow eosinophils. A tentative explanation for this discrepancy is offered on the basis of histochemical evidence showing that a fraction of the eosinophilic cells do not stain for phospholipase B activity. The number of these cells is approximately equal to that of the precursor immature cells of the eosinophilic leukocyte system. The evidence indicates that in normal conditions a major portion, if not all, of the phospholipase B activity of the organs studied is contributed by the eosinophilic leukocytes present in the tissue.

INTRODUCTION

Recent histochemical studies (1) have shown that the eosinophilic leukocytes present in various rat organs contain phospholipase B (E.C. 3.1.1.5) activity. The presence of this enzyme in rabbit leukocytes had been previously reported by Elsbach et al. (2,3). It was therefore of interest to determine what proportion of the total phospholipase B activity of a given tissue is contributed by the eosinophils. This problem has been investigated for the spleen, bone marrow, peritoneal cells and uterus of the rat using a combination of histological and biochemical methods and the results are reported in the present paper.

MATERIALS AND METHODS

Animals

Male Osborne-Mendel rats, 175-200 g, were used for the spleen, bone marrow and peritoneal cells studies. The animals were anesthetized with ether and killed by decapitation to obtain spleen and bone marrow. Live animals

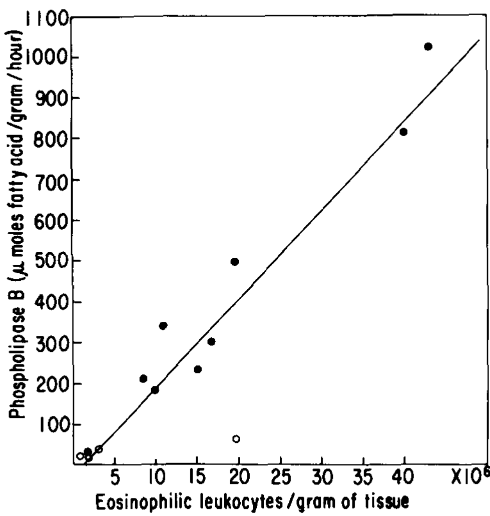

FIG. 1. Phospholipase B activity and eosinophilic leukocytes counts in rat spleen: ● control rats; ○ dexamethazone-treated rats. The number of eosinophilic leukocytes per gram of tissue was computed from the counts of cells/mm^2 as explained under Methods.

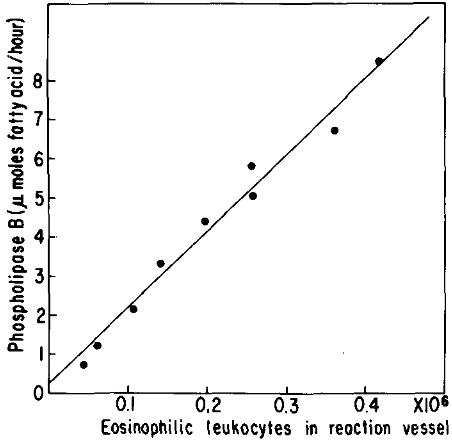

FIG. 2. Phospholipase B activity of spleen cells. On the ordinate phospholipase B activity expressed in micromoles of fatty acid split from lysolecithin per hour: on the abscissa the number of eosinophilic leukocytes present in the reaction vessel as estimated by hemocytometric methods.

TABLE I

Phospholipase B Activity and Eosinophilic Leukocyte Counts in Rat Spleen

Treatment and Rat No.	Phospholipase B $y^{a,b}$	Eosinophils $x^{a,c}$
Control		
32	41	2.89
33	304	16.78
34	816	39.98
35	1022	43.23
36	217	8.47
37	237	15.00
38	500	19.48
39	344	10.96
40	187	9.80
Dexamethasone[d]		
D1	39	1.69
D2	21	0.74
D3	26	1.75
D4	67	19.8

[a]Correlation coefficient for x,y = 0.926, t=8.15 > P 0.01; equation of the regression line, $y=-18.8 + 21.3 x$; confidence interval of the slope, 99% = 21.3 ± 8.1.

[b]Calculated as micromoles of fatty acid split from lysolecithin per gram of tissue per hour.

[c]Expressed as millions of eosinophilic leukocytes in one gram of tissue.

[d]Rats D1, D2, D3 and D4 were injected with 1 mg of dexamethasone at 2 AM and 5 PM on two successive days and used on the morning of the third day.

under light ether anesthesia were used for the preparation of peritoneal cells suspensions. Female rats, (Sprague-Dawley derived, Charles River, CD) were used for the uterus studies. The animals, received when 7 weeks old and 135 g average body weight, were kept for five weeks in a room with controlled illumination providing alternating cycles of light (14 hr: 7 AM-9 PM) and darkness (10 hr: 9 PM-7 AM). Daily vaginal smears were made, and 14 rats with regular 4 day cycles were selected for use. The animals were killed in different phases of the cycle (3 in proestrus, 5 in estrus, 3 in the first and 3 in the second day of diestrus) as shown by the vaginal smears taken in the morning immediately before anesthesia with ether and decapitation.

Tissue Preparation for Cell Counts and Enzymatic Assay

Estimation of the phospholipase B activity and the number of eosinophilic leukocytes required different techniques for the various tissues:

Spleen. For this tissue two methods were used. The first involved counting of the eosinophils in histological sections and testing for phospholipase activity in homogenates from the same organ. The central part of the spleen, 200-300 mg wet weight, was carefully isolated by cutting the organ perpendicularly to its long axis and fixed in neutral formalin; the remainder of the organ was used for homogenization and testing. After paraffin embedding, 40 successive sections 6 μ thick were cut from the fixed specimen, every fifth section being transferred to glass slides for staining. Various staining procedures were tried; in our experience the best results, in terms of differentiation of the eosinophils from the surrounding tissue and the red cells for counting, were obtained by treating the hydrated sections with Zenker's solution for 10 min followed by rinsing in water for 10 min and staining for 10 min in 0.083% phloxine in 0.083 M K H_2PO_4. After differentiation for 5 min in alcohol-acetone-water (1:1:1) the sections were dehydrated and mounted in Permount.

TABLE II

Phospholipase B Activity of Spleen Cells

Rat No.	Phospholipase B $y^{a,b}$	Cells in reaction vessel: Eosinophils x^{a}	Nucleated[c]	Total
1	2.18	0.107	3.877	---
2	5.85	0.256	9.753	21.953
3	5.05	0.256	14.620	29.698
4	1.24	0.063	10.560	33.823
5	3.32	0.142	8.314	20.028
6	8.50	0.417	26.125	99.990
7	6.75	0.362	19.277	57.400
8	4.43	0.197	33.600	91.630
9	0.74	0.045	30.291	79.282

[a]Correlation coefficient for x,y = 0.982, t = 14.1 >P 0.01; equation of the regression line, $y = 0.2 + 19.7 x$; confidence interval of the slope, 99% = 19.7 ± 4.8.

[b]Calculated as micromoles of fatty acid split from lysolecithin per hour.

[c]Expressed as millions of cells present in reaction vessel.

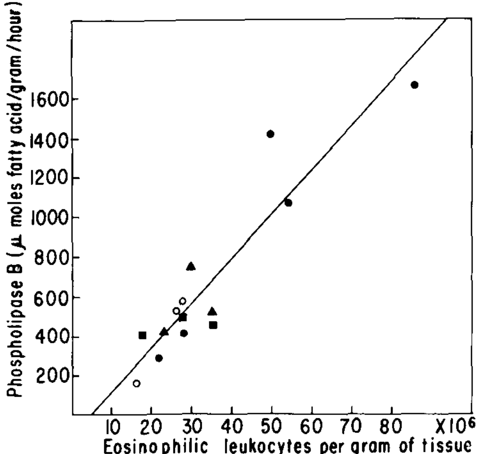

FIG. 3. Phospholipase B activity and eosinophilic leukocytes counts in rat uterus. The symbols indicate the phases of the estrus cycles as judged by vaginal smears: ○ proestrus; ● estrus; ▲ diestrus day 1; ■ diestrus day 2. The number of eosinophilic leukocytes per gram of tissue was computed from the counts of cells/mm^2 as explained under methods.

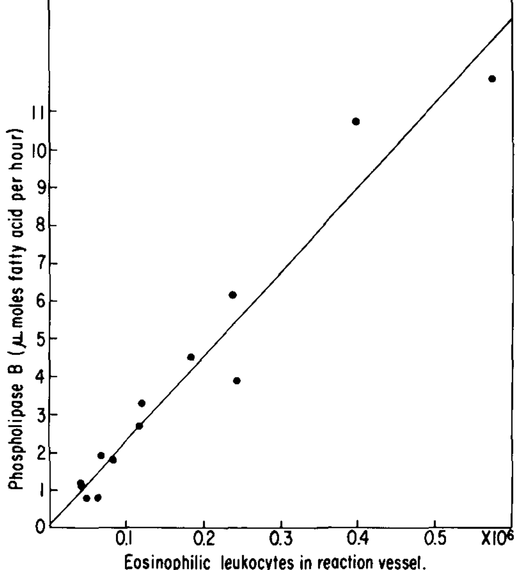

FIG. 4. Phospholipase B activity of peritoneal cells. On the ordinate phospholipase B activity expressed in micromoles of fatty acid split from lysolecithin per hour:on the abscissa the number of eosinophilic leukocytes present in the reaction vessel determined by conventional counting methods.

The second procedure involved preparation of free cells suspensions. The spleen was first sectioned transversally with a sharp blade into fine slices, 1 mm thick or less, and these were transferred to a watch glass containing a few milliliters of cold isotonic saline. After teasing the slices into smaller fragments not exceeding a few cubic millimeters, the preparation was transferred to a glass homogenizer (A. H. Thomas, No. 4288-B, size C, i.d. 2.50 cm) fitted with teflon pestle having a diameter of 2.37 cm. Cold saline was added to bring the volume to 20 ml and the tissue fragments were dispersed by 20 gentle strokes of the pestle. A first crop of free cells was decanted and filtered through a single layer of cheese cloth. Another 20 ml of cold saline were added to the heavier fragments in the homogenizer and a tighter fitting pestle, i.d. 2.43 cm, was used to obtain a second crop of cells to be decanted and pooled with the first. The cells were then subdivided into aliquots for testing and counting and separated by centrifugation for 6 min at 600 x *g*. Routinely half of the cells were used for counting after resuspension in 1 ml of isotonic saline and half were used for testing after dispersion in 3 to 5 ml of reaction medium.

Uterus. The entire organ was removed from the abdominal cavity and freed of the surrounding peritoneal tissue. The two horns were severed at their insertion on the body of the uterus: one horn was minced with scissors and homogenized first in a teflon glass homogenizer in 15 ml of cold reaction medium and then transferred to a glass-glass homogenizer and finely dispersed by careful hand-stroking. The other horn was cut in three pieces corresponding to the distal, middle and proximal parts and the three fragments were fixed, embedded and cut at 6 μ for staining. With this tissue, best results were obtained with the procedure suggested by Archer (4) using Wrights stain in place of the Leishmann stain as originally recommended. Staining with phloxine as described for the spleen sections was found applicable and used occasionally.

Peritoneal Cells. The cells were harvested by injection of 50 ml of isotonic saline in the peritoneal cavity of lightly anesthetized rats and withdrawal within a few minutes of about 45 ml of the administered fluid by drainage through a No. 16 needle. Routinely, one half of the cells was used for counting and one half for the enzymatic tests after centrifugation at 600 x *g* for 6 min and resuspension in isotonic saline or reaction medium. Only one cell harvesting was made from each rat.

Bone Marrow. The tibiae of the rat were used for the preparation of marrow cells suspensions. The bones were quickly removed, freed of surrounding tissue and chipped at their extremities with small pliers to expose the

TABLE III

Phospholipase B Activity and Eosinophilic Leukocyte Counts in Rat Uterus

Rat No.	Ovarian cycle phase[a]	Phospholipase B $y^{b,c}$	Eosinophils $x^{b,d}$
U 10	P	532	26.1
U 13	P	163	16.0
U 15	P	571	27.6
U 23	E	408	28.8
U 24	E	290	21.7
U 5	E	1067	54.2
U 6	E	1414	49.6
U 17	E	1658	86.2
U 25	D1	425	23.1
U 32	D1	524	34.8
U 33	D1	757	29.6
U 7	D2	488	27.5
U 1	D2	456	35.1
U 9	D2	404	17.5

[a]Ovarian cycle phase: P, proetrus; E, estrus: D1, diestrus day 1; D2, diestrus day 2.

[b]Correlation coefficient for x,y = 0.922, t = 8.27 $>$ P 0.01; equation of the regression line, y = -83 + 21.6 x; confidence interval of the slope, 99% = 21.6 ± 7.9.

[c]Calculated as micromoles of fatty acid split from lysolecithin per gram of tissue per hour.

[d]Expressed as millions of eosinophilic leukocytes in 1 g of tissue.

marrow cavity. The bone was then inserted into a short section of plastic tubing, i.d. 3/32 in. mounted on a syringe adapter and flushed with 2-3 ml of cold isotonic NaCl. The extruded marrows were collected in the glass homogenizer already described for the spleen slices, added with cold isotonic saline to a final volume of 22 ml and dispersed by 20 strokes of the loose fitting pestle, i.d. 2.37 cm. After passage through a single layer of cheese cloth to remove larger particles, aliquots were centrifuged for counting and testing. Usually one half of the cells were centrifuged and resuspended in 1 ml of isotonic NaCl for counting: the other half, after centrifugation and resuspension in 5 ml of reaction medium, was used for the enzymatic tests.

Femural bone marrow resuspended in a small volume of rat serum, 0.5 ml, was used in preparing smears for staining with Giemsa, and sulfide and phloxine after incubation with lysolecithin. The latter procedure was developed in order to estimate the percentage of eosinophilic cells showing lysolecithinase activity and is essentially identical with the method already reported for tissue sections (1) except that staining with sulfide was done in 50% cold acetone instead of cold isotonic NaCL and was followed by counter staining for 10 min with 0.1% phloxine in 0.1 M phosphate buffer pH 6.9. After differentiation for 3 min in methanolic acetate (220 ml of 1.0 M acetic acid + 40 ml of 1.0 M Na OH + 100 ml methanol to 500 ml with water) the specimen were rapidly dehydrated in the higher alcohols and mounted.

Cell Counts

Cell suspensions from bone marrow, spleen and peritoneal fluid were counted by standard hematological methods in the improved Neubauer chamber. Total cell and nucleated cells counts were performed after dilution in formalin-citrate and Randolph stain respectively. Eosinophils were counted by both the Randolph and Discombe methods and the two procedures gave identical results.

The eosinophilic leukocytes of the spleen and uterus sections were counted with a microscope provided with a stage allowing progression of the slide in orthogonal directions by small steps, 200 μ. To insure scanning of the entire section without omissions or duplications in cell counting, the microscope eyepiece was fitted with a reticulum lens reduced by application of black masking tape to a square field. The area of the field was adjusted to the stepwise motion of the stage and the magnification selected for counting: in most instances a 25x objective and a 10x ocular and a counting field of 400 x 400 μ were used. The point counter attachment for microscope stage manufactured by the Carl Zeiss Co., No. 474035, and the counting reticulum supplied by Graticules Ltd., England, are distributed in the U.S. by Brinkman Instruments, Westbury, New York.

For the spleen the method of Rytomaa (5)

TABLE IV

Phospholipase B Activity of Peritoneal Cells

Rat No.	Phospholipase B y[a,b]	Cells in reaction vessel[a,c] Eosinophils x	Nucleated
2	3.22	0.121	0.458
3	1.78	0.083	0.353
4	0.75	0.063	0.441
5	11.76	0.573	1.998
6	10.68	0.396	0.952
7	4.47	0.181	0.582
8	1.87	0.066	0.158
9	1.07	0.040	0.631
10	1.13	0.040	0.320
41	6.10	0.236	1.155
42	0.76	0.048	0.638
43	2.67	0.116	0.440
44	3.82	0.242	0.869

[a]Correlation coefficient for x,y = 0.946, t = 13.9 $>$ P 0.01; equation of the regression line, y = 0.1 + 22.1 x; confidence interval of the slope, 99% = 22.1 ± 4.9.

[b]Calculated as micromoles of fatty acid split from lysolecithin per hour.

[c]Expressed as millions of cells present in reaction vessel.

was used: counts were routinely performed over the entire area of a cross-section which was then projected on paper for estimation of its area by means of a planimeter. The number of cells per unit volume of tissue was calculated according to the formula of Floderus (6):

$$\frac{\text{cells}}{\text{mm}^3} = \frac{\text{cells}}{\text{mm}^2} \times \frac{1000}{a+d+2h}$$

where a = 6, thickness of the section in microns; d = 10, mean diameter of the eosinophils in microns; h = 0.5, smallest observable particle in microns.

For the uterus, two nonconsecutive sections, at least 12 μ apart, of each part of the organ (distal, middle, proximal) were counted and their area measured. The average of the six experimental values thus obtained and relating eosinophilic population to unit volume, was taken for comparison with the enzymatic data. This procedure was adopted after preliminary experiments had shown that the enzymatic content per unit weight of tissue is uniform throughout the length of the horns when discrete fragments of tissue are used.

Double stained bone marrow smears (Giemsa and phospholipase B) were counted with the aid of a red filter to increase the contrast between the black-stained, phospholipase-positive cells and the red-stained, phospholipase-negative elements which showed only very faintly under these conditions of illumination. Examination of the same area under normal light provided an easy means of counting all cells showing eosinophilic staining. Two hundred and fifty to 500 cells were counted from two or more smears: a given area of the preparation was scanned in its entirety by following a square wave pattern of progression of the slide under the counting reticulum.

Differentiation of the eosinophils on Giemsa smears was performed by scanning the stained preparations until a total of 200 to 350 cells had been counted. A crisscross pattern of scanning was adopted and two to three smears were counted in each case. Cells showing both azurophilic and eosinophilic granulations were classified as immature eosinophils: this group thus includes the eosinophilic promyelocytes 1 and 2, also distinguished by the presence of nucleoli, and some myelocytes. All cells showing exclusively eosinophilic granules in a pink cytoplasm and different nuclear shapes (round, ring, indented) were grouped as mature eosinophils.

Measurement of Phospholipase B Activity

The enzymatic tests were performed according to the procedure previously described (7) involving treatment with trypsin to insure a sustained rate of hydrolysis upon addition of lysolecithin. On the basis of preliminary trials the concentrations of trypsin to be used routinely with cell suspensions were established at 60 and 30 μg/ml: with tissue homogenates lower concentrations, 30 μg and 15 μg/ml, were

TABLE V

Phospholipase B Activity and Eosinophilic Leukocytes of Rat Bone Marrow

Rat No.	Phospholipase B $y^{a,b}$	Cells in reaction vessel[c] Eosinophils x^{a}	Nucleated	Total
01	1.44	0.091	4.229	10.220
3	5.80	0.456	7.360	11.274
4	4.87	0.344	9.174	20.441
05	4.87	0.250	2.765	5.897
31	6.80	0.498	4.194	7.297
32	1.10	0.079	6.359	9.127
33	2.64	0.166	5.225	9.367
34	3.99	0.312	4.159	6.995
35	6.28	0.561	5.448	9.126
36	2.97	0.189	5.950	11.980
37	2.37	0.189	7.852	10.519
38	5.20	0.396	5.689	8.491
39	2.77	0.192	6.102	10.123
40	1.75	0.121	5.947	8.508

[a]Correlation coefficient for x,y - 0.961, t = 11.98 $>$ P 0.01; equation of the regression line, y = 0.6 + 11.5 x; confidence interval of the slope, 99% = 11.5 ± 2.9.

[b]Calculated as micromoles of fatty acid split from lysolecithin per hour.

[c]Expressed as millions of cells present in reaction vessel.

sufficient to insure maximal activity readings. The technical details and justification for this procedure have been reported (7).

RESULTS AND DISCUSSION

Spleen. The values of the phospholipase B activity of spleen homogenates and the corresponding counts of eosinophilic leukocytes in histological sections are presented in Table I. The regression and correlation coefficients and the confidence interval of the regression line are given at the bottom of the Table: the same data are presented graphically in Figure 1. Table II and Figure 2 show the results obtained with spleen cells suspensions. These results indicate a close correlation between the eosinophilic population and the phospholipase B activity. It is relevant that the regression coefficients of the two series are very close showing that the same poopulation of eosinophilic cells was present in the cell suspensions and in the spleen homogenates. This point had been tested in preliminary experiments, which showed that at least two thirds of the enzymatic content of the splenic tissue was recovered in the cell suspensions. It is also apparent from the data that the proportionality between enzyme and cells holds over a wide range reflecting the considerable variation in eosinophilic content of the spleen usually encountered in these animals. On the whole our data are in agreement with the estimates of Browaeys and Wallon (8) regarding the number of eosinophils per 100 g body weight of the rat. These values are somewhat higher than those calculated by Rytomaa (5) for animals with relatively small spleens and lower cell counts per unit area of the tissue. In our experiments when the size of the spleen was small, lower enzymatic activity and cell counts were observed. It is possible that these variations are due to some unknown environmental factor or to strain differences.

Statistical analysis showed no correlation between enzymatic activity and the counts of nucleated or total cells in the spleen cell suspensions (regression coefficients 0.28 and 0.03 respectively, t values below 1 in both cases). To ascertain if a portion of the total phospholipase activity, at least, could be present independently of the eosinophils, four rats were treated with dexamethasone to reduce the number of these cells in the peripheral tissues: as shown in Table I and Figure 1, such treatment resulted in a proportional decrease in enzymatic activity. The 95% confidence limits of the regression lines in these and all the following data (Fig. 1-4) contain the origin, thus supporting the conclusion that the organ content of phospholipase B depends essentially on the number of eosinophilic leukocytes and that additional sources of the enzyme, if present at all, are in comparison too small to be detected.

Uterus. The relationship between enzymatic content and the eosinophilic population of the uterus is shown in Figure 3 drawn from the data of Table III. Animals in different phases of the ovarian cycle were used in these experi-

TABLE VI

Phospholipase B Activity of Eosinophilic Leukocytes of Rat Bone Marrow

Rat No.	Phospholipase B[a] A	Eosinophils[b] B	Fraction of eosinophils double stained C	Activity per million cells D=A/Bq	Activity per million cells E=A/BC
31	6.80	0.498	0.75	13.6	18.1
34	3.99	0.312	0.63	12.8	20.3
36	2.97	0.189	0.80	15.7	19.6
38	5.20	0.396	0.85	13.1	15.4

[a]Calculated as micromoles of fatty acid split from lysolecithin per hour.
[b]Expressed millions of cells in the reaction vessel.

ments and are identified by different symbols. It is apparent that the higher values in both phospholipase B and eosinophilic counts coincide with the estrus phase and the lower values correspond to the late dietrus-proestrus phase.

A relationship between ovarian cycle and eosinophilic leukocyte population of the uterus has already been established (5,9). Our data are in general agreement with the previous reports indicating a large number of eosinophils in the uterus at the height of the estrus. Two animals judged by vaginal smears to be in the estrus phase, however, showed low values of both cell and enzymatic content. This might reflect some difficulty in the interpretation of the smears or that the animals in question were no longer following a regular ovarian cycle. In any case proportionality between enzyme and cells was maintained.

These results show the relationship between enzymatic content and cell number already observed for the spleen. The regression coefficients for the two organs do not differ significantly. A rather large experimental error, probably due to shrinkage of the tissues upon fixation and embedding and the presence of the peritoneal tissue which complicates the procedure of isolating the uterine horns and the measurement of the section areas, is reflected in the large confidence interval for this series. The uterine tissue is particularly compact and does not lend itself to teasing and preparation of cell suspensions, therefore experiments with isolated cells were not possible.

Peritoneal Cells. The data for these cells and their statistical correlation are shown in Table IV and Figure 4. The similarity to the results with spleen cells suspensions is apparent. No correlation is found between enzymatic activity and the nucleated cell or mast-cell counts of these preparations.

Bone Marrow. The experimental values for the cells counts and the phospholipase B activity of bone marrow cells suspensions are summarized in Table V. From these data a good correlation between eosinophils and enzymatic activity can be shown. However, the calculated activity per million eosinophilic cells (i.e., the slope of the regression line) is significantly lower than the values found in the other tissues (11.6 for the marrow and 19.7 and 22.1 respectively, for the spleen and the peritoneal cells). To ascertain if this could be due to the presence of eosinophilic cells lacking in phospholipase B activity, some of the marrows were double stained with eosin and the enzymatic histochemical procedure (see Methods). The results are shown in Table VI, columns A, B and C. The activities per million cells calculated on the basis of the total eosinophil count or the percentage of these cells exhibiting phospholipase B activity are compared in columns D and E, respectively. It is apparent that omission of the phospholipase B-negative eosinophils from the calculation brings the activity per million cells of the marrow within the range observed for the peripheral tissues.

In order to interpret these data two additional approaches were tried. The first consisted of staining the smears for phospholipase B and then with Giemsa to determine if cells other than the eosinophilic leukocytes might contain the enzyme. In all of our studies on marrow, and other tissues, we have found a positive stain for the enzyme only in cells which also exhibited the characteristic eosinophilic granulation. The other cells of the leukocytic or erythrocytic series were uniformly negative. This agrees well with the observed statistical correlation between eosinophils and enzyme in all preparations containing different proportions of these cells and the lack of correlation with regard to the total or nucleated cell counts. The second approach involved differential counting of the eosinophils. This study could not be carried out on double stained smears, phospholipase B and Giemsa, because enough of the structural detail of the cells is

TABLE VII

Mature and Immature Eosinophils of Rat Bone Marrow and Phospholipase B Activity

Rat No.	Eosinophils		Phospholipase B activity per million eosinophils, y[a]	
	Total	Mature, x[a]	Total	Mature
31	351	246	13.6	19.5
32	230	145	14.0	22.2
33	339	189	15.8	20.0
34	247	177	12.8	17.9
36	291	221	15.7	20.8
37	209	151	12.6	17.4
38	213	157	13.1	17.8
39	267	192	14.4	20.1
40	197	135	14.4	21.0

[a]Correlation coefficient for mature eosinophils (x) and phospholipase B activity (y): 0.99, $t = 19.0 > P\ 0.01$; equation of the regression line, $y = 0.2 + 18.1\ x$; confidence interval of the slope, 99% = 18.1 ± 3.2.

altered by the procedures to preclude a satisfactory identification of the various cell types. As an alternative, Giemsa stained smears of the bone marrows were examined and scored for the presence of immature and mature eosinophils according to the criteria summarized under Methods. If the mature cells are used in the calculation of the activities for million cells, the average enzymatic content approximates the values found for the spleen and peritoneal cells and the corrected values for the double stained marrow cells (Table VII).

On the whole these results support the conclusion that a direct relationship between eosinophilic cells and phospholipase B activity exists in spleen, uterus, peritoneal fluid and bone marrow of the rat. The excellent agreement of the experimental data for the spleen obtained by two different methods, histological sections and cell suspensions, shows that all results by either of the two procedures are valid. For the bone marrow, while the relationship between eosinophils and enzyme can also be considered as proven, the lower activity per unit cell remains to be interpreted. At present we favor the explanation, suggested by the experiments with double staining and the immature and mature cell counts, that the immature cells, about one fourth of the total eosinophil population of the marrow, contain little or no phospholipase B activity. This can be proved in future experiments by shifting of the composition of the marrow from younger to older cells.

Our results confirm the presence of phospholipase B activity in leukocytes first reported in the rabbit by Elsbach et al. (2,3). The combined histochemical and biochemical evidence from our studies in the rat shows that in this animal the enzyme is more specifically localized in the eosinophilic cells. Studies now in progress will extend this investigation to other tissue of the rat and other species.

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Variation of Triglycerides and Fatty Acid Methyl Esters in Silkworm Eggs During Embryonic Development

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ABSTRACT

Neutral lipid composition of silkworm eggs showed remarkable differences with stages of embryonic development, such as one day after oviposition, diapausing, before incubation and before hatching. Large amounts of fatty acid methyl esters were found in lipids of eggs just before hatching, and fatty acid compositions in both triglycerides and fatty acid methyl esters varied with the lapse of time of the embryonic development. Polyunsaturated fatty acids were present in large amounts in fatty acid methyl ester fractions from eggs just before hatching and absent in those from diapausing and 1 day old eggs, while a large amount of polyunsaturated fatty acids was present in triglycerides in diapausing and 1 day old eggs and only small amounts were present in eggs just before hatching. The methyl esters were not formed as artifacts from free fatty acids and glycerides in silkworm lipids during the extraction procedure.

INTRODUCTION

Considerable work has been done on the fatty acid composition of whole lipids (1-3) and of triglyceride fractions (4,5) of silkworm. Studies on the fatty acids in triglycerides of insect eggs have been made on aphid, *Euceraphis punctipennis Zett* (6), on grasshopper, *Aulocara elliotti* (7) and on grasshopper, *Melanoplus bivattatus* (8), but the comparison of the fatty acid compositions of eggs in various stages has not been reported. The purpose of the present paper is to answer the question as to whether or not significant differences in lipid composition of eggs occur as embryonic development proceeds.

During the course of our study on lipid composition of silkworm eggs in various stages, significant amounts of fatty acid methyl esters were found especially in the lipid of eggs just before hatching. Concerning the occurrence of fatty acid methyl esters in animals, Leikola et al. (9) reported that significant amounts were present in the human pancreas, and that the methyl esters were not artifacts from solvents or caused by autolysis post mortem. The presence of fatty acid methyl esters in the pancreas and their fatty acid composition were reported by Lough and Garton (10). Recently, Sloan et al. (11) and Jackson et al. (8) have reported on the presence of fatty acid methyl esters and their fatty acid composition in eggs of the grasshopper, *Melanoplus bivittatus* (Say).

The present paper deals with the existence of fatty acid methyl esters in egg lipid and with the variation of the fatty acid composition in both the triglycerides and in fatty acid methyl ester fractions during embryonic development. Additionally, it was confirmed that the fatty acid methyl esters were not derived from free fatty acids in silkworm lipids as an artifact or through esterification with solvents during the extraction procedure.

MATERIALS AND METHODS

Animals

Silkworm eggs, 1 day old, in diapausing, before incubation (chilled treatment) and just before hatching (late stage) were taken from two kinds of F_1 hydrid between two races, J. 122 X C. 115 and J. 124 X C. 124.

One day old eggs were collected within 12 to 24 hr after laying, diapausing eggs kept at 25 C for a month after laying, eggs chilled at 5 C for six months after laying, and eggs just before hatching together with a few newly hatched larvae.

Each of the materials in the above mentioned groups was homogenized with chloroform-methanol (2:1 v/v) and the solution was filtered after allowing it to stand for one day at 5 C. After evaporation of the solvent under reduced pressure, the residue was dissolved in ether. The solution was refluxed for 2 hr, dried over anhydrous Na_2SO_4 and the ether was evaporated. Lipid samples were stored at a temperature below 5 C. These procedures were carried out under nitrogen.

Thin Layer Chromatography

Each lipid sample was analyzed by thin layer chromatography (TLC) on Silica Gel G using the following two solvent system: petroleum ether-ethyl ether-acetic acid (90:10:1 and

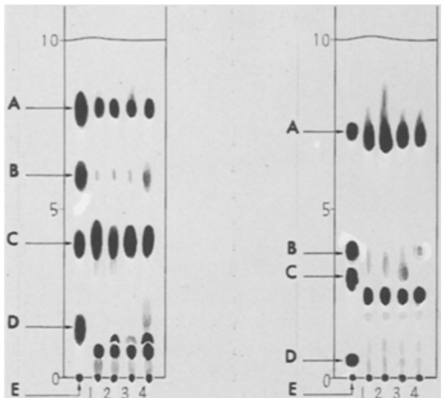

FIG. 1. TLC analyses of lipids in silkworm eggs. Right: TLC Solvent, petroleum ether-ethyl ether-acetic acid (90:10:1). A, Cholesteryl oleate. B, Methyl oleate. C, Triolein. D, Oleic acid. E, Standard mixture A. 1, 1-Day-old eggs. 2, Diapause eggs. 3, Chilled eggs. 4, Late stage eggs. Left: TLC Solvent, petroleum-ether-ethyl ether-acetic acid (60:40:1). A, Tripalmitin. B, 1,3-Dipalmitin. C, 1,2-Dipalmitin. D, 1-Monopalmitin. E, Standard mixture B. 1, 1-Day-old eggs. 2, Diapause eggs. 3, Chilled eggs. 4, Late stage eggs.

FIG. 2. TLC analyses of fractions by silica gel column of lipids in silkworm eggs. I, 1-Day-old eggs. II, Diapause eggs. III, Chilled eggs. IV, Late stage eggs. A, Cholesteryl oleate. B, Methyl oleate. C, Triolein. D, Oleic acid. E, Standard mixture A.

60:40:1). The detection was by application of concentrated H_2SO_4 followed by charring.

The standard mixtures A and B shown in Figure 1, were Mixture TLC-1 consisting of cholesteryl oleate, methyl oleate, triolein and oleic acid, and Mixture TLC-8 consisting of tripalmitin, 1,3-dipalmitin, 1,2-dipalmitin and 1-monopalmitin purchased from Applied Science Laboratories Inc. Trilaurin, tripalmitin, tristearin, methyl laurate and methyl linolenate were also used as standards for the identification of triglycerides and fatty acid methyl esters. The quantitative measurement of TLC spots was carried out by a photodensitometer (Atago Model 8, Atago Optical Works Co. Ltd.).

Fractionation of Lipids by Column Chromatography

The hexane solution of each lipid sample (about 100 ml) was chromatographed on silica gel column (Mallinckrodt, analytical reagent, 100 mesh, 6 g) with 30 ml of hexane (Fraction 1), 60 ml of 15% benzene-hexane (Fraction 2, 30 ml; 3, 30 ml), 150 ml of 5% ether-hexane (Fraction 4, 30 ml; 5, 30 ml; 6, 30 ml; 7, 60 ml), 120 ml of 15% ether-hexane (Fraction 8), 60 ml of 30% ether-hexane (Fraction 9), 60 ml of 50% ether-hexane (Fraction 10), and 60 ml of 90% ether-hexane (Fraction 11) successively. Relative weight percentages of these fractions are shown in Table I. Each fraction was analyzed by TLC on Silica Gel G using petroleum ether-ethyl ether-acetic acid (90:10:1) system as shown in Figure 2.

Identification of Linolenic Acid Methyl Ester

Fraction 4, eluted by 5% ether-hexane from lipid in late stage eggs shown in Figure 2, was rechromatographed with the same solvent system as mentioned in the fractionation of lipids by column chromatography. Then the ester fraction was chromatographed again using solvents of gradually increasing ratios of ether to hexane. The substance eluted with 2% ether-hexane by the last chromatography gave one spot on TLC and one peak by GLC. This substance was analyzed by mass spectrometry using Hitachi RMU-6 (single focusing) and Japan Electron Optics Lab. Co. Model JMS-OIS (double focusing) spectrometers. The identification was further substantiated by IR and NMR spectra of the substance and its $LiAlH_4$ reduction product.

Investigation on Extraction Procedure

Silkworm larvae 48 hr old (4th instar) were used to investigate the possibility of formation of fatty acid methyl esters as artifacts from free fatty acids during the extraction procedure, since significant amounts (5-10%) of fatty acid methyl esters were found in the larvae of this stage. [Further studies of methyl esters in larvae will be published in a forthcoming paper.] A solution of 1-^{14}C-palmitic acid was placed in a

TABLE I

Fractionation of Lipids in Silkworm Eggs on Silica Gel Column (Relative Wt %)

Fraction No.	Main component	Developmental stage of egg			
		1 Day old	Diapause	Chilled	Late stage
1	Hydrocarbons	0.8	0.9	1.2	0.8
2+3	Sterol esters	2.1	0.8	1.5	0.6
4	Fatty acid esters	0.4	0.4	0.7	3.6
5+6	Triglycerides	44.7	46.5	49.6	45.4
7+8	Sterols and free fatty acids	8.2	11.9	15.4	16.8
9+10+11	Mono- and diglycerides and phospholipids	43.8	39.5	31.6	32.8

beaker and the solvent was evaporated. Then haemolymph and the organs without inclusion of the digestive tracts from two larvae and methanol were added successively. The mixture was homogenized after standing for two days at 5 C. Thereafter, the methanol was evaporated at 60 C. The extract obtained was developed by TLC using a petroleum ether-ethyl ether-acetic acid (90:10:1) system and each zone corresponding to standard samples was cut off. The same procedures were carried out on the extractions with chloroform-methanol (2:1 v/v) and chloroform-ethanol (2:1 v/v). The radioactivities of each sample are shown in Table III.

Nonlabelled standard free fatty acids, monoglycerides, 1,3-diglycerides and triglycerides were added to both the haemolymph and the organs of larvae, respectively. Each sample was homogenized and extracted with chloroform-methanol (2:1 v/v) by the same procedure mentioned above. Each extract was analyzed by gas chromatography.

Fatty Acids From Triglycerides

Each triglyceride fraction obtained by column chromatography was saponified with 95% methanol containing 5% KOH at 80 C for 2 hr under a stream of nitrogen. After removal of the unsaponifiable fraction with ether, the aqueous layer was acidified with 3 N H_2SO_4 and extracted with ether repeatedly. The ether extract obtained was washed with distilled water several times and dried over anhydrous Na_2SO_4. Free fatty acids were obtained after evaporation of ether under a stream of nitrogen. Methyl esters of fatty acids were prepared with diazomethane in ether.

Gas Liquid Chromatography

A Shimadzu Gas Chromatograph Model 4APF with hydrogen flame ionization detector was used in the present study. The column for fatty acid methyl esters was a 2.0 m x 4 mm i.d. glass column packed with 15% Diethylene Glycol Succinate on Celite, 60-80 mesh. The column temperature was 180 C; the carrier gas (N_2) flow rate was maintained at 60 ml/min. The standard samples were C_{10}-C_{16} mixture (Shimadzu) and methyl laurate, palmitate, stearate, oleate and linolenate (Sigma).

RESULTS

Typical thin layer chromatograms of lipids obtained from eggs in each stage are presented in Figure 1. On the chromatogram using petroleum ether-ethyl ether-acetic acid (90:10:1) solvent system, the main spots showing the same

TABLE II

Fatty Acid Composition of Triglycerides and Fatty Acid Methyl Esters Fractions From Silkworm Eggs (Relative Wt %)

Fatty acids	1 Day old		Diapause		Chilled		Late stage	
	Triglycerides	Methyl esters	Triglycerides	Methyl esters	Triglycerides	Methyl esters	Triglycerides	Methyl esters
C_{12}	0.3	1.9	1.1	0.5	2.0	Trace	Trace	Trace
C_{14}	0.5	1.3	1.1	0.5	1.2	Trace	2.0	Trace
C_{16}	13.6	47.2	12.6	57.1	11.9	32.5	27.6	22.6
C_{18}	2.8	15.5	0.9	22.3	1.3	16.3	11.7	20.6
$C_{18:1}$	28.6	34.1	7.2	19.6	15.0	23.1	26.4	18.4
$C_{18:2}$	6.9	0	8.9	Trace	8.9	11.8	5.3	17.1
$C_{18:3}$	47.3	0	68.2	Trace	59.7	16.3	27.0	21.8

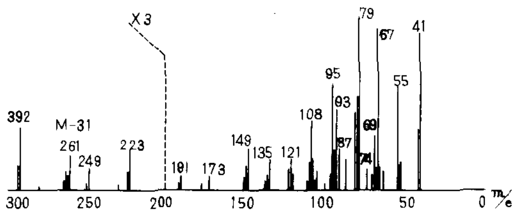

FIG. 3. Mass spectrum of linolenic acid methyl ester from silkworm egg in late stage.

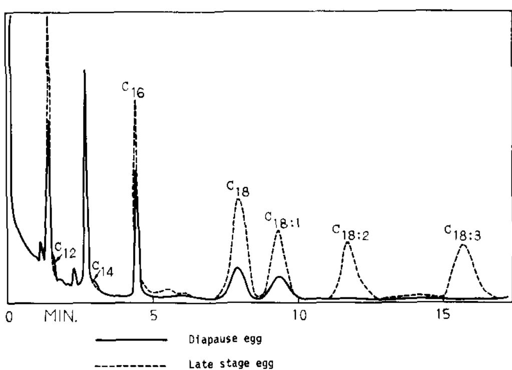

FIG. 4. GLC analyses of fatty acid methyl ester fractions in lipids from silkworm eggs.

R_f values as sterol oleate and triolein were found in lipids from eggs of all four stages. Large amounts of lipids showing the same R_f value as methyl oleate were found in late stage eggs, but only trace amounts were found at other stages.

No distinctly different chromatogram was obtained by a TLC using petroleum ether-ethyl ether-acetic acid (60:40:1) system. In this case, considerable amounts of lipids showing the same R_f values as 1,3- and 1,2-dipalmitin were found in lipid samples of chilled and late stage eggs. Although standard methyl esters were not distinguishable from each other, methyl esters were separated from triglycerides using the 90:10:1 ratio but not using the 60:40:1 ratio.

Relative weight percentages of the 11 fractions obtained by silica gel chromatography of the lipids in each stages and thin layer chromatograms of these fractions are shown in Table I and Figure 2, respectively. For chromatography of neutral lipids from rat liver, beef liver and yeast, it was reported by Barren and Hanahan (12) that the most reproducible solvents for lipid fractionation and the lipids eluted by these solvents are as follows: hexane, hydrocarbons; 15% benzene-hexane, sterol esters; 5% ether-hexane, triglycerides plus fatty acids; 15% or 20% ether-hexane, free sterol; 30% ether-hexane, diglycerides; 50% ether-hexane, unidentified components; and 90% to 100% ether-hexane, monoglycerides. In the present study, similar elution patterns were obtained from 1 day old, diapausing and chilled eggs. In late stage eggs, Fraction 4, eluted by 5% ether-hexane, consisted of triglycerides and an unknown component showing the same R_f value as standard fatty acid methyl esters. Trace amounts of the unknown component were detected also in 1 day old, diapausing and chilled eggs.

The substance purified by repeated silica gel chromatography of Fraction 4 from late stage eggs gave one peak in GLC, showing the same relative retention time as that of methyl linolenate. This substance gave m/e 292(M^+) and 261(M^+ - OCH_3) in mass spectrometry as shown in Figure 3, and the molecular ion gave $C_{19}H_{32}O_2$ composition corresponding to methyl linolenate. The NMR and IR spectra of this substance were also identical with those of methyl linolenate and the spectra of the alcohol produced by $LiAlH_4$ reduction were identical with the corresponding alcohol.

Typical gas chromatograms of fatty acid methyl ester fractions obtained from lipids of diapausing and late stage eggs are shown in Figure 4. The retention times of five peaks of fatty acid methyl esters in diapausing eggs and seven peaks of those in late stage eggs coincided with those of standard samples, such as methyl laurate, myristate, palmitate, stearate, oleate, linoleate and linolenate.

By densitomeric determination of TLC (Fig. 2), the amounts of methyl esters (Fraction 4) were found to be about 3% of the neutral lipid in late stage, but less than 0.3% in other stages.

The relative quantities of fatty acid methyl esters from 1 day old, diapausing, chilled and late stage eggs were calculated from peak areas on the gas chromatograms, and the fatty acid compositions of the methyl esters and of the triglycerides from egg lipids at each developmental stage are summarized in Table II. The main fatty acids in the methyl esters obtained from lipids were C_{16} in 1 day old and diapausing eggs, C_{16} and $C_{18:1}$ in chilled eggs, and C_{16}, C_{18}, $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$ in late stage eggs. There were only trace amounts of $C_{18:2}$ and $C_{18:3}$ in methyl ester fractions in diapausing eggs, but both compounds increased in chilled and late stage eggs. These esters were not found in 1 day old eggs. The main fatty acid in triglycerides were $C_{18:3}$ in 1 day old, diapausing and chilled eggs, and C_{16}, $C_{18:1}$ and $C_{18:3}$ in late stage eggs.

Only a trace of 1-^{14}C-palmitic acid was incorporated into fatty acid esters as presented

in Table III. Thus, it can be concluded that the formation of fatty acid esters from free fatty acid does not occur as an artifact during the extraction procedure. By the addition of free fatty acids and glycerides to both haemolymph and the organs of larva, followed by extraction with chloroform-methanol, it was made clear that free fatty acids were not esterified and the glycerides were not transesterified. It was confirmed by gas chromatography that fatty acids were not esterified by refluxing with different solvents, such as methanol, chloroform-methanol (2:1 v/v), methanol-water (95:5 v/v), chloroform-ethanol (2:1 v/v) and ethanol. Fatty acid methyl esters were not transesterified by refluxing with chloroform-ethanol (2:1 v/v), ethanol, chloroform-isopropanol (2:1 v/v) and isopropanol.

TABLE III

Conversion of 1-C^{14}-Palmitic Acid to Methyl Esters During Extraction Procedures From Silkworm (% of Total cpm Collected)

Fractions from TLC	Solvents for extraction		
	MeOH	MeOH-$CHCl_3$	EtOH-$CHCl_3$
(Front)			
1 Hydrocarbons and sterol esters	0.01	0.03	0.01
2	0.09	0.12	0.06
3 Fatty acid methyl esters	0.38	1.08	0.20
4	0.06	0.06	0.02
5 Triglycerides	0.10	0.13	0.07
6 Fatty acids and sterol	96.59	96.97	98.73
7	2.77	1.61	0.91
(Start)			

DISCUSSION

In the present study, the existence of about 3-4% of fatty acid methyl esters in neutral lipid of late stage eggs and of trace amounts in eggs at other stages was shown by careful fractionation of lipids.

More recently, the existence of significant amounts of fatty acid methyl esters in lipids of 5 day old 5th instar larva of the silkworm has been demonstrated by us. Therefore, the increase of the amounts of fatty acid methyl esters in late embryonic development may be closely related to fatty acid metabolism in post-embryonic development.

Furthermore, variation in fatty acid composition of triglycerides and fatty acid methyl esters in lipids from silkworm eggs was observed as embryonic development proceeded. The fatty acid composition of total lipids of silkworm eggs presented by Nakasone and Ito (1) is similar to that of triglycerides from 1 day old silkworm eggs but dissimilar to that of triglycerides from late stage eggs.

The main fatty acids of triglycerides from silkworm eggs were C_{16}, $C_{18:1}$ and $C_{18:3}$. As embryonic development proceeded, $C_{18:3}$ decreased and C_{16} and C_{18} increased. In fatty acid methyl esters from silkworm eggs, $C_{18:3}$ and $C_{18:2}$ increased strikingly and C_{12}, C_{14} and C_{16} decreased during this period of development. Total amounts of fatty acid methyl esters in chilled, diapausing and 1 day old eggs were small. In other words, polyunsaturated fatty acids, which play an important role in silkworm nutrition (13), were abundantly present in fatty acid methyl ester fractions from late stage eggs and absent in those from diapausing and 1 day old eggs, while a large amount of polyunsaturated fatty acids in triglycerides was present in 1 day old, diapausing and chilled eggs, and a small amount in triglycerides of late stage eggs.

There may have been a shift of $C_{18:2}$ and especially $C_{18:3}$, neither of which can be synthesized by insects, from the triglyceride pool into the methyl ester pool. The usual fatty acid transport route in insects has been reported as protein-bound diglycerides (14,15).

With regard to fatty acid methyl esters, it was demonstrated that no methyl esters of fatty acids were formed from either free fatty acids or glycerides in silkworm lipids during the extraction procedure. From the aforegoing results we are inclined to believe that the methyl esters in silkworm lipids are not artifacts, except for the possibility that the formation of methyl esters may be induced during the extraction procedure from enzyme-bound fatty acid or some other reactive forms. Both the biogenesis and the role of methyl esters remain for future investigation.

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Polymorphism of Glyceryl Ethers and Ether Esters

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ABSTRACT

Phase behavior has been studied by thermal and diffraction methods for 1- and 2-palmityl and stearyl ethers of glycerol and for 14 trialkyl glyceryl ethers, dialkyl monoacyl glyceryl ethers and monoalkyl diacyl glyceryl ethers, all of which were saturated trichain substituted glycerol compounds containing one or more of the following chains: palmityl (Py), palmitoyl (P), stearyl (Sy) and stearoyl (S). The monoalkyl glyceryl ethers resemble monoglycerides in crystallization behavior but with significant differences. Isomeric 1- and 2-ethers are very close in melting point. The 1-ethers show, besides a stable form, two other forms which transform reversibly to each other. The 2-ethers are polymorphic but with only one clearly established melting level. All trichain compounds were polymorphic also, most being dimorphic, each exhibiting a metastable α form, typically more stable than that of related triglycerides. Forms other than α were labeled I, II, etc., in order of decreasing melting point and were typically obtained from solvent. Polymorphic behavior showed some rather large departures from that of related triglycerides and appeared generally more sensitive to impurities. The two triethers, PyPyPy and SySySy were dimorphic each with a stable form much resembling metastable α in diffraction pattern, hence presumed to be of a new (more dense) hexagonal type of cross sectional structure. Three dialkyl monoacyl compounds PyPyP, SySyS and SyPyS and also three monoalkyl diacyl compounds PPyP, SSyS and SPyS were dimorphic, with Form I a stable, nontilted, somewhat β'-like form. PySyS and PSyP, which were trimorphic, showed such a β'-like form as a Form II, i.e., a second highest melting point. The stable phase of four compounds, namely PyPP, SySS, PySyS (all trimorphic) and dimorphic PySS (and possibly that of SyPP) could be called a β phase. Presence of an alkyl group on the 2 position of glycerol, in all but the PySyS, prevented β structure. PSyP and SyPP exhibited triple chain length structures, not encountered in saturated mixed triglycerides with less than four carbons difference in the acyl chains. SyPP was exceptional in showing four forms.

INTRODUCTION

There is increasing evidence for the occurrence of esterified glyceryl ethers in natural fats especially in those of animal origin. Even trialkyl ethers have been reported (1). Such compounds have attracted biochemical interest and the syntheses of several trialkyl glyceryl ethers, dialkyl monoacyl glyceryl ethers and monoalkyl diacyl glyceryl ethers have been recently reported (2-4).

The crystallization behavior of these compounds has not been extensively explored. This subject should be of interest particularly in relation to the rather extensively studied crystallization of triglycerides. Accordingly, polymorphic studies of several ethers and ether esters have been investigated by thermal and diffraction methods. Since this study was undertaken, related studies by thermal and infrared methods have been reported (5).

An extensive and excellent review of The Biochemistry of Lipids Containing Ether Bonds has appeared (6).

EXPERIMENTAL PROCEDURES

The glyceryl ethers were made after the manner of Baumann and Mangold (7). From

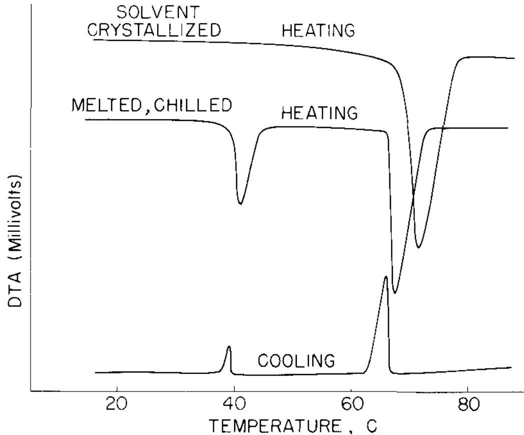

FIG. 1. DTA curves for 1-octadecyl ether of glycerol.

TABLE I

Analyses of Glyceryl Ethers

Preparation	% 1-Monoether[a]		Hydroxyl No.		Carbon, %		Hydrogen, %		Sapon. No.		Total fatty acid	
	Exp.[b]	Theory	Exp.	Theory	Exp.	Theory	Exp.	Theory	Exp.	Theory	Exp.	Theory
Monoethers[c]												
1-Palmityl	96.1	100.0	353	354								
1-Stearyl	97.4	100.0	315	326								
2-Palmityl	0.1	0.0	355	354								
2-Stearyl	1.3	0.0	325	326								
Trichain compounds												
PyPyPy					80.2	80.0	13.6	13.7				
SySySy					80.3	80.6	13.8	13.8				
PyPP					77.1	77.2	12.4	12.7	139	141.4	63.6	64.6
SySS					78.6	78.0	12.7	12.9	125.5	127.9	64.0	64.8
PPyP					77.2	77.2	12.5	12.7	141	141.4	62.8	64.6
SSyS					78.3	78.0	12.7	12.9	125	127.9	64.0	64.8
PySS					78.0	77.8	12.7	12.8	131	132.1	66.4	67.0
SPyS					78.3	77.8	13.0	12.8	130.5	132.1	66.3	67.0
SyPP					77.4	77.5	12.7	12.8	135.5	136.6	61.7	62.4
PSyP					77.3	77.5	12.8	12.8	136	136.6	62.0	62.4

[a]Periodic acid analysis.
[b]Experiment.
[c]>99% by TLC.

TABLE II

Comparison of Melting Points of Different Preparations

Preparation	L&S	B&M (2,3)	O,P,A&S (4)
Monoethers			
1-Palmityl	63.7	65.5	63.0
1-Stearyl	70.3	71.5	71.0
2-Palmityl	63.5		63.0
2-Stearyl	71.0		71.0
Trichain compounds			
PyPP	57.9		53-56
SySS	65.4	61.5-62	
PPyP	56.9		57.5-58.0 (61-62?)
SSyS	65.2		63.5-64.0
SyPP	55.8	53.5-54	53-54
PySS	61.0	61-61.5	
PSyP	60.7		
SPyP	59.6		
PyPyPy	47.8	47-48	
SySySy	57.3	57.5-58	

C_{16} and C_{18} alcohols of 98.5+% chain length purity, alkyl methane sulfonates were prepared. Isopropylidene glycerol or benzylidene glycerol, according to whether the goal was the 1- or 2-ether, was reacted either with K or KOH. The product was then reacted with the alkyl methane sulfonate to give the desired ether. Analyses appear in Table I.

From the appropriate monoether, diesters were obtained by reacting with acyl chloride in pyridine plus isooctane solution at 100 C. Reflux continued from 12 to 18 hr using an air condenser and heating mantle. The acyl chlorides were prepared from acids of 99% purity as indicated by gas liquid chromatography of the methyl esters. The crude ether diesters in isooctane were washed three times each with: (a) H_2O, (b) 5% K_2CO_3 in 60% to 80% ethanol and (c) H_2O, with hexane added to insure diester solution in the upper layer. After drying with Na_2SO_4 the sample was placed on a Silica Gel (5% H_2O) column (40 g to 1 g of sample) and was eluted in 30 to 40 fractions with the single solvent mixture, Skelly F-benzene (2:1 v/v). Fractions were selected and pooled according to results of thin layer chromatography (TLC) with hexane-ethyl-ether-acetic acid (80:20:1) on Silica Gel G. Yields were above 90% usually. Final purification was effected by 10 C crystallization (1:10) from hexane. All samples showed a single spot by TLC. Semimicro chemical analyses are reported in Table I.

Diethers were prepared as reported (8). These were converted to triethers by reacting with KOH then with alkyl methane sulfonate. Triethers were purified as above for monoethers. Again single spots were obtained by TLC.

Analytical data appear in Table I. In Table II are listed comparative melting point results for recent preparations by three different laboratories.

Phase behavior was examined by a combination of three methods:

1. Capillary melting point with complete melting point (CMP) obtained at a rate of 0.5 C, and rapid melting point (RMP) obtained by thrusting in at specified temperature, with the RMP being the mean of the lowest clear point and the highest point with solid remaining.

2. Differential thermal analysis (DTA) by a DuPont 900 unit employed for heating curves of solvent crystallized and melt crystallized samples and cooling curves of melt. DTA was valuable for checking melting level and purity of phase and for indication of transformation tendency of metastable phase.

3. X-ray diffraction by film technique with Ni-filtered CuK_α radiation and 5 or 10 cm sample to film distance (5 cm for phase identification, 10 cm for long spacing determination).

Thermal and diffraction data on our synthesized compounds are recorded in Tables III, IV and V.

RESULTS AND DISCUSSION

Mono Ethers

In contrast to the monoglyceride case, where the 2-compounds are relatively lower melting, 1- and 2-monoethers have nearly equal complete melting points.

The 1-hexadecyl and 1-octadecyl ethers exhibit very similar behavior. Each has a higher melting stable Form I melting about 5 C above

TABLE III

Thermal and Diffraction Data for Monoalkyl Glyceryl Ethers

	1-Palmityl			1=Stearyl			2-Palmityl			2-Stearyl		
	Form III	II	I	III	II	I	III	II	I	III	II	I
Thermal data	TP[a] 27.5	MP 57.8	MP 63.7	TP 41	MP 65.7	MP 70.3	---	MP 61	MP 63.5	---	MP 69.9	MP 71.0
Diffraction data (A) Short spacings	4.11 VS 3.65 S	4.16 M	4.40 VS 3.88 S 3.68 S	4.13 S 3.67 M	4.16 S (+ weak lines)	4.43 S 3.87 S 3.65 S	4.18 S 3.98 S 3.90 M	4.37 W 4.21 M 3.89 S	4.42 W+ 4.24 M 4.06 M+ 3.91 M	4.55 W 4.48 W 4.10 S 3.86 M 3.77 M	4.33 W 4.15 W 3.90 M	4.42M 4.22M 4.07M 3.90M+
Long spacings	44.3	48.3	41.5	46.9	51.7	44.3	37.5	36.8	37.3	41.1	40.5	40.9

[a]Transformation point Form III ⟶ Form II; VS, very strong; S, strong; M, medium; W, weak; VW, very weak.

TABLE IV

Thermal and Diffraction Data of Trialkyl Glyceryl Ethers and Monoalkyl Diacyl Glyceryl Ethers Chains of Single Length

	PyPyPy		SySySy		PyPP			SySS			PPyP		SSyS	
	α	I	α	I	α	II[a]	I (β)[b]	α	II[a]	I (β)[b]	α	I (β')	α	I (β')
Melting points	42.1	47.8	51	57.3	43.7	53.2	57.9	53.3	62.0	65.2	44.0	56.9	53.2	65.4
Diffraction data Short spacings	4.11 S	4.01 S 3.84 VW	4.09 VS	4.01 S 3.84 W	4.11 VS	4.32 S 4.15 W+ 3.90 W+ 3.70 W+	5.33 VW 4.58 VS 3.87 W 3.73 W	4.11 VS	4.44 S 4.27 S 3.94 M 3.73 M	5.28 VW 4.57 S 3.83 M 3.69 M	4.13 VS	5.41 W+ 4.16 S 3.85 M- 3.67 M	4.11 VS	5.41 W 4.12 VS 3.83 M 3.66 M
Long spacings	45.9	46.0	51.2	50.8	45.6	42.0	40.4	51.4	46.7	45.0	44.2	44.4	49.6	48.8

[a]From acetone.

[b]From hydrocarbon.

an α-like Form II. As in the 1-monoglyceride case (9), the lower melting form transforms reversibly to a low temperature Form III. By differential thermal analysis (DTA) the exothermal transition from III to II can be observed below the exothermal melting of Form II (Fig. 1). With the 1-octadecyl compound, the reversibility of the Form II→Form III transition can be followed by x-ray diffraction but the metastable 1-hexadecyl forms are too fleeting; nevertheless diffraction data could be obtained at 0 C for Form III of 1-hexadecyl ether and at 45 C for Form II, the latter in mixture with Form I. Metastable forms appear more fleeting than corresponding monoglyceride forms. A striking contrast between these ethers and the 1-monoglycerides is the variation in long spacings of different polymorphs for the ethers in contrast to the remarkable constancy for 1-monoglycerides.

The 2-monoethers unlike the 2-monoglycerides (10) are polymorphic. Nevertheless only one characterizing thermal point was observed by DTA and only minor melting point differences. All forms of a given homolog are of nearly equal long spacing. Stable Form I is obtained from solvent, Form II by melting and chilling, and a probable but not well characterized Form III is also obtained by melting and chilling, with distinguishing conditions for II and III not understood. Metastable forms transform slowly to Form I at room temperature.

Trichain Compounds

The behavior of the trichain ethers and ether-esters showed many similarities to that of corresponding triglycerides (Table VII) but there was much individuality and there were some surprises.

Nomenclature of Polymorphs. In approach to a nomenclature for the polymorphs observed, the first consideration is clarity and the second is correlation with familiar triglyceride nomenclature. There is no confusion in calling lowest melting forms with single 4.1 A short spacings by the name α. All other forms are designated by Roman numerals according to our understanding of melting level, with I being highest. Forms which clearly have strong 4.6 A short spacings and tilted chains are given the secondary designation β. Forms with strong 4.1 A short spacings and other reasonably strong short spacings are referred to in the text as β′-like. Two forms which are of triple chain length are so designated as, e.g., I-3; all other forms are of double chain length structure.

α Forms. All trichain compounds explored were polymorphic. As with triglycerides, a low melting α form with perpendicular chains was

TABLE V

Thermal and Diffraction Data of Monoalkyl Diacyl Glyceryl Ethers Chains of Mixed Lengths

	PySS		SPyS		SyPP				PSyP		
	α	I (β)	α	I	α	III-3[a]	II[a]	I (β)[b]	α	II[c]	I-3[d]
Melting points	48.6	61.0	48.2	59.6	45.8	54.9	55.1	55.8	47.1	55.7	60.7
Diffraction data											
Short spacings	4.11 S	5.34 W 4.60 S 3.86 M 3.70 M	4.11 S	5.41 W 4.15 S 3.78 W 3.66 W	4.13 S	4.46 S 4.31 M 4.10 M 3.98 M+ 3.86 M+	4.27 S 4.08 M 3.86 S	4.57 S+ 4.06 S 3.72 M	4.13 VS	5.40 W 4.11 S 3.83 W 3.67 M	4.43 W 4.26 W 4.11 M 3.99 W 3.76 M
Long spacings	49.9	46.4	48.5	47.5	48.0	62.8	43.6	38.5	45.5	45.5	63.1

[a]From 1:10 hexane.
[b]From 1:100 hexane.
[c]From 1:20 hexane.
[d]From acetone.

TABLE VI

Thermal and Diffraction Data for Dialkyl Monoacyl Glyceryl Ethers

PyPyPy		SySyS		PySyS			SyPyS	
α	I	α	I	α	II	I (β)	α	I
Melting points								
41.7	54.5	51.0	62.7	46.7	56.0	57.8	47.7	55.2
Short spacings								
4.13 S	5.41 W 4.14 S 3.86 W 3.69 W	4.13 S	5.41 W 4.14 S 3.86 W 3.69 W	4.13 S	5.41 W 4.14 S 3.79 W 3.67 W	4.53 VS 4.12 W 3.93 M 3.73 W- 3.63 W	4.13 S	5.41 4.14 S 3.79 W 3.67 W
Long spacings								
46.0	44.3	50.5	49.2	49.0	47.0	43.2	49.5	48.2

common to all compounds. It was obtained by chilling the melt and was normally more stable than the corresponding triglyceride form. In general, α mp was lower by a little over 1 C per each replacement of -CO- group by -CH_2- group. There also appeared to be a contraction of short spacings, hardly outside of experimental error from about 4.14 A for triglycerides to about 4.11 or 4.12. Long spacing magnitude appeared to be affected by the position of alkyl with respect to acyl chains in the molecule as will be discussed later.

Other Forms. The other forms were normally obtained from solvent. The nature of the polymorphs showed a greater sensitivity to impurity than normally found with triglycerides; more types and more minor variations of diffraction pattern were observed.

Trialkyl Glyceryl Ethers

The triethers, PyPyPy and SySySy, are dimorphic with a low melting rather stable α form, not transforming in 3 hr within 5 C of the melting point. The α forms melt some 4 C lower than those of the comparable equal chain length triglycerides.

For the samples that were most highly purified, as judged not so much by the necessary conditions of adequate elemental analysis and single spot on TLC but especially by melting point level, there was only one form observed in addition to metastable α. This high melting polymorph from hexane, Form I, is remarkable in the superficial resemblance of its diffraction pattern to that of α. Long spacings are nearly identical and short spacings differ only slightly. Form I shows a single strong spacing at 4.01 A, as compared with about 4.10 for α. Other Form I spacings are very weak or considerably smaller or both. Yet Form I and α differ in mp by 6 C. One is forced to conclude that the two structures are very similar, that both have ⊥ chains and hexagonal subcells but that Form I is more densely packed by a factor of about $(4.10/4.01)^2$ ~1.04. In support of the hexagonal arrangement, significant spacings appear at 4.01S, 2.31M and 2.00W for Form I in accordance with the required ratio $1:1/\sqrt{3}:1/2$. Single crystals of α phase for detailed structure study have seemed out of the question, but perhaps isostructural single crystals of a lower homolog of PyPyPy are realizable and might shed light on hexagonal packing of long chains in general.

Monoalkyl Diacyl Glyceryl Ethers

The monoether diesters are not so simple a matter to discuss except for the rather uniformly behaving α forms. These compounds are discussed as two homologous pairs and two isomeric pairs.

Homologs PyPP and SySS. The compounds are trimorphic with typical α phases melting only about 1 C below corresponding phases of tripalmitin and tristearin. Stable forms obtained by crystallization from hexane 1:10 are clearly of β type melting about 8 C below the triglyceride levels. Intermediate phases obtained by crystallization from acetone 1:10 are not familiar β' types but are suggestive of stable monostearin phase (whose subcell has been described as M||) (12).

Great difficulty was encountered in sufficiently purifying SySS for it to behave homologously with respect to PyPP. Each of two preparations (one at Hormel and one at this laboratory) gave β PyPP, but only one of four preparations, the highest melting, gave β SySS.

Homologs PPyP and SSyS. The behavior of these homologs was closely comparable to that of diether monoesters. For some reason the alkyl chain in the 2 position seems very influential toward dimorphism with α and β'-like structures, and with the latter probably non-

TABLE VII

Thermal and Diffraction Data for Triglycerides (for Comparison) (11)

PPP			SSS		
α	β'	β	α	β'	β
Melting points					
45.0	56.5	66.0	54.7	64	73.3
Short spacings					
4.14 S	4.18 VS 3.78 S	5.24 M 4.61 VS 3.84 S 3.68 S	4.14 S	4.18 VS 3.78 S	5.24 M 4.61 VS 3.84 3.68 S
Long spacings					
45.8	42.5	40.8	50.6	47.0	45.1

tilted. One is reminded also of the β' stability of PSP and SPS (13) and is tempted to speculate that the different chain in the middle position militates against the "in-line" arrangement of the chains on the 1 and 2 positions of glycerol, as is characteristic of β phase (14).

The Isomeric Pair PySS and SPyS. The members of this pair were dimorphic. Metastable α forms are of nearly equal melting point. PySS shows a stable β phase with tilted chains. SPyS shows stable β'-like phase with probably non-tilted chain; again a compound with differing middle chain resisting β formation. The unsymmetrical compound melts higher than the symmetrical one, not an unheard of situation, but unusual.

The Isomeric Pair SyPP and PSyP. It is concluded that PSyP is trimorphic with (a) α phase, (b) stable triple chain length Phase I which is β'-like and (c) an intermediate-melting double chain length β'-like phase reminiscent of, e.g., PyPyP stable phase. (An indication of a β phase in the first solvent crystallization has not been supported by later efforts.)

SyPP has been the most difficult of these compounds to characterize satisfactorily from a polymorphic standpoint. As finally characterized, it is unique in being tetramorphic. The highest melting Form I, β-like, but possibly never obtained pure, was crystallized from 1:100 dilution in hexane at 1 C. Two other phases of almost equal melting points were obtained in different regions of the crystallization vessel with sample at 1:10 dilution in hexane at 43 C. One of these was of triple chain length. The other was of double chain length and β'-like. The fourth form was a normal α form obtained by chilling the melt.

Dialkyl Monoacyl Glyceryl Ethers

The behavior of dialkyl monoacyl compounds, PyPyP, SySyS, PySyS and SyPyS, all prepared at the Hormel Institute, can be quickly summarized. It is quite similar to that of the dimorphic single chain length 2-ether diesters, PPyP and SSyS, except that PySyS shows a third form. All compounds showed α and β'-like polymorphs with slight differences in the shorter spacings of the higher polymorph according to whether the compound was of mixed or unmixed chain lengths. The stable forms were as received and had been crystallized from solvent (ethanol and acetone) (3). This β'-like form was only slightly shorter than α in long spacings. PySyS alone showed a β-like form from α held one week just under the α mp.

Comparison With Triglycerides

It is of interest to compare behavior of these ethers and ether esters to that of the familiar triglycerides. An ether group in the 1 position, whether Sy or Py, still permits β formation in SySS or PySS as in SSS. The same group in the

TABLE VIII

On Stability of α Phase Stored
1 Week at T C, Near α MP

Compound	T C	Phase
PyPyPy	38	α
SySySy	49	α
PyPP	43	Mainly α
SySS	49	α
PPyP	38	I
SSyS	43	α + I
PySS	49	α + I
SPyS	49	I
SyPP	43	α
PSyP	43	Mainly α
PyPyP	38	I
SySyS	49	α
PySyS	43	I (β)
SyPyS	43	Mainly α

TABLE IX

Comparison of α Long Spacings of Monoalkyl Diacyl Glyceryl Ethers

Isomer	α LS (A)	Difference (A)	Suggested partially schematic configuration
SySS	51.4	+1.8	1[a] Sy —o—o— S 2; o— S 3
SSyS	49.6		2 Sy —o—o— S 1; o— S 3
PySS	49.9	+1.4	1 Py —o—o— S; o— S
SPyS	48.5		2 Py —o—o— S; o— S
SyPP	48.0	+2.5	1 Sy —o—o— P; o— P
PSyP	45.5		2 Sy —o—o— P; o— P
PyPP	45.6	+1.4	1 Py —o—o— P; o— P
PPyS	44.2		2 Py —o—o— P; o— P
PySyS	49.0	-0.5	1 S —o—o— Sy 2; o— Py 3
SyPyS	49.5		1 S —o—o— Py 2; o— Sy 3

[a]o, glyceryl carbon; S, stearoyl chain; Sy, stearyl chain.

2 position eliminates β, except for PySyS, even though SSS and SPS are notable β formers.

Sy in PSyP not surprisingly eliminates β, metastable in PSP, and introduces a triple structure.

Sy in SyPP permits β, introduces a triple structure, and apparently creates uncertainty as to which of three phases (other than α) the compound will exhibit.

It becomes apparent that the -CO- group present in triglycerides induces compounds of that class to crystallize much after the fashion of hydrocarbons with the same three (hexagonal, O⊥ and T||) subcells. Absence of one or more -CO- groups, especially in the 2 position, in a trichain glyceryl compound, influences the compound away from the T|| arrangement.

Phase Transformation

As a group, the glyceryl ethers and ether esters transform slowly. DTA curves for 10 mg samples of α forms obtained by chilling the

melt uniformly showed no transformation during the run in contrast with curves for related triglycerides which show substantial transformation during the run to higher polymorphs, except in the cases of SPS and SPP. Efforts at substantially transforming α phase by storage one week near the α mp were successful only with PPyP, SPyS, PyPyP and PySyS as indicated in Table VIII.

Comparison With Infrared Studies

It is difficult to compare conclusions based on diffraction plus thermal data with those from infrared plus thermal data, but some comment is in order with regard to our degree of agreement with Oswald et al. (5) on PyPP, SyPP, PPyP and SSyS investigated by both laboratories.

It can be concluded that results are in close agreement as to the dimorphic behavior of PPyP and SSyS, our I and α forms corresponding to Oswald's A and B Forms.

Conclusions seem to be in considerable agreement for PyPP, our I and α corresponding to Oswald's A and C. Our II Form does not resemble β' tripalmitin as nearly as Oswald states for his B form.

We agree with Oswald in regarding SyPP as tetramorphic. His D and our α are surely the same. But we have no experience with these or similar compounds of a form with "melting point of 61-62 C, which quickly changed to 57-58 C (Form B)." Form I, our highest melting (about 56 C), is regarded as most stable, certainly near the melting point, in contrast to Oswald's "stable A form....melting point of 53-54 C." It is unfortunate that the specter of the "vitreous form" of triglycerides was raised again in Forms D and E. We agree that the different polymorphs of SyPP are hard to disentangle.

Variation in Long Spacings of α With Isomerism

No crystal of an α polymorph of any long chain compound, adequate for detailed crystal structure study, has been prepared. Any hint about α-type structure is therefore of interest. There are details of behavior of α polymorphs of the monoalkyl diacyl glyceryl ethers which are suggestive; they involve long spacing difference between isomers. The data are isolated in Table IX.

If one takes a simplistic approach, it is possible to set up a logical system in accordance with the direction of long spacing difference between isomers.

Suppose the following rules apply:

1. A first and second chain on a glyceryl group are oppositely oriented and in a straight line (as is known in β phase triglyceride structures).

2. There is a strong tendency for acyls (or alkyls) of a given molecule to lie side by side and conversely for acyls to avoid alkyls (as supported by occurrence of triple chain length structure in PSyP, but not PSP). Of course, in double chain length structures such as we deal with here, there must be some S chains adjacent to Sy chains.

3. The long chains are perpendicular to end group planes as has appeared typically of α crystals.

Application of these rules to the SySS-SSyS pair leads to an approximate Sy+S length for each isomer. However in SySS the second S on the third glyceryl position tends to maintain the structure length while in SSyS, the second S tends to shorten the structure length. Similar considerations hold for other isomeric monoether diester pairs. Furthermore such an argument does not lead to a distinction in the pair PySyS-SyPyS, whose α long spacings agree within experimental error.

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Exceptional Occurrence of Odd-Chain Fatty Acids in Smelt (*Osmerus mordax*) From Jeddore Harbour, Nova Scotia

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ABSTRACT

The fatty acid composition of smelt, *Osmerus mordax*, from several areas in Eastern Canada is reported. Smelt from Jeddore Harbour, N.S., were unusual in having up to 10% of C_{15}, C_{17} and C_{19} acids. These acids were similar in structure to odd-chain acids occurring in other marine lipids, notably mullet, and were distributed among all body and gonad lipids. Possible reasons for their occurrence are discussed.

INTRODUCTION

Odd-numbered fatty acids generally account for only 1-2% of total fatty acids in fish lipids (1-4). The sole exception to this rule so far described is the mullet, *Mugil cephalus*, which normally contains from 10-25% odd-numbered acids, depending on area and season of catch (2,4-6). Recently, however, we have unexpectedly observed that smelt, *Osmerus mordax*, obtained from one specific location in Nova Scotia contained relatively large amounts of odd-chain acids. Since the occurrence of these acids in any quantity is unusual, we have examined in some detail the lipids and fatty acids of smelt of various local origins. Particular attention has been paid to the distribution of monoethylenic fatty acid isomers of any one chain length.

EXPERIMENTAL PROCEDURES

Smelt were gill-netted at various dates in Nova Scotia and New Brunswick as follows: Jeddore Harbour, N.S. (Lot No. 1) February 6, 1969 (wt 31-39 g); Jeddore Harbour, N.S. (Lot No. 2) March 3, 1969 (wt 33-48 g); Digby, N.S. March 11, 1969; Pictou, N.S. February 24, 1969; Miramichi, N.B. March 7, 1969. The fish were frozen in a domestic freezer and usually were sent to the laboratory for analysis within one day of catch. Heads, tails and internal organs other than gonads were discarded, and bodies and gonads were pooled separately by sex for analysis.

Lipids were extracted by the method of Bligh and Dyer (7). Samples of lipids were transesterified (8) and the methyl esters analyzed by gas liquid chromatography (GLC) on packed columns as described previously (9). Methyl esters from body lipids of Jeddore Lot No. 1 and of Digby, Pictou and Miramichi samples were fractionated by thin layer chromatography (TLC) on silver nitrate-silicic acid plates and the fractions obtained analyzed by open-tubular GLC (10). Lipids from Jeddore Lot No. 2 were subjected to silicic acid column chromatography (9) and the fatty acids of the major fractions analyzed by packed-column GLC.

RESULTS AND DISCUSSION

All the fish analyzed were mature, and would have spawned in May or June, 1969. Details of their lipid content and total fatty acid composition are shown in Table I. Data for Jeddore Lot No. 1 are averaged from three analyses of individual males and two of individual females; those for Jeddore Lot No. 2 are from a pool of lipid extracted from four of each sex; those for Digby, Pictou and Miramichi are from a pool of six, eight and six fish (not segregated by sex) respectively. This intersex pooling appeared permissible since the sex differences in fatty acid composition of body lipids in Jeddore Lot No. 1 fish were not obvious; in Jeddore No. 2 body lipids (Table I) the males contained more 16:0 and 22:6 (chain length:double bonds) in the body lipids than did the females, which may have been due to their higher phospholipid content (Table II, Ref. 9).

The main point of interest is the relatively large amount of odd-chain acids in the body lipids of Jeddore smelt (in Lot No. 1 fish amounting to over 10% of the total) as compared to smelt from other areas. Although the level of these acids dropped in the Lot No. 2 fish, both 15:0 and 17:1 levels were still unusually high. The distribution of odd-chain acids in Jeddore smelt was fairly similar to that found in mullet from the Gulf of Mexico (5), the main difference being that smelt contained less 15:0 than did the mullet. However, wide differences occurred in the composition of mullet oils examined by different groups (4-6). This may represent local or seasonal variations; so this difference between smelt and mullet

TABLE I

Fatty Acid Composition and Lipid Content of Smelt (*Osmerus mordax*) From Various Areas in Eastern Canada (Wt%)

	Bodies						Gonads	
	Jeddore			Digby	Pictou	Miramichi	Jeddore	
	No. 1	No. 2					No. 2	
Fatty acid		Male	Female				Male	Female
12:0	Trace	Trace	0.1	Trace	Trace	Trace	0.4	0.1
13:0	Trace	---	---	---	---	---	0.4	0.1
14:0	2.8	3.0	3.6	2.8	2.8	2.7	2.0	3.5
14:1	0.1	Trace	0.5	0.1	0.3	0.5	0.4	0.4
15:0	2.9	1.7	1.7	0.3	0.8	0.7	1.5	2.9
15:1	1.5	0.9	1.0	0.2	Trace	0.2	0.6	1.6
16:0	15.9	20.2	15.9	16.7	19.0	19.0	14.4	14.8
16:1	10.9	10.4	11.6	8.3	11.2	9.1	7.8	13.1
16:2ω7	0.4	0.7	0.4	0.7	0.1	0.6	0.2	0.4
16:3ω4	0.2	Trace	0.1	0.4	0.3	0.6	0.3	0.5
16:4ω1	0.5	Trace	0.1	Trace	0.2	Trace	0.1	0.3
17:0	0.9	0.4	0.7	1.0	1.0	0.6	0.2	0.5
17:1	6.0	2.8	3.9	0.8	0.6	0.6	2.9	7.0
17:2	Trace	Trace	0.3	Trace	Trace	Trace	Trace	0.2
18:0	2.7	2.7	2.4	2.0	2.8	3.0	2.1	0.5
18:1	20.0	21.4	23.9	23.5	21.3	20.3	25.4	23.3
18:2ω6	0.8	0.4	0.7	0.8	1.4	0.9	1.2	1.0
18:3ω3	0.2	0.4	0.3	0.3	0.4	0.4	0.4	1.2
18:4ω3	0.6	0.9	0.7	1.3	1.0	0.7	0.7	1.0
19:1	0.9	0.1	0.6	0.3	0.3	0.4	1.1	0.2
19:4ω5	1.0	Trace	---	---	---	---	---	---
20:0	0.5	---	0.3	---	---	---	Trace	0.3
20:1	0.9	0.9	2.5	1.2	1.6	0.7	2.5	0.4
20:2ω6	0.2	Trace	0.4	0.1	0.2	Trace	0.2	0.2
20:4ω6	2.0	2.1	2.0	1.8	1.2	1.4	8.6	2.6
20:4ω3	0.3	Trace	0.1	Trace	Trace	0.2	Trace	0.2
20:5ω3	12.4	11.1	10.9	20.7	13.1	11.6	13.5	12.7
21:5ω2	Trace	---	---	Trace	Trace	---	---	0.1
22:1	Trace	---	---	0.2	Trace	---	---	---
22:5ω3	0.6	0.6	0.9	0.5	0.8	0.5	4.4	1.0
22:6ω3	14.4	19.1	14.3	15.6	19.5	25.5	8.8	9.9
24:1	Trace	---	---	---	---	---	---	---
Lipid content (%)	2.3	1.1	1.4	2.0	1.3	2.3	0.6	0.7
Total odd-chain acids	13.2	5.9	8.2	2.6	2.7	2.5	6.7	12.6

fatty acids may be unrelated to species difference. Otherwise, the major odd-chain acids described by Sen and Schlenk (5) (15:0, 17:0, 17:1, 17:2, 19:1, 19:4 ω5) were present in roughly similar proportions in the smelt. 19:4 ω5 was obvious only in Jeddore Lot No. 1, and it may be associated with the higher levels of C_{15} and C_{17} acids.

The low levels of 20:1 and the virtual absence of 22:1 from smelt from all locations is curious. Smelt are anadromous and inhabit an inshore or estuarine environment. These fish are reported to have a diet consisting of amphipods, euphausiids, mysids, shrimp, marine worms (*Nereis*) and any small fish that are available such as small herring, mummichogs and silversides (11). Of these, at least certain euphausiids and shrimp (12) and mature herring (13) contain appreciable amounts of long chain monoenes and polyenes. We can offer no explanation at present, other than a species peculiarity, for the failure of these acids to be deposited in the smelt triglycerides.

Since monoene acids (15:1, 17:1 and 19:1) made up a considerable portion of the odd-chain acids, their isomer distribution was analyzed (the principal 17:1 isomers indicated by open tubular GLC in Jeddore smelt fatty acids were verified by oxidative fission) and compared with that of fish from other areas

TABLE II

Lipid Composition of Smelt Bodies and Gonads (Jeddore Lot No. 2)

Lipid origin	Body		Gonad	
	Male	Female	Male	Female
Lipid composition (% total by weight)				
Hydrocarbons	0.8	0.6	---	3.5
Sterol esters	1.0	1.1	1.0	2.6
Triglycerides	56.1	62.4	58.0	74.2
Free fatty acid)	)	)	---	---
)	6.5)	5.7)		
Cholesterol)	)	)	30.0	1.6
Cardiolipin	1.6	2.0	)	0.6
Cephalins	11.7	11.0	) 11.0	3.3
Lecithins	19.4	16.5	)	12.5
Sphingomyelins	2.9	0.7	)	1.7

(Table III). In all cases the Δ^9 isomer predominated in both even and odd-chain acids up to C_{18}, but the C_{19} and C_{20} acids contained larger amounts of Δ^{11} and Δ^{13} isomers. Similar results were obtained by Sen and Schlenk (5) for mullet oil odd-chain monoenes, and by Ackman and Castell (10) for herring oil even-chain monoenes. If desaturation in marine species occurs in the $\Delta^{9\text{-}10}$ position of saturated acids, as it does in terrestrial species (14), then these results are consistent with monoenes up to C_{18} arising mainly through desaturation of the corresponding saturated acids, whereas the C_{19} and higher monoenes are formed more through chain elongation.

The detailed composition of smelt body lipid, Jeddore Lot No. 2 fish, was investigated using silicic acid chromatography (Tables II and IV). Lipid composition was as expected for a fish of moderate fat content; i.e., triglycerides were present in fairly large amounts and the major phospholipid was lecithin. The fatty acid distribution among lipid fractions is shown in Table IV. Odd-chain fatty acids were found in all fractions. The only point of interest in their distribution was that they tended to follow the

TABLE III

Monoene Isomer Distributions in Body Lipids From Smelt of Various Origins

Fatty acid[a]		Jeddore No. 1	Digby	Pictou	Miramichi
14:1ω7	(Δ^7)	29	---	---	---
14:1ω5	(Δ^9)	71	100	100	100
15:1ω8	(Δ^7)	31	23	50	35
15:1ω6	(Δ^9)	69	77	50	65
16:1ω9	(Δ^7)	3	5	13	11
16:1ω7	(Δ^9)	93	90	84	85
16:1ω5	(Δ^{11})	4	4	3	4
16:1ω3	(Δ^{13})	---	1	---	---
17:1ω10	(Δ^7)	Trace	6	6	9
17:1ω8	(Δ^9)	93	71	86	77
17:1ω6	(Δ^{11})	7	23	8	14
18:1ω9	(Δ^9)	76	70	79	74
18:1ω7	(Δ^{11})	23	29	19	23
18:1ω5	(Δ^{13})	1	1	2	3
19:1ω10	(Δ^9)	11	22	33[b]	22
19:1ω8	(Δ^{11})	73	59	60	65
19:1ω6	(Δ^{13})	16	19	7	13
20:1ω11	(Δ^9)	21	18	44	13
20:1ω9	(Δ^{11})	38	46	54	56
20:1ω7	(Δ^{13})	41	36	2	31

[a]Per cent isomer distribution in monoenes of each chain length.
[b]May include a small amount of ω12 isomer.

TABLE IV

Distribution of Odd-Chain and Major Even-Chain Acids in Principal Smelt Body Lipids (Wt%)

Fatty acid	Jeddore Lot No. 2					
	Male body			Female body		
	Triglycerides	Cephalins	Lecithins	Triglycerides	Cephalins	Lecithins
14:0	4.6	1.2	1.8	4.9	0.6	2.0
15:0	1.7	0.7	2.0	2.2	0.3	2.2
15:1	1.1	0.2	---	1.5	---	0.1
16:0	14.4	18.0	29.8	14.3	12.7	27.3
16:1	15.6	4.4	5.9	15.6	2.9	5.8
17:0	0.6	1.9	0.9	0.4	0.8	0.7
17:1	4.4	2.0	1.7	5.4	1.6	2.0
18:0	1.7	7.6	2.9	1.6	4.7	0.9
18:1	29.5	18.2	13.1	27.9	17.1	12.5
18:2ω6	0.8	0.8	0.4	1.2	0.7	0.4
19:1	0.3	0.7	0.2	0.8	0.7	0.2
19:4ω5	Trace	0.7	---	---	---	---
20:1	2.9	1.3	0.6	3.3	0.8	0.9
20:5ω3	10.4	7.8	12.7	9.6	10.3	15.8
22:6ω3	7.0	27.5	23.6	6.0	42.0	24.8
Other	5.0	7.0	4.4	5.3	4.8	4.4
Total odd-chain acids	8.1	6.2	4.8	10.3	3.4	5.2

distribution of the next higher even-chain acids. Thus, triglycerides contained large amounts of 18:1 and 17:1, and lecithins contained large amounts of 16:0 and 15:0. In general, fatty acid distribution was similar to that observed in other fish lipids (9).

The composition of smelt gonad lipids was also examined, since mullet roe lipids have been reported to contain large amounts of wax esters rich in odd-chain acids and alcohols (15). Smelt gonads, however, contained mainly triglycerides with some phospholipids, and no unusual lipids in any quantity (Table II).

Most of this work was undertaken to establish that smelt lipids conform to usual fish lipid patterns with the exception of the occurrence of odd-chain acids in Jeddore smelt. Lipid types and compositions in Jeddore smelt were otherwise normal, and as smelt from other areas had a normal fatty acid composition it seems unlikely that the Jeddore smelt would have any unusual capacity to synthesize or deposit odd-chain acids. A more probable explanation is that there is some local peculiarity in the diet of Jeddore smelt, although the trophic level where this occurs is uncertain. Although none of the data provide any concrete information about whether the odd-chain acids were of endogenous or exogenous origin, their distribution suggests that they could have arisen from a diet rich in propionate (16) or related short-chain acids, rather than from a specific source such as algal heptadecane (17). The fairly general distribution of odd-chain acids through the fish lipids may also indicate that they do not have any specific metabolic role and that their occurrence in unusual proportions is accidental.

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Phylogeny of Lipase Specificity[1,2]

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ABSTRACT

Lipase preparations from 19 animal species, representing nine of the animal phyla, were studied. Acetone powders were prepared from the pancreas, hepatopancreas, gastrointestinal tract or whole animal, and the preparations were screened for lipolytic activity by tributyrin agar clearing. The pH optimum of each preparation was determined by using both tributyrin and corn oil emulsions. The positional specificity at the pH optimum of each lipase preparation was determined from the fatty acids released by a 2-5 min reaction with cocoa butter and lard. The vertebrate lipases behaved analogously to hog pancreatic lipase by showing a high specificity for the 1 and 3 positions of long chain triglycerides. Invertebrate preparations that did hydrolyze long chain triglycerides did so randomly, with no strong positional or fatty acid specificity.

INTRODUCTION

The 1,3-positional specificity of pancreatic lipase has offered an almost ideal means for investigating the external fatty acid chains in triglycerides (1,2). Obviously, there would be an advantage in having a number of lipases, each of which possessed a particular positional or fatty acid specificity.

Lipases have been found in many species of animals, plants and microorganisms (3). In general, the lipases that have been investigated attack glycerides randomly or show the 1,3-positional specificity characteristic of hog pancreatic lipase. The exceptions reported are a plant lipase, isolated from the oat pericarp (4,5), and a lipase isolated from the mold, *Geotrichum candidum* (6). The oat lipase was reported to have a high specificity for the 2 position of tributyrin (4), while the *Geotrichum candidum* lipase was shown to be specific for fatty acids containing *cis*-9-unsaturation, regardless of its position in the triglyceride molecule (7).

Our study was undertaken with the hope of isolating lipases that would be useful in elucidating glyceride structure. To be systematic, the study was made of the digestive lipases in organisms selected from the phyla of the animal kingdom.

EXPERIMENTAL PROCEDURES

Animal Lipases

Mammalian and shark pancreatic glands were obtained through the Iowa State University Veterinary Diagnostic Laboratory; from Pel-Freez Biologicals, Inc., Rogers, Ark.; or from the Harborton Marine Laboratory, Harborton, Va.

Invertebrates were obtained live from either Pacific Bio-Marine Supply Company, Venice, Calif., or the Lemberger Co., Oshkosh, Wisc. The digestive glands, gastrointestinal tract, or whole animal was used as a lipase source.

The tissues were homogenized in a Waring Blender and mixed with an equal volume of cold acetone to prepare stable acetone powders (8).

Tributyrin Agar Clearing

The acetone powders were screened for lipolytic activity by using tributyrin agar clearing. The plates and agar (without emulsifier) were prepared according to the procedure of Ellinghausen and Sandvik (9). For the assay, 10 mg of an acetone powder were mixed with 1.0 ml of 0.025 N ammonium hydroxide (8). The enzyme suspensions were serially diluted (1:1 until a final dilution of 1:4096 was obtained. With a Warburg pipette, 0.025 ml of each dilution was delivered into individual wells cut in chilled tributyrin agar with a No. 3 cork borer. The agar plates were incubated for 48 hr at 37 C. Tributyrinase activity was detected by a zone of clearing around each well. The last dilution capable of producing clearing was termed the lipase titer.

pH Optimum

The optimum pH for each preparation was determined by using tributyrin and Mazola corn oil emulsions. An emulsion was prepared by

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[2]Presented at the AOCS-AACC Joint Meeting, Washington, D.C., April 1968.

[3]Now with Campbell Soup Company, Camden, N.J.

TABLE I

pH Optima of Animal Lipases vs. Tributyrin and Corn Oil

Species (Common name)	Tributyrin pH Optimum	Tributyrin Activity[a]	Corn oil pH Optimum	Corn oil Activity[a]
Vertebrates				
Homo sapiens (man)[b]	8.0	24.9	9.0	2.8
Rhesus sp. (monkey)[b]	10.0	44.0	11.0	1.3
Suis scrofa domestica (hog)[b]	8.0	72.8	9.0	1.6
Didelphis marsupialis (oppossum)[b]	8.5	21.2	8.0	1.6
Gallus sp. (chicken)[b]	7.0	10.6	10.0	1.3
Chelydra serpentina (turtle)[b]	8.0	6.2	11.0	0.9
Squalus acanthius (shark)[b]	6.0, 9.0	0.2, 0.2	NR[c,d]	---
Ciona intestinalis (sea squirt)[e]	NR	---	5.0	0.02
Invertebrates				
Cambarus virilis (crayfish)[f]	4.0, 9.0	1.1, 0.8	4.0	0.7
Dendrostomum pyroides (peanut worm)[e]	6.0	0.9	6.0	0.06
Lumbricus terrestris (earthworm)[e]	9.0	2.9	5.0, 10.0	0.01, <0.01
Chaetopterus variopedatus (parchment tube worm)[e]	9.0	0.03	8.0	<0.01
Aplysia californica (sea hare)[f]	7.0	0.02	6.0	<0.01
Strongylocentrotus purpurtus (sea urchin)[e]	8.0	0.4	9.0	0.04
Pisaster giganteus (sea star)[f]	8.0	0.04	NR[d]	---
Parastichopus parvimensis (sea cucumber)[e]	7.0	0.06	NR[d]	---
Metridium senile (anemone)[d]	10.0	0.4	NR[d]	---
Dysidea amblia (sponge)[g]	9.0	0.01	6.0[h]	<0.01
Tetrahymena pyriformis (protozoan)[g]	9.0	0.03	8.0	<0.01

[a]Microequivalents 0.0100 N NaOH/mg acetone powder/hr.
[b]Pancreas used as lipase source.
[c]NR, no reaction observed.
[d]No reaction observed when lard was substituted for corn oil.
[e]Entire gut used as lipase source.
[f]Hepatopancreas used as lipase source.
[g]Whole animal used as lipase source.
[h]Hydrolyzed lard slightly; pH optimum 6.0.

homogenizing 2.0 ml of the substrate, 100 ml of 1.25% gum acacia (warmed to 45 C) and 6.0 ml of 1% $CaCl_2$ for 5 min in a Waring Blender. After adjusting the pH of the emulsion to 7.0 with 1% KOH, 5.4 ml of the emulsion was transferred to 150 ml x 15 mm screw-capped test tubes. The emulsion in each tube was buffered at the appropriate pH by adding 2.6 ml of Universal Buffer (10,11). For the assay, 10-100 mg of an acetone powder was mixed with 1.0 ml of 0.025 N NH_4OH. The lipase suspension was then added to the emulsion. The tributyrin emulsion was incubated for 15 min to 24 hr, while the corn oil emulsion was incubated for 6 to 48 hr. The reaction was stopped by adding 1.0 ml of 20% H_2SO_4. The fatty acids were extracted with 15 ml of diethyl ether-methanol (2:1 v/v). A 5.0 ml aliquot of the organic layer was removed and mixed with 50 ml of methanol. The fatty acids were titrated with .01 N NaOH to an end point of pH 9.0, using the Beckman Model K automatic titrator.

Electrophoresis

The acetone powder extract was prepared by mixing 80 mg of the powder with 1.0 ml of .025 N NH_4OH. The sample was prepared for disc-gel electrophoresis by mixing 30-40 μl of the acetone powder extract with 460-470 μl of water and 500 μl undiluted sample gel. Two disc-gel columns were prepared for each sample, according to the instructions given by the Canal Company. The columns were developed by a current of 2.5 ma per column, using the Canal Company Model 1400 constant rate source. Following the electrophoresis, the gels were removed from the glass tubes. One column was stained, while the other column, containing the same sample as the first, was placed on tributyrin agar, covered with a glass plate, which gently pressed the gel column against the agar, and incubated at 37 C. After 10 to 18 hr, active fractions were indicated by clear zones on the tributyrin agar.

TABLE II

Protein Fractions Possessing Lipolytic Activity on Tributyrin Agar

Lipase source	No. of active fractions	Distance migrated, cm
Man	2	0.55, 2.37
Monkey	1	0.90
Hog	1	1.36
Opossum	1	1.40
Chicken	2	1.34, 1.95
Turtle	2	1.00, 2.70
Shark	1	1.20
Ciona	NR[a]	---
Crayfish	1	1.17
Peanut worm	1	1.60
Earthworm	2	1.37, 2.77
Tubeworm	1	3.16
Sea hare	NR	---
Sea urchin	1	2.25
Sea star	1	1.77
Sea cucumber	1	3.00
Anemone	1	0.19
Sponge	NR	---
Protozoan	NR	---

[a]NR, no reaction observed.

Positional Specificity

Emulsions were prepared as described for the determination of the pH optimum, except that 1.8 g of cocoa butter or lard or 2.0 ml of corn oil were used for each 100 ml of 1.25% gum acacia. The emulsion was buffered at the pH corresponding to the pH optimum of the particular preparation. After the acetone powder (10 mg/1.0 ml .025 N NH_4OH) was added, the mixture was agitated for 2-5 min in a water bath at 37 C. The reaction was stopped, and lipids were extracted as described previously. The fatty acids were separated from the glycerides by a hexane-0.5% aqueous Na_2CO_3 extraction (12). The free fatty acids were recovered and converted to methyl esters (13). Samples of the whole fat were also converted to methyl esters. The fatty acid composition was determined by gas chromatography by using 15% ethylene glycol succinate on Chromasorb P (45/60 mesh). The carrier gas was helium, and the 6 ft x 0.25 in. column was maintained at 185 C.

RESULTS AND DISCUSSION

The animals selected for study, the pH optima exhibited by the acetone powders, and the digestive organs studied are shown in Table I. In all instances, the activity of the animal lipases was greater when the short chain triglyceride, tributyrin, was used as the substrate than when a long chain triglyceride, corn oil, was used. This finding is consistent with the observations of other workers (2,14). Of all the lipases investigated, three exhibited more than one pH optimum. The lipolytic activity shown by the preparations isolated from the invertebrates was considerably less than the activity

TABLE III

Relative Percentages of Fatty Acids Hydrolyzed From Cocoa Butter by Animal Lipases

Lipase source	Time, min	pH	Fatty acids		
			C_{16}	$C_{18:1}$	C_{18}
---	---	---	29.8[a]	35.6[a]	34.6[a]
Man	3	8.0	45	7	48
Monkey	2	8.0	47	5	48
Hog	2	7.0	48	7	45
Opossum	2	8.0	52	3	45
Chicken	2	7.0	51	9	40
Turtle	2	8.0	57	3	40
Shark	5	9.0	NR[b]	---	---
Ciona	5	5.0	30	35	35
Crayfish	5	4.0	52	17	31
Peanut worm	5	6.0	32	31	37
Earthworm	5	7.0	33	39	27
Tube worm	5	9.0	34	32	34
Sea hare	5	7.0	NR	---	---
Sea urchin	5	8.0	34	32	34
Sea star	5	8.0	NR	---	---
Sea cucumber	5	7.0	NR	---	---
Sea anemone	5	10.0	NR	---	---
Sponge	5	9.0	NR	---	---
Protozoan	5	9.0	NR	---	---

[a]Relative percentages of fatty acids present in cocoa butter.
[b]NR, no reaction.

TABLE IV

Relative Percentages of Fatty Acids Hydrolyzed From Lard by Animal Lipases

Lipase source	Time, min	pH	Fatty acids					
			C_{14}	C_{16}	$C_{16:1}$	C_{18}	$C_{18:1}$	$C_{18:2}$
---	---	---	1.6[a]	28.5[a]	2.7[a]	13.4[a]	43.8[a]	10.0[a]
Man	3	8.0	Trace	8	2	14	61	15
Monkey[b]	2	8.0	Trace	6	4	10	67	11
Hog	2	7.0	Trace	8	5	9	60	18
Opossum	2	8.0	Trace	6	2	10	66	15
Chicken	2	7.0	Trace	8	2	11	60	20
Turtle	2	8.0	0	2	Trace	4	80	14
Shark	5	9.0	NR[c]	---	---	---	---	---
Ciona	5	10.0	Trace	37	Trace	7	56	Trace
Crayfish	5	4.0	Trace	19	1	13	59	9
Peanut worm	5	6.0	Trace	20	1	10	65	3
Earthworm	5	7.0	8	34	Trace	18	40	Trace
Tubeworm	5	9.0	NR	---	---	---	---	---
Sea hare	5	7.0	NR	---	---	---	---	---
Sea urchin	5	8.0	Trace	26	2	14	53	5
Sea star	5	8.0	NR	---	---	---	---	---
Sea cucumber	5	7.0	NR	---	---	---	---	---
Sea anemone	5	10.0	NR	---	---	---	---	---
Sponge	5	9.0	NR	---	---	---	---	---
Protozoan	5	9.0	NR	---	---	---	---	---

[a]Relative percentages of fatty acids in lard.

[b]Gave an unidentifiable peak between $C_{18:1}$ and $C_{18:2}$; relative per cent, 2%.

[c]NR, no reaction.

shown by the pancreatic lipases. Four preparations (shark, sea star, sea cucumber and anemone) did not hydrolyze corn oil. These four were assayed with lard and 1-monoolein substrates. None of them showed any activity with lard, and only the shark pancreatic extract showed any activity with 1-monoolein. Probably these preparations contain esterases active against soluble or partially soluble substrates such as tributyrin but inactive against emulsified substrates such as corn oil. Presumably these species do have lipases, but their lipase may be inactivated by the preparation method or inactive against gum acacia emulsions. Of all the pancreatic preparations only shark contained no lipase. Perhaps sharks produce digestive lipase in some other organ. The sea squirt yielded an unusual lipase active against corn oil but inactive against tributyrin.

The electrophoretic results are shown in Table II. In all instances, bands whose positions corresponded with the areas of clearing produced by the unstained columns on tributyrin agar could be located in the stained gel columns. A few preparations possessed two active fractions on tributyrin agar. In these

TABLE V

Relative Percentages of Fatty Acids Hydrolyzed From Corn Oil by Animal Lipases

Lipase source	Time, min	pH	Fatty acids			
			C_{16}	C_{18}	$C_{18:1}$	$C_{18:2}$
---	---	---	11.6[a]	1.6[a]	27.4[a]	59.4[a]
Man	3	8.0	19	Trace	26	55
Man	3	10.0	19	1	24	56
Hog	2	5.0	18	2	27	53
Chicken	2	5.0	16	1	26	57
Ciona	5	5.0	33	Trace	14	53
Crayfish	5	5.0	22	2	26	50
Peanut worm	5	6.0	19	1	24	56
Earthworm	5	7.0	15	2	29	54
Sea urchin	5	8.0	27	1	23	49
Sea anemone	5	10.0	15	5	26	53

[a]Relative percentages of fatty acids in corn oil.

instances, the active protein fraction located nearer the origin on the gel column possessed the greater activity.

Those preparations containing two active bands may contain significant amounts of two lipases, although it is likely that one of the active bands may be an esterase active against the partly soluble tributyrin. This is especially probable for the chicken and turtle preparations which also show large differences in the pH optima for tributyrin and corn oil. Earthworm gut contains two active bands and shows two pH optima against corn oil, so this preparation probably contains two lipases. One may have come from the gut contents.

The natural fats, cocoa butter and lard, were employed as substrates in the determination of the positional specificity of all the lipases in this study. Cocoa butter has been shown to contain almost exclusively oleic acid on the β position and mostly stearic and palmitic on the α positions (15). Lard is unique among the naturally occurring fats in that palmitic acid, a saturated fatty acid, is found almost exclusively on the 2 position of the triglycerides in this fat (15). In the specificity studies, hog pancreatic lipase was used as a reference because this particular lipase has been well characterized in regard to its positional specificity (16). The relative percentages of fatty acids hydrolyzed from cocoa butter and lard by the lipase preparations are shown in Tables III and IV, respectively. As long as the time was kept short, the percentage of fatty acids released did not change significantly with hydrolysis time, indicating that the system was measuring the specificity on the original substrate. The times reported were selected to give as short a reaction time as possible consistent with sufficient hydrolysis for accurate measurements. The percentages of all the fatty acids released by the pancreatic lipases were all similar, showing a strong discrimination against the oleyl group on the 2 position of cocoa butter and the palmityl group on the 2 position of lard. This indicates that they have a high specificity for the 1 and 3 positions of triglycerides similar to that already established for hog pancreatic lipase. The invertebrate lipases showed no evidence of such a positional specificity.

In some instances there appeared to be acyl group preferences as well as positional specificities. In cocoa butter some lipases released significantly more palmitic acid than stearic acid although they are both on the 1 and 3 positions. In lard, the invertebrate lipases appeared to discriminate against linoleyl groups for these lipases released less linoleic acid than other acids that are concentrated on the same (1 and 3) positions. These lipases were also tested against corn oil in which the linoleyl group is concentrated on the 2 position. The results in Table V show that there was again a slight discrimination against linoleyl groups.

These results suggest that the digestive lipases of the lower animals have little positional specificity, although they may possess weak acyl group specificity. With the development of vertebrates, the pancreas appeared as a specialized tissue. Apparently the strong 1,3-specificity of the digestive lipases began with their production in the pancreas. The lack of activity of shark pancreas may indicate that the pancreas of some of the lower vertebrates do not have the function of lipase production.

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Reactions of Fatty Aldehydes With Fatty Alcohols: Formation of Acetals, Hemiacetals and Alk-1-enyl Alkyl Ethers[1]

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ABSTRACT

A convenient method for the synthesis of acetals of fatty aldehydes and fatty alcohols by transacetalation between fatty aldehyde dimethyl acetals and fatty alcohols is described. The acetals undergo decomposition to the alk-1-enyl alkyl ethers during GLC. Equimolar mixtures of fatty aldehydes and fatty alcohols show hemiacetal structure as evidenced by IR spectra in KBr discs but are dissociated completely into their components in solution and during TLC. They do not undergo dehydration and conversion to alk-1-enyl alkyl ethers during GLC under conditions of dealcoholization of acetals but are dissociated into aldehydes and alcohols.

INTRODUCTION

Since plasmalogens yield fatty aldehydes on mild acid hydrolysis, the biosynthesis of plasmalogens has been postulated to be via condensation of fatty aldehydes with the α-hydroxyl group of glycerol in diglycerides and lysophosphatides, followed by dehydration of the hemiacetal intermediate (1,2). The condensation of fatty aldehydes with short chain alcohols to form the acetals and their catalytic decomposition to the corresponding alk-1-enyl alkyl ethers has recently been described (3,4). This paper describes a convenient method for the synthesis of acetals derived from fatty aldehydes and fatty alcohols and their decomposition to the corresponding alk-1-enyl alkyl ethers. Further, hemiacetal formation between fatty aldehydes and fatty alcohols is examined by gas liquid chromatography (GLC), thin layer chromatography (TLC) and infrared (IR) spectroscopy to determine the possibility of their dehydration to the alk-1-enyl alkyl ethers.

EXPERIMENTAL PROCEDURES AND RESULTS

Materials

The fatty aldehydes were synthesized by oxidation of their mesylates by dimethylsulfoxide (5) and their purity was established by GLC (6). The fatty aldehyde dimethyl acetals (DMA) were prepared by a modification of Gray's method (7). The aldehydes were refluxed with 5% methanolic HCl, cooled (5-10 C) and neutralized with methanolic KOH and then evaporated to dryness. Extraction of the residue with ethyl ether and evaporation of the solvent yielded pure DMA.

Acetals of Fatty Aldehydes and Fatty Alcohols

The synthesis of acetals derived from alcohols other than methanol is usually effected by refluxing the mixture of the aldehyde and alcohol in benzene solution in the presence of an acid catalyst, *p*-toluene sulfonic acid (9,10). The direct condensation of fatty aldehydes and fatty alcohols by this method is not only time-consuming but gives rise to highly colored by-products from which it is difficult to obtain the acetal in pure form. The purification of these acetals by silicic acid column chromatography is difficult because of decomposition occurring in these columns. These acetals can, however, be synthesized in pure form by a modification of the transacetalation procedure described by Piantadosi et al. (11). The synthesis of 1,1-ditetradecoxytetradecane serves to illustrate the procedure applicable for the synthesis of other acetals.

In a 100 ml, round bottom ground-neck flask are placed 2.6 g (0.01 mole) of tetradecanal DMA, 4.28 g (0.02 mole) of 1-tetradecanol and 40-50 mg of *p*-toluenesulfonic acid or sulfosalicylic acid. The flask was heated under vacuum (20-30 mm) at 60-65 C for 30 min with occasional gentle shaking to mix the contents, and cooled to room temperature. The contents of the flask were dissolved in ethyl ether and washed once with 5% sodium carbonate solution and dried over a mixture of anhydrous sodium carbonate and sodium sulfate. The product obtained after filtration and evaporation of the ether solution was recrystallized from acetone to yield 3.2 g of the desired acetal, mp 33-33.5 C. TLC of the acetal (Silica Gel G-toluene) showed a single spot free from the starting materials.

Analysis. Calculated for $C_{42}H_{86}O_2$: C, 80.95; H, 13.80. Found: C, 80.81; H, 13.67.

[1]Presented at the AOCS Meeting, San Francisco, April 1969.

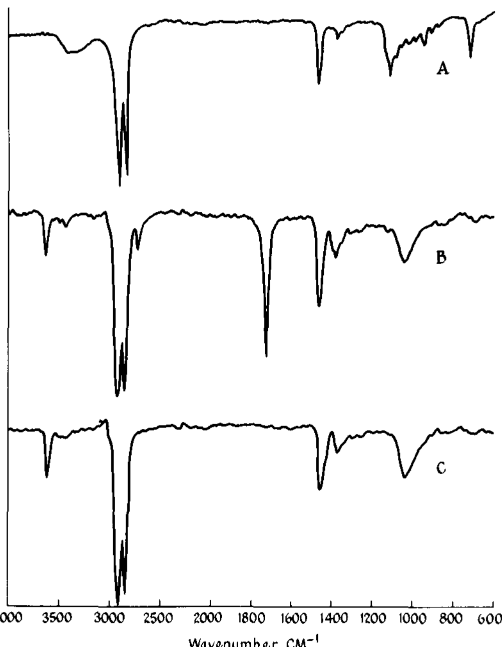

FIG. 1. IR spectra of hemiacetal of tetradecanal and 1-tetradecanol in KBr disc (A), in methylene chloride solution (10%) (B), and of 1-tetradecanol in methylene chloride (C).

1,1-Dioctadecoxyoctadecane was similarly prepared from octadecanal DMA (2.56 g) and 1-octadecanol (4.32 g). The product was recrystallized from a mixture of ethyl ether and methanol; yield 4.05 g, mp 47-48 C.

Analysis. Calculated for $C_{54}H_{110}O_2$: C, 80.14; H, 13.59. Found: C, 80.02; H, 13.44.

In the spectra of acetals of long chain aldehydes and alcohols, the absorption band at 1180-1185 cm^{-1} present in fatty aldehyde DMA (8,10) and other acetals described by Bergmann and Pinchas (12) was absent. In the long chain acetals, the band at 1114-1117 cm^{-1} was less intense than in the DMA and a broad band at 1060-1070 cm^{-1} was present instead of the sharp bands 1065 cm^{-1} and 1048 cm^{-1} shown by DMA.

Hemiacetals of Fatty Aldehydes and Fatty Alcohols

The hemiacetals of the above fatty aldehydes and fatty alcohols were prepared by melting a mixture of equimolar amounts of the aldehyde and the corresponding alcohol and allowing the melt to solidify at room temperature as described by Erickson and Campbell (13).

Figure 1 shows the IR spectra of the hemiacetal prepared from tetradecanal and tetradecanol both in KBr disc and in methylene chloride solution (10%) as well as that of 1-tetradecanol. In the IR spectra (KBr disc) the carbonyl bands 2700 cm^{-1} and 1730 cm^{-1} disappeared and a prominent C-O-C absorption at 1100 cm^{-1} appeared in the hemiacetal. A wide OH band at 3400 cm^{-1}, different from the sharp OH absorption at 3620 cm^{-1} of the alcohol was present. The OH absorption at 1040 cm^{-1} of the alcohol was absent in the spectra of the hemiacetals. On the other hand, in methylene chloride solution, the absorption bands of the hemiacetals (KBr disc) disappeared and, instead, bands characteristic of aldehyde (2700 cm^{-1} and 1730 cm^{-1}) and alcohol (3620 cm^{-1} and 1040 cm^{-1}) appeared indicative of complete dissociation into the aldehyde and alcohol. Complete dissociation of the hemiacetals was also observed in solutions of higher concentration (> 50%) as well as in solutions of nonpolar solvents such as cyclohexane.

When the hemiacetals were subjected to TLC under the same conditions as the acetals, two spots of equal intensities corresponding to the components aldehyde and alcohol were observed, again showing complete dissociation.

Alk-1-enyl Alkyl Ethers

Since the fatty aldehyde dimethyl acetals have been shown to decompose to the corresponding alk-1-enyl methyl ethers in aluminum columns during GLC (3,14), the decomposition of the long chain acetals to the corresponding alk-1-enyl alkyl ethers by the same procedure was attempted. GLC was performed at 170 C with aluminum columns packed with Gas-chrom P coated with 15% ethylene glycol succinate (EGS). The solid acetals were injected either in ether solution or liquid melts and the alk-1-enyl alkyl ether peak was collected by preparative GLC as described previously (3). TLC of the product (Silica Gel G-toluene) showed one spot having an R_f similar to that of the injected acetal but whose IR spectrum was different from that of the acetal. The characteristic vinyl ether doublet around 1660 cm^{-1} indicated to be a mixture of *cis* and *trans* isomers (15,16) of the alk-1-enyl alkyl ether. This absorption band was not as strong as that present in the alk-1-enyl methyl ethers (3,4), perhaps due to the fact that -O-CH=CH-group is situated in the center of the molecule. Absorption bands characteristic of *trans* double bond (937 cm^{-1}) and -O-CH=group (1270 cm^{-1}) were also present in the spectra of the alk-1-enyl alkyl ethers. These compounds yielded the corresponding aldehydes and alcohols on hydrolysis with 1:1 aqueous HCl.

The hemiacetals were injected into the GLC

columns as liquid melts, since they were dissociated in solution. Each of them gave two peaks, one corresponding to the aldehyde and the other to the alcohol. The products collected by preparative GLC were analyzed by TLC and IR. TLC showed the products to be aldehyde and alcohol. IR spectra did not show any -O-CH=CH absorption at 1660 cm^{-1}. It is concluded that under the GLC conditions used for the acetals, the hemiacetals were also completely dissociated into the aldehydes and alcohols without any occurrence of dehydration.

DISCUSSION

The acetals of fatty aldehydes and fatty alcohols are best prepared by transacetalation involving the dimethyl acetals of fatty aldehydes and fatty alcohols in the presence of an acid catalyst, rather than the direct condensation of the aldehydes and alcohols. As the dimethyl acetals, these acetals undergo decomposition to the corresponding alk-1-enyl alkyl ethers during gas chromatography.

Hemiacetal formation between fatty aldehydes and fatty alcohols has not been previously studied in detail. Hemiacetals from the following pairs were reported to be formed: 1-heptanol, heptanal; 1-octanol, octanal; 1-nonanal, nonanol; 1-decanol, decanal; 1-undecanol, undecanal; and 1-decanol, octanal (17). Zaar (18) prepared the addition products of 1-dodecanol and dodecanal and 1-dodecanal and dodecanal. Erickson and Campbell (13) reported formation of a hemiacetal between 1-dodecanal and dodecanal.

In the present study, hemiacetal formation between fatty aldehydes and fatty alcohols has been examined by IR spectroscopy, TLC and GLC. On melting and cooling equimolar mixtures of fatty aldehydes and fatty alcohols, hemiacetal structures are evident by IR spectra in KBr discs, but solution spectra even in nonpolar solvents show no hemiacetal structure. The hemiacetals also showed evidence of complete dissociation to the component aldehydes and alcohols in TLC and GLC under conditions in which acetals were stable. Unlike decomposition of the acetal to the alk-1-enyl alkyl ether during GLC, the hemiacetal did not undergo any dehydration to yield the same alkenyl ether. Attempts to dehydrate them by other methods are in progress.

The dehydration of hemiacetals as a possible biosynthetic pathway of plasmalogen synthesis has been postulated (1,2). In view of the instability of the hemiacetals in solution, and the formation of alk-1-enyl alkyl ethers from acetals, it is tempting to postulate that acetals may serve as better precursors in plasmalogen biosynthesis by some dealcoholization mechanism than hemiacetals.

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Accumulation of Acidic Phospholipids in a Case of Hyperlipidemia With Hepatosplenomegaly

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ABSTRACT

The results of studies made on an adult patient with hepatosplenomegaly, hyperlipidemia and accumulation of acidic phospholipids in the liver are presented. Storage of a lipoid substance was demonstrated histologically in lymph nodes, the liver and the bone marrow. Lipid analysis of the biopsy specimen from the liver revealed marked elevations of free cholesterol and two classes of acidic phospholipids: phosphatidyl inositol and another one which was tentatively identified as lysobisphosphatidic acid. Electron microscopic examination showed cytoplasmic inclusions with concentrically laminated structure.

INTRODUCTION

Marked abnormalities in the phospholipid composition of the human liver have rarely been found in common hepatic diseases. During our study on the lipid composition of biopsy specimens of the liver (Adachi et al., manuscript in preparation), a patient with the unusual feature of altered hepatic phospholipid composition characterized by an elevation of acidic phospholipids was encountered. Further investigation showed lipid storage in other tissues, in the bone marrow and in lymph nodes which were not enlarged.

In this report, data are presented on the lipid analysis of the liver biopsy specimen together with some histological and electron microscopic findings. The possible relation of this new type of phospholipidosis to adult Niemann-Pick disease (1-3) and foam cell syndrome (4-8) are discussed.

CASE REPORT

A 52-year-old Japanese male who had been suffering from fatigability, pain in the epi-

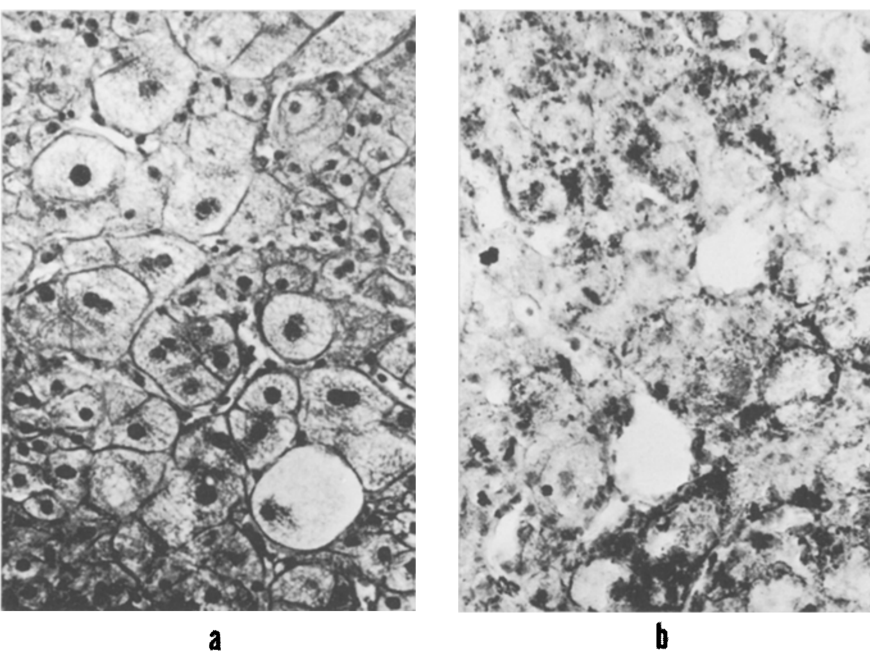

FIG. 1. Microscopic section of the liver (formalin-fixed). (a) Hematoxylin-eosin staining. (b) Sudan III staining. (See text.)

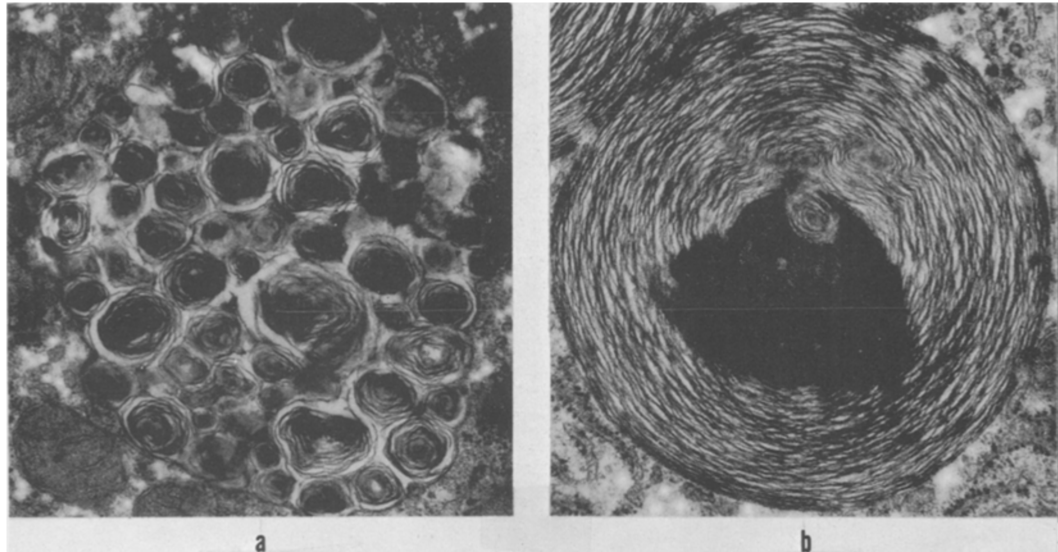

FIG. 2. Electron micrograph of a liver parenchymal cell. (a) An inclusion composed of multiple small particles containing some internal membranes. (b) Large membraneous lipid bodies with laminated structure (a, X22,000; b, X33,000; reduced approximately 23%).

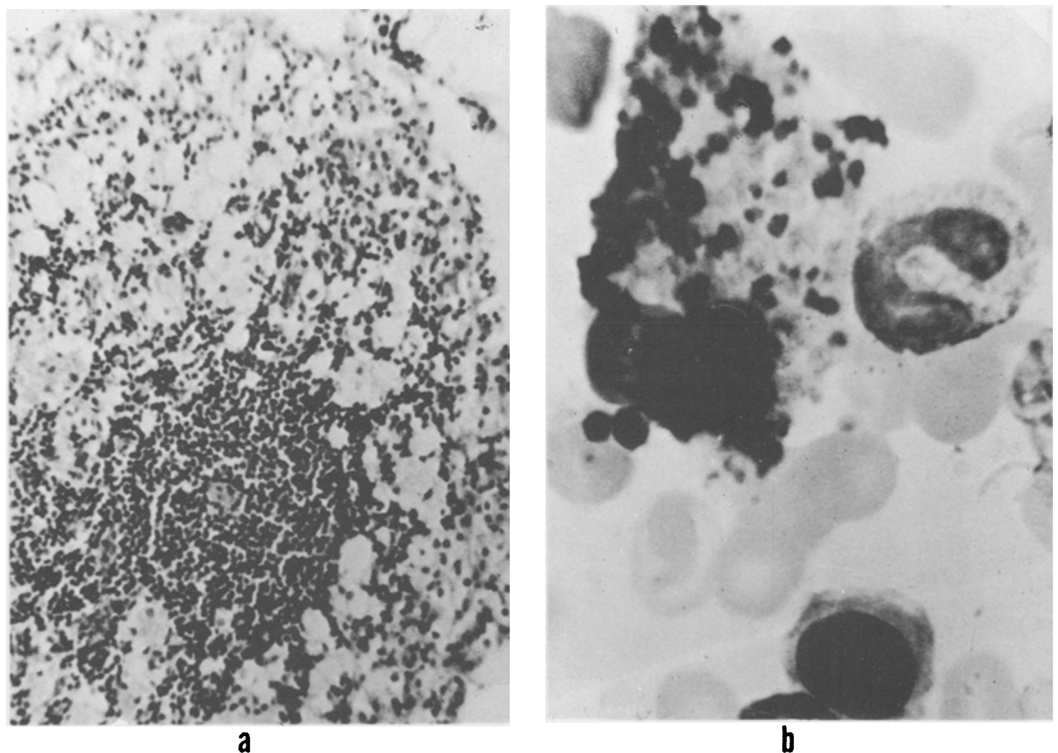

FIG. 3. Lipid storage in RES cells. (a) Microscopic section of a lymphnode (formalin-fixed, H-E staining). (b) May-Giemsa staining of the bone marrow obtained by usual aspiration biopsy.

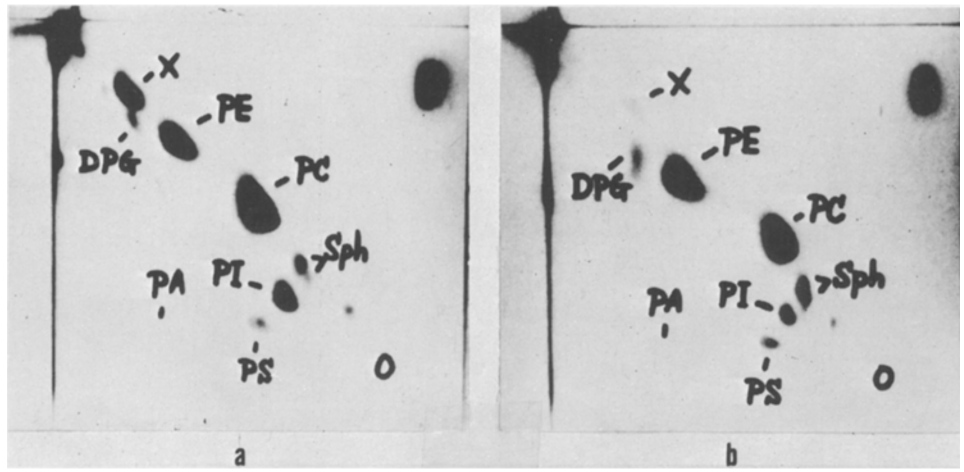

FIG. 4. Thin layer chromatography of liver lipids. (a) Liver lipids of the present case (400 μg spotted). (b) Normal liver lipids (300 μg spotted). TLC developed in the first dimension (vertical) with chloroform-methanol-28% aqueous ammonia-water (120:80:10:5) and in the second dimension (horizontal) with chloroform-acetone-methanol-acetic acid-water (100:40:30:20:12). Spots visualized by charring.

gastrium, shortness of breath and palpitation for the preceeding 14 months was admitted to Osaka University Hospital on February 7, 1969 for the purpose of laparoscopic examination. His prior illnesses included appendicitis at the age of 12 and pleurisy at the age of 25. He had no history of diabetes mellitus. He did not ingest alcoholic drinks nor any other known hepato-toxic agents. For several months prior to the admission to this hospital, the patient had been consuming a low fat diet. His intelligence was normal. There was a history of well-defined, round corneal opacities in both eyes since childhood. The patient stated that his mother had had the same kind of corneal opacities. Three siblings are normal.

The liver was palpable three finger breadths below the costal margin, and the spleen two finger breadths. Lymph nodes and tonsils were not enlarged. Laboratory tests showed a moderate retention of sulfobromophthalein (17%), an elevation of serum alkaline phosphatase activity (18 King-Armstrong units) and a slight increase of serum glutamic-oxaloacetic transaminase activity (82 Karmen units). Erythrocyte sedimentation rate was markedly increased (120 mm/60 min). Hematologic findings were: red blood cell count, 4,230,000/mm^3; hemoglobin, 13.8 g/100 ml and white blood cell count, 8,100/mm^3 with neutrophilic leucocytes 61%, eosinophiles 4%, basophiles 2%, monocytes 6% and lymphocytes 27%. Lipid analysis of fasting serum showed a total serum cholesterol of 408 mg/100 ml, total phospholipids 355 and triglycerides 259. The globulin was moderately increased, with a total serum protein of 8.6 g/100 ml. Albumin was 45.7%, α_1-globulin 8.4, α_2 15.7, β 9.6 and γ 20.6 on serum electrophoresis. An electrocardiogram was borderline normal. X-ray examination of the chest demonstrated a small aged tuberculous lesion in the right apical lung field and a few calcified shadows in the left hilus and in the left middle lung field.

Every time the patient was subjected to even a small surgical operation, he had a fever and leucocytosis, usually sustained for more than a week, and unresponsive to antibiotics.

HISTOLOGICAL OBSERVATIONS

Biopsy specimens of the liver were obtained with a Vim-Silverman type needle in the laparoscopic visual field (9). One of the specimens was used for histological examination and the other, weighing 65 mg, for lipid analysis. Six months later, the liver biopsy was performed again and the sample was also examined by electron microscopy.

Microscopic section of the liver showed enlarged hepatocytes with irregular vacuolization (Fig. 1a) which were grouped mainly in the central zone of the lobules and interlaced with thin collagen fibers. Numerous small sudanophilic particles were noted in the enlarged hepatocytes around the vacuolated areas (Fig. 1b). On electron micrograph, the cytoplasm of hepatocytes were filled with multiple small inclusions with concentrically laminated membraneous structure (Fig. 2). Although

TABLE I

Lipid Composition of the Liver

	52-year-old male (This case)		31-year-old male	25-year-old male	27-year-old male
	Biopsy specimen				
Disease	Feb. 7, 69	July 14, 69	Chronic hepatitis	Normal	Normal
Triglyceride	0.83[a]	2.13	2.17	0.43	0.53
Cholesterol (ester)	0.22	0.35	0.20	0.18	0.37
Cholesterol (free)	0.68	0.84	0.12	0.17	0.19
Phospholipid	3.50	5.32	2.17	2.71	3.11
Phospholipid composition					
Phosphatidyl choline	46.9[b]	38.3	48.2	48.0	48.8
Phosphatidyl ethanolamine	17.8	17.2	26.6	30.1	29.1
Diphosphatidyl glycerol	2.4	2.4	4.7	4.3	5.7
Sphingomyelin	4.2	5.7	7.5	6.4	5.8
Phosphatidyl inositol	12.6	9.7	5.7	5.8	5.6
Phosphatidyl serine	3.3	2.4	5.0	3.8	3.8
Lyso-bis-phosphatidic acid	9.2	21.0	0.5	0.1	0.7

[a]Per cent of the wet weight of the liver.

[b]Per cent of the total phospholipid.

there was no marked proliferation of reticulo-endothelial (RES) cells in the liver, lipid bodies filled also the cytoplasm of these RES cells.

A section of scalene lymph nodes showed a storage of sudanophilic lipid substance in the peripheral zone of the node (Fig. 3a). RES cells in the bone marrow contained numerous small granules deeply stained blue-black with May-Giemsa staining (Fig. 3b). Some of the RES cells were enlarged and vacuolated.

Vacuolated lymphocytes were also present in the peripheral blood, making up about 10% of the medium and small sized lymphocytes.

EXPERIMENTAL PROCEDURES

Lipids were determined by quantitative thin layer chromatographic (TLC) and colorimetric procedures, essentially as described by Rouser et al. (10-12). Triglyceride, cholesterol, cholesterol ester and phospholipid were separated on TLC with petroleum ether-diethyl ether 85:15. Triglyceride and cholesterol ester were determined by ester linkage determination after transesterification with 6% (v/v) H_2SO_4-methanol (13). Phospholipid analysis was carried out on a silicic acid plate spread in 0.01 M magnesium acetate (20 g Silica Gel H Merck suspended in 60 ml of 0.01 M magnesium acetate for 5 plates). For phosphorus analysis, spots were visualized by charring at 180 C for 30 min after spraying with 20% aqueous solution of ammonium sulfate, containing 4% H_2SO_4 (14).

Fatty acids were analyzed as their methyl esters by gas liquid chromatography (GLC). Spots of phospholipids on TLC were visualized by spraying with 0.01% rhodamine 6G in ethanol, and samples methanolyzed with 14% BF_3-methanol at 100 C for 30 min. GLC analysis was carried out using Shimadzu GC-4A apparatus, equipped with a flame ionization detector. A 3 mm i.d. x 6 ft column, packed with 10% ECNSS-S on 100-120 mesh Gaschrom P and another column, packed with 3% SE-30 on 80-100 mesh Gaschrom Q, were used. Argon was used as a carrier gas and the instrument operated at 185-190 C.

RESULTS AND DISCUSSION

Results of the lipid analysis are shown in Table I. Free cholesterol, triglyceride and the total phospholipid were increased. Phosphorus analysis disclosed an increase of phosphatidyl inositol and a relative decrease of phosphatidyl ethanolamine. An acidic phospholipid (X) which usually comprises less than 1% of the total phospholipid in normal liver showed a marked increase (Fig. 4). Fatty acid to phosphorus ratio was about 2:1 and the major fatty acids were oleic and docosa-hexaenoic (22:6, ω-3) acids (Table II).

The acidic phospholipid that increased in

TABLE II

Fatty Acid Composition of Liver Phospholipids

Fatty acid[a]	Phosphatidyl choline, %	Phosphatidyl ethanolamine, %	Lyso-bis-phosphatidic acid, %
14:0	0.3	0.1	0.1
15:0	0.1	0.1	0.3
16:0	31.0	19.9	3.2
16:1	3.7	2.1	4.0
18:0	13.9	25.7	3.3
18:1	16.0	9.3	38.2
18:2	16.8	10.6	13.1
18:3	2.0	1.0	2.0
20:0	0.4	---	---
20:2	0.9	---	0.6
20:3	2.7	1.5	1.1
20:4	4.0	11.0	1.3
22:2	1.6	2.3	1.3
22:5	---	2.5	2.9
22:6	6.6	13.9	28.5

[a]Carbon chain length: number of double bonds.

this case is apparently the one which was found in Niemann-Pick disease and a case of amaurotic idiocy, and is identified as lysobisphosphatidic acid (15). The present case is different because the cells involved in the liver were predominantly hepatic parenchymal cells. The lack of increase of sphingomyelin in this case is similar to some of the cases previously reported (15).

Dusendschon (1), Pfändler (2) and Terry et al. (3) reported an adult lipidosis resembling Niemann-Pick disease. According to Terry et al. (3), the increase in sphingomyelin was not as large as is usually seen in cases of the classical Niemann-Pick disease, while other lipids also showed considerable increases in their case. Although the findings in the present case are different from those encountered in classical Niemann-Pick disease, the elevation of an acidic phospholipid both in this case and in Niemann-Pick disease suggests the possibility that there is a common metabolic defect. Another similarity is shown by the presence of hyperlipidemia. Crocker and Farber (16) summarized 18 cases of Niemann-Pick disease and reported that some of the patients showed transient hyperlipidemia during the course of their illness, while cases of Gaucher's disease and Tay-Sachs disease did not show such a tendency.

A storage disorder of unknown etiology involving RES cells in the bone marrow, together with the presence of foam cells, had been reported by several authors (4-8). In these cases, the lipid granules in the cytoplasm of histiocytes were light or stained deeply blue with Wright or Giemsa. The spleen was also involved in most of the cases, while morphological changes in the liver were not described. The bone marrow findings in the present case are similar to those reported in these cases of foam cell syndrome.

The final diagnosis for the present case is impossible at this stage. However, this case seems to fit into the spectrum of diseases that includes the foam cell syndrome and Niemann-Pick disease. Accurate analysis of the lipid composition of the liver and other tissues in additional cases may shed light on the pathogenesis of this spectrum of diseases.

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Specificity of Lipase From Several Seeds and *Leptospira pomona*[1]

ABSTRACT

The specificities of lipase preparation from oats, *Leptospira pomona* DM_2H, corn, soybeans, safflower seed and pinion nuts were examined by using cocoa butter, lard and corn oil as substrates. Oat lipase exhibited an acyl group specificity, favoring the hydrolysis of linoleyl groups and discriminating against stearoyl groups. The *L. pomona* lipase exhibited a tendency to hydrolyze acyl groups on the 1 and 3 positions of glycerol preferentially. The hydrolysis by the other seed lipases was nonspecific.

A search for lipase of unusual specificities that might be useful in triglyceride structure analysis led to the investigation of lipases from oats, corn, soybeans, pinion nut and safflower seed and from the microorganism, *Leptospira pomona* DM_2H. Oat lipase had been reported to have a specificity for the 2 position of triglycerides (1,2).

Oat lipase was prepared by soaking oats in buffer or water (1). Filtrates of the culture medium were used for the *L. pomona* DM_2H (3). The other seed lipases were prepared by blending the seed with a small amount of water and preparing an acetone powder (4). Cocoa butter and lard were randomized by heating to 60 C with 0.05% sodium methoxide for 3 hr. The randomized fats were washed free of catalyst. Gel electrophoresis and determinations of pH optima and enzyme specificities were carried out as described previously (5). The specificities of the lipases were determined by measuring the release of fatty acids from natural and modified fats. The reaction times were kept short enough so the percentages of fatty acid released were independent of the hydrolysis time. The inclusion of 1% mono- and diglyceride in the substrate did not change the percentage of fatty acids released, indicating that the reaction was uninfluenced by the accumulation of hydrolysis products. Blank determinations with heated enzyme gave no detectable fatty acids.

The oat lipase had a pH optimum at 7.4 and gave one active band on gel electrophoresis. Contrary to previous reports (1), oat lipase was very active against long chain triglyceride emulsions without the addition of oatmeal. The results in Table I indicate that oat lipase releases much less stearic acid than a random attack on the triglycerides would produce. This effect could not be attributed to the positional distribution of the stearoyl groups, for similar results were obtained with randomized fats. There is a preferential release of the linoleic acid in lard and randomized lard.

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TABLE I

Percentages of Fatty Acids Released From Cocoa Butter and Lard by a 2 Min Reaction at pH 7.4 With Oat Lipase

Substrate	Fatty acids					
	C_{14}	C_{16}	$C_{16:1}$	C_{18}	$C_{18:1}$	$C_{18:2}$
Cocoa butter[a]	---	29.8	---	34.6	35.6	---
Cocoa butter[b]	---	42.8	---	5.2	52.0	---
Randomized cocoa butter[b]	---	30.6	---	15.7	53.7	---
Lard[a]	1.6	27.3	1.1	15.4	45.7	8.9
Lard[b]	1.1	25.6	0.6	3.4	46.6	22.7
Randomized lard[b]	7.2	30.1	Trace	2.4	38.6	21.7

[a]Percentages determined by chemical hydrolysis.
[b]Percentages resulting from enzymatic hydrolysis.

TABLE II

Percentage of Fatty Acids Hydrolyzed From Fats by *L. pomona* Lipase Using a 5 Min Reaction Time

Substrate	pH	Fatty acids					
		C_{14}	C_{16}	$C_{16:1}$	C_{18}	$C_{18:1}$	$C_{18:2}$
Cocoa butter[a]	---	---	29.8	---	34.6	35.6	---
Cocoa butter[b]	5.0	---	58	---	23	19	---
Cocoa butter[b]	11.0	---	54	---	27	19	---
Corn oil[a]	---	---	11.6	---	1.6	27.4	59.4
Corn oil[b]	5.0	---	28	---	4	21	48
Lard[a]	---	1.6	27.3	1.1	15.4	45.7	8.9
Lard[b]	5.0	1	15	1	18	51	14
Lard[b]	11.0	1	16	1	17	53	12

[a]Percentage determined by chemical hydrolysis.
[b]Percentages resulting from enzymatic hydrolysis.

The results for *L. pomona* DM_2H lipase specificity are given in Table II. Low amounts of oleic acid are released from cocoa butter and low amounts of palmitic acid are released from lard. These acids are concentrated on the 2 positions of these fats so these results indicate that this enzyme shows preference for the 1 and 3 positions of triglycerides. This specificity is not nearly as exact as that of hog pancreatic lipase, and the enzyme resembles those from *Staphylococcus aureus* and *Aspergillus flavus* reported by Alford et al. (6). The *L. pomona* DM_2H lipase exhibited only one active fraction on electrophoresis. It gave pH optima at 6.0 and 10.0 against tributyrin and at 4.0 and 11.0 against corn oil.

Lipase activity was demonstrated in the acetone powders of the corn, safflower, soybean and pinion nut. The specificity of these lipases was tested against cocoa butter at pH 6.5-7.0. The determination was complicated by contamination of the lipases with the natural oils of the species which were hydrolyzed along with the cocoa butter. The presence of these natural oils was shown by the release of large amounts of linoleic acid by the enzymes and of linolenic acid as well by the soybean enzyme. The amounts of the various fatty acids released were consistent with a random attack of the lipases simultaneously on the natural oils and the cocoa butter.

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[Received July 18, 1969]

Phospholipid Complexes With Molybdate

ABSTRACT

Phosphorus-containing organic-compounds combine directly with molybdate to form complexes analogous to phosphomolybdate. These complexes are extractable by organic solvents such as ethyl acetate or butanol. The ability of chloroform-ethanol mixtures in extracting only the phospholipid-molybdate complexes but not those of water-soluble phosphates may find some useful applications.

Phospholipid and organic phosphorus of any type is usually assayed as inorganic phosphate after oxidation with perchloric acid. Most colorimetric methods are based on the fact that the phosphomolybdate complex is reduced to molybdenum blue more rapidly than in a solution of molybdate (1).

To our knowledge, phospholipid-molybdate complexes have never been described in the literature, and the formation of blue spots on paper or thin layer chromatograms of organic phospho-derivatives sprayed with the Hanes-

TABLE II

Percentage of Fatty Acids Hydrolyzed From Fats by *L. pomona* Lipase Using a 5 Min Reaction Time

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Phospholipid and organic phosphorus of any type is usually assayed as inorganic phosphate after oxidation with perchloric acid. Most colorimetric methods are based on the fact that the phosphomolybdate complex is reduced to molybdenum blue more rapidly than in a solution of molybdate (1).

To our knowledge, phospholipid-molybdate complexes have never been described in the literature, and the formation of blue spots on paper or thin layer chromatograms of organic phospho-derivatives sprayed with the Hanes-

TABLE I

Extractability of Organic Solvents on Representative Phospho-derivatives and Their Molybdate Complexes

Compounds and assays	Treatment	Ethyl acetate		Chloroform-Ethanol,4:1	
		Extract	Aqueous layer	Extract	Aqueous layer
Glycerophosphate (2 μmoles)					
Total phosphorus (μmoles)	1	0.05	1.95	0.03	1.95
Total phosphorus (μmoles)	2	1.97	0.01	0.02	1.97
Total phosphorus (μmoles)	3	1.96	0.00	0.02	1.99
Absorbancy at 820 mμ	3	0.115	---	---	0.095
Inorganic phosphate (0.1 μmole)					
Total phosphorus (μmoles)	1	0.00	0.10	0.00	0.10
Total phosphorus (μmoles)	2	0.10	0.00	0.00	0.10
Total phosphorus (μmoles)	3	0.10	0.00	0.00	0.10
Absorbancy at 820 mμ	3	0.520	---	---	0.515
Phospholipids[a] (1 μmole)					
Total phosphorus (μmoles)	1	0.98±.02	0.00	0.97±.01	0.01
Total phosphorus (μmoles)	2	0.99±.03	0.00	0.98±.03	0.01
Total phosphorus (μmoles)	3	0.99±.02	0.00	0.99±.01	0.01
Absorbancy at 820 mμ	3	0.420±.020	---	0.405±.020	---
Phosphorus-free lipids[b] (5-50 μmoles)					
Absorbancy at 820 mμ	3	0.005	---	0.005	---

[a]Phospholipids tested were pure fractions of phosphatidylethanolamine and phosphatidylinositol from yeast, phosphatidylcholine from egg yolk and sphingomyelin from brain isolated by column chromatography on silicic acid (11,12).

[b]The following phosphorus-free lipids were tested: triolein, cholesterol and brain cerebroside.

Isherwood molybdate reagent (2) is thought to be due to a spontaneous release of inorganic phosphate by the acid of the reagent. However, this explanation is not consistent with the spontaneity and sensitivity of the reaction of the Zinzadze's molybdenum blue reagent (3) producing intense blue spots at room temperature with phospholipids (4), or even with phosphonolipids (5) which do not release inorganic phosphate even by prolonged treatment with concentrated sulfuric acid at 160 C (6). This was also recognized earlier by Dittmer and Lester (4) who stated that the chemistry of the reaction is not known.

We were able to show that phosphorus-containing organic compounds combine directly with molybdate to form complexes analogous to phosphomolybdate. These complexes, as phosphomolybdate itself (1), are extractable by organic solvents such as ethyl acetate or butanol. In addition, phospholipid-molybdate complexes are extractable by chloroform-ethanol (4:1 v/v), which does not extract the analogous complexes of water-soluble phosphates.

Experimental data indicating the formation of phosphomolybdate and phosphomolybdenum blue analogs of organic phosphates are depicted in Table I. These data refer to tests carried out by placing the test tubes in boiling water for 10 min, cooling and extracting the aqueous media with an equal volume of the organic solvents indicated. The aqueous media (final volume: 5 ml) contained either 1.2 N $HClO_4$ alone (Treatment 1) or perchloric acid plus 0.25% ammonium molybdate (Treatment 2) or perchloric acid, ammonium molybdate and 1,2,4-aminonaphtholsulfonate (Treatment 3). A uniform, thin dispersion of the lipids into these media was effected by dissolving them first in 0.1 ml of methanol. The distribution of the products into the organic and aqueous layers of the binary systems were checked by total phosphorus determinations (7) after digestion with 12 N $HClO_4$ (180 C, 1 hr) and occasionally by additional specific assays mentioned in the text.

As shown in Table I, water-soluble phosphates became extractable by ethyl acetate only after treatment with ammonium molybdate either alone (Treatment 2) or in the presence of reducing agent (Treatment 3). Furthermore, glycerol assay carried out by the periodate-chromotropic acid method (8) indicated the absence of free or bound glycerol in the aqueous layers of the ethyl acetate extraction of the glycerophosphate samples submitted to Treatments 2 and 3.

The formation of molybdate complexes with organic phosphates is strongly supported by the analytical data (Table I) of tests involving phospholipids. The blue color of the turbid blue media produced by phospholipids subject to Treatment 3 is quantitatively extracted by chloroform-ethanol (4:1 v/v) into the chloroform layer while, under the same conditions the blue color formed by water-soluble phosphates is retained in the aqueous layer. Molybdenum assay (9) showed that the chloroform layers obtained from inorganic phosphate and glycerophosphate subject to Treatments 2 and 3 were essentially molybdenum-free, while the corresponding to phospholipid samples respective chloroform layers were found to contain molybdenum in a molar ratio to phosphorus 5:1. Furthermore, thin layer chromatographic examination confirmed the lack of noticeable hydrolysis of the lipids recovered from these chloroform layers, most probably because perchloric acid, when dilute, is a rather mild hydrolytic agent, being widely used as a deproteinizing agent even when acid-labile phosphates are involved (1).

Phosphorus-free lipids alone or mixed with water-soluble phosphates, and mixtures of phospholipids with water-soluble phosphates were submitted to Treatments 2 and 3 in order to secure that the mentioned solubility effects and the production of blue color is not related to the presence of lipid material in general.

As shown in Table I, the intensity of blue color produced with phospholipids is approximately 10% of the color produced by an equivalent amount of inorganic phosphate. When water-soluble organic phosphates are involved, the respective figure is 1%. These data are consistent with the well-known superior sensitivity of phospholipids over the water-soluble organic phosphates against the Zinzadze's reagent on thin layer chromatograms.

These findings, besides their theoretical interest, may obviously find several useful applications in the preparation or purification of phosphorus compounds, free of other water-soluble material, and in the determination of organic phospho-derivatives directly, without digestion. For instance, although there is no apparent advantage in applying such a technique for estimating the phosphorus content of lipid fractions, the method has certain obvious advantages in assaying serum phospholipids directly (without extraction) or after thin layer chromatographic separation of phospholipids.

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Nonconversion of 5,11,14-Eicosatrienoic Into Arachidonic Acid by Rats

ABSTRACT

Methyl ester of [U-^{14}C] 5,11,14-20:3 was administered to essential fatty acid deficient rats. In contrast to a report by Takagi, it was found that the trienoic acid was not converted into arachidonic acid.

Several years ago Takagi reported that rats deficient in essential fatty acids (EFA) converted 5,11,14-20:3 efficiently into 5,8,11,14-20:4 (arachidonic) acid (1). This conversion may proceed either by desaturation between the existing double bonds or by partial degradation and resynthesis. However, no indication for such processes has been found

The formation of molybdate complexes with organic phosphates is strongly supported by the analytical data (Table I) of tests involving phospholipids. The blue color of the turbid blue media produced by phospholipids subject to Treatment 3 is quantitatively extracted by chloroform-ethanol (4:1 v/v) into the chloroform layer while, under the same conditions the blue color formed by water-soluble phosphates is retained in the aqueous layer. Molybdenum assay (9) showed that the chloroform layers obtained from inorganic phosphate and glycerophosphate subject to Treatments 2 and 3 were essentially molybdenum-free, while the corresponding to phospholipid samples respective chloroform layers were found to contain molybdenum in a molar ratio to phosphorus 5:1. Furthermore, thin layer chromatographic examination confirmed the lack of noticeable hydrolysis of the lipids recovered from these chloroform layers, most probably because perchloric acid, when dilute, is a rather mild hydrolytic agent, being widely used as a deproteinizing agent even when acid-labile phosphates are involved (1).

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These findings, besides their theoretical interest, may obviously find several useful applications in the preparation or purification of phosphorus compounds, free of other water-soluble material, and in the determination of organic phospho-derivatives directly, without digestion. For instance, although there is no apparent advantage in applying such a technique for estimating the phosphorus content of lipid fractions, the method has certain obvious advantages in assaying serum phospholipids directly (without extraction) or after thin layer chromatographic separation of phospholipids.

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TABLE I

Radioactivity of Fatty Acid Methyl Esters (%)

2-Acyls of PC, by GLC		C_{20} of PC + PE, by paper chromatography	
Region	%^{14}C	Region	%^{14}C
16:0	0.3	Front	0.05
18:1	0.8	20:5	0.07
18:2	0.5	5,8,11,14-20:4	0.34(0.22)[a]
18:3	$<$0.1	5,11,14-20:3	99.07
5,11,14-20:3	96.8	18:1+16:0	0.35
5,8,11,14-20:4	1.2(1.6)[b]	---	0.07
22:3	0.2	---	0.03
22:5	0.3	origin	0.03

[a]The value in parentheses was found in this area from a model mixture of [U-^{14}C]5,11,14-20:3 and nonradioactive 18:1 and 20:4 methyl esters.

[b]The value in parentheses was found in this area from a model mixture of [U-^{14}C]5,11,14-20:3 and nonradioactive rat liver fatty esters.

with comparable acids such as 9,15-18:2 and 7,13-20:2 (2). In the work reported here, [U-^{14}C]5,11,14-20:3 was used to elucidate its metabolism by tracer techniques. We did not find any evidence for conversion of 5,11,14-20:3 into arachidonic acid.

Methyl ester of [U-^{14}C]5,11,14-20:3 had been prepared from labeled ginkgo lipids (3) and was 100% pure according to gas liquid chromatography (GLC). The trienoic acid was all-*cis* according to IR analysis of nonradioactive preparations from ginkgo (4). The ester (11.8 mg, 17.6 x 10^6 dpm, in 270 μl methyl oleate) was administered by stomach tube to three male Holzman strain rats which had been on a fat free diet (5) for 37 weeks. The animals were killed by chloroform anesthesia 12 hr after feeding and the liver lipids were extracted and fractionated into classes as previously described (6). From the radioactivity administered, 0.1% was found in cholesterol esters; 0.3% in triglycerides; 3.6% in phosphatidyl ethanolamine (PE) and 10.7% in phosphatidyl choline (PC).

PC was selectively hydrolyzed with phospholipase A (snake venom, *Crotalus adamanteus*, Ross Allen Reptile Institute). Two-acyls and 1-acyls contained 93.6% and 6.4%, respectively, of the radioactivity in PC. Methyl esters of the fatty acids from position 2 were subjected to GLC. The fractions were collected and counted in a scintillation counter (Table I).

It is well known that some trailing of radioactivity can occur in such chromatographic separations. Therefore, the correctness of 1.2% ^{14}C in 20:4 (Table I) must be questioned. For this problem, it was advantageous to employ a chromatographic system in which 20:4 migrates ahead of 20:3.

PC and PE were combined and their acids transesterified in CH_3OH + HCl. The methyl esters were subjected to GLC on a phase of low polarity (7) and were collected according to chain length. About 2 mg C_{20} esters was streaked on siliconized Whatman No. 1 paper (8) and the chromatogram was developed over a length of 28 cm with acetonitrile + H_2O (3:1 v/v). After exposure to iodine vapors, the chromatogram was cut according to regions to determine their radioactivity (Table I). Recoveries of counts were $>$90%.

According to the blank values of these methods (Table I) not more than 0.1% of the radioactive 5,11,14-20:3 incorporated in the rat may have been converted to arachidonic acid. Such a low value is within the experimental error or may be explained by regular syntheses through the acetate pool.

The results are in striking contrast to the conversion claimed by Takagi (1) but they are in accord with the nonconversion of 9,15-18:2 and 7,13-20:2 (2). Takagi used Wister strain rats which had been on an EFA deficient diet for 14 weeks and they each received 0.2 g/day of 5,11,14-20:3 ethyl ester or of ethyl linoleate for five days. According to the wording of the publication, the amount may also have been 1.0 g/day per rat (1). The level of arachidonic acid had then increased from 1.8% to 9.0% and to 12.2%, respectively, of total liver fatty acids.

In our experiments, 12 hr were given for conversion. This period had proven satisfactory in numerous other experiments with rats to demonstrate conversions of radioactive highly unsaturated fatty acids (7). Similar to these, 5,11,14-20:3 was efficiently incorporated, in particular into the 2 position of PC. However, 5,11,14-20:3 was less amenable to conversions

than any of the other polyunsaturated acids and its desaturation to 5,8,11,14-20:4 acids could not be demonstrated.

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[Received April 2, 1970]

On the Biosynthesis of Glycerol Ethers in Mitochondria

ABSTRACT

Mitochondria from mouse preputial glands incubated with 1-^{14}C-myristic acid and appropriate co-factors produced, after $LiAlH_4$ reduction, a compound that resembled a glycerol ether. However, close examination of this material showed that it was not an ether, and we tentatively identified the original compound as a phosphorylated β-keto (or β-hydroxy) fatty acid.

The biosynthesis of glycerol ethers by microsomal preparations of tumors has recently been described (1,2). Since the mouse preputial gland contains large quantities of glycerol ethers (3,4) in both the neutral lipids and phospholipids, we chose it as a normal tissue for comparison with the neoplasms. Preliminary studies by Sansone (5) revealed that the mitochondria of this tissue contain glycerol ethers. During the course of incubating labeled myristic acid (14:0) with mouse preputial gland mitochondria, we isolated a product that behaved in many ways like a glycerol ether. However, close examination of the compound revealed that it was not a glycerol ether, and we tentatively identified it as a phosphorylated β-keto (or possibly β-hydroxy) fatty acid. A recent report (6) on the biosynthesis of alkyl glycerol ethers in liver mitochondria prompted us to report our negative results.

Mitochondria from mouse preputial glands were prepared by the procedure of O'Brien and Kalf (7). The homogenity of the preparation was established by electron microscopy. Whole sonicated mitochondria were incubated with 1-^{14}C-myristic acid, Mg^{++}, CoASH, ATP, NADH, NADP, and α-glyceryl phosphate in 0.1 M phosphate buffer (pH 7.0) for periods up to 4 hr. Total lipids were extracted by the procedure of Bligh and Dyer (8). Phospholipids were separated from neutral lipids on a micro column of silicic acid. Thin layer chromatography (TLC) of the phospholipids revealed a major and a minor area of radioactivity. In an acidic solvent system, the major area was found near the solvent front. However, in a basic system, this lipid moved only slightly off the origin. All of the alkyl glycerol ether radioactivity was found in the major component, which we tentatively identified on the basis of its chromatographic behavior as an acidic phospholipid. A portion of the major phospholipid fraction was subjected to lithium aluminum hydride reduction (9), and the reaction products were separated by TLC. Radioactivity was observed in the region of the alkyl glycerol ethers. We prepared the isopropylidene derivative of the product isolated from the alkyl glycerol ether region and subjected it to further TLC. Again, the radioactivity migrated with the isopropylidene derivative of an authentic alkyl glycerol ether. However, the conditions for the preparation of the derivative of the unknown lipid were more severe than for the authentic compound, and, at best, only a 60% yield was obtained. Under the conditions we employed, the authentic compound was derivatized in less time than the unknown material, and in a quantitative yield. We isolated the isopropylidene derivative of the unknown and frac-

than any of the other polyunsaturated acids and its desaturation to 5,8,11,14-20:4 acids could not be demonstrated.

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tionated it by preparative gas liquid chromatography (GLC). A series of authentic isopropylidene derivatives of alkyl glycerol ethers (12:0, 14:0, 16:0 and 18:0) were added as carriers. All of the radioactivity appeared in the region of a 12:0 derivative.

Examination of the literature revealed that mitochondrial preparations similar to the one we used add acetate to preformed fatty acids, and the β-hydroxy intermediate accumulates (10). If this were so in our case, lithium alumnium hydride reduction would produce 1,3-dihydroxyhexadecane. This compound, like the 1,2-alkane diols (11), would migrate on TLC in the region of alkyl glycerol ethers and form isopropylidene derivatives, but at a slower rate than glycerol ethers. On GLC, this material would elute with the 12:0 ethers, since it contains one carbon and one oxygen less than the expected 14:0 ether. The structures of the isopropylidene derivatives of 1,3-dihydroxyhexadecane and 14:0 alkyl glycerol ether are given below.

```
H3C   CH3                     H3C   CH3    CH3
   \ /                           \ /       |
    C                             C       (CH2)13
   / \                           / \       |
  O   O                         O   O      O
  | H |                         |   |      |
H2C- C -C-(CH2)12-CH3         H2C---C------CH2
     H                              H

      I                             II
```

Since our substrate was 1-^{14}C-myristic acid, it would have been impossible for it to have been oxidized to lauric acid and incorporated into a radioactive 12:0 ether. Moreover, a complete oxidation followed by de novo synthesis would have resulted in the incorporation of radioactivity into all of the ether fractions obtained by GLC.

On the basis of these data, we concluded that our mitochondrial system had not produced alkyl glycerol ethers. Rather, it seemed that the radioactivity had been incorporated into a phosphorylated derivative of either a β-keto or a β-hydroxy fatty acid.

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[Received December 29, 1969]

Detection of Lithocholic Acid in Multiple Sclerosis Brain Tissue

ABSTRACT

By methods previously employed for detecting lithocholic acid in brains of guinea pigs afflicted with experimental allergic encephalitis, the same acid was detected in a coronal specimen of human brain obtained on autopsy of a multiple sclerosis patient. The specimen contained several well-defined demyelinated plaque areas. No lithocholic acid was detected in a somewhat larger coronal specimen of normal human brain.

The observation that lithocholic acid is present in the brain of guinea pigs afflicted with experimental allergic encephalomyelitis (EAE) (1) prompted us to examine brain tissue from a multiple sclerosis patient for this acid. Some pathologists consider EAE to be an experimental counterpart of the naturally occurring human disease multiple sclerosis (2,3). Lithocholic acid is a toxic substance (4), and is mildly demyelinating on intracerebral injection (this laboratory's unpublished results). The occurrence of this compound in a tissue which has undergone demyelination could provide a

tionated it by preparative gas liquid chromatography (GLC). A series of authentic isopropylidene derivatives of alkyl glycerol ethers (12:0, 14:0, 16:0 and 18:0) were added as carriers. All of the radioactivity appeared in the region of a 12:0 derivative.

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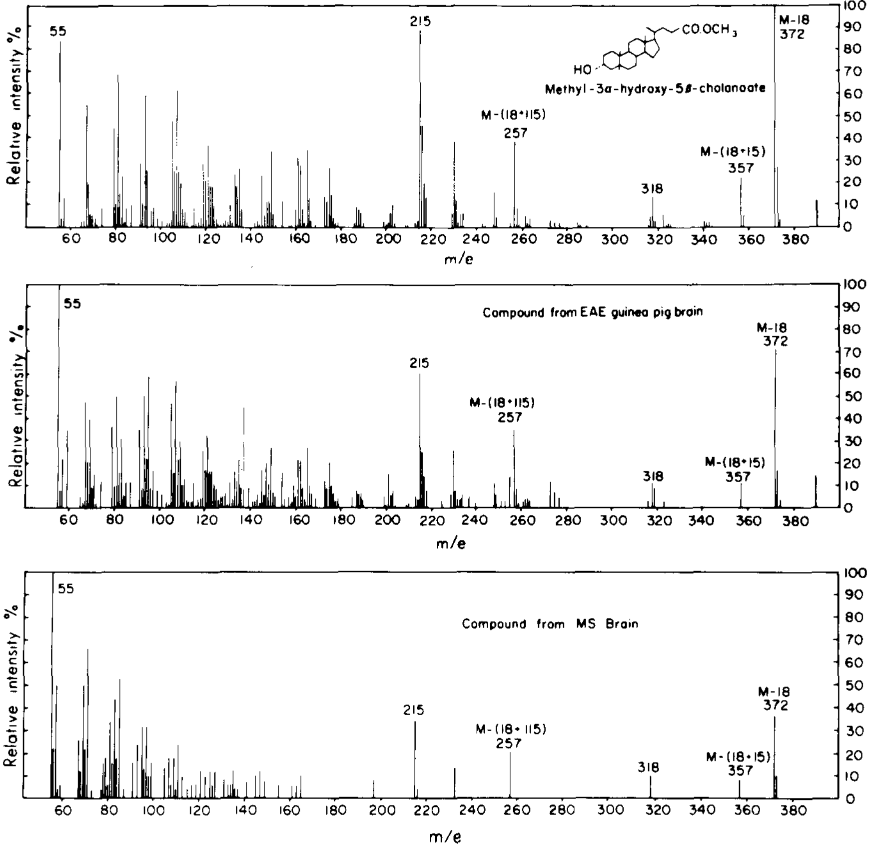

FIG. 1. Mass spectra of the isolated material, the material from EAE brain, and a methyl lithocholate standard.

clue regarding the immediate cause of demyelination within the tissue.

Formalin-fixed coronal sections were obtained within 48 hr after death. The section from the multiple sclerosis patient had 12 areas of gross demyelination and weighed 8.5 g. The section from a control patient weighed 13.9 g. Each sample was minced with a scissors and 1 μc of an ethanol solution of 4-^{14}C-cholesterol (specific activity: 0.15 mc/mg) was added. The tissue was then autoclaved for 4 hr at 120 C in 10 ml of 5% NaOH in 50% ethanol. Nonsaponifiable and saponifiable fractions were prepared from the autoclaved mixture in the usual manner. An aliquot (1/10) of the saponifiable (acid) fraction was tested for radioactivity by scintillation spectrometry. No radioactivity was detected; therefore any lithocholic acid found was not produced by degradation and alkaline-thermal isomerization of cholesterol during the isolation procedure (an unlikely but remote possibility). Thin layer chromatography (TLC) of 1/20 of the acidic fraction of each sample was carried out on Silica Gel G using the solvent system trimethyl pentane-ethyl acetate-acetic acid (75:45:1). When the plate was sprayed with 50% H_2SO_4 (v/v in water) and heated, a faint spot with the same R_f (0.33) as the lithocholic acid standard was seen in the multiple sclerosis sample. No spot was seen in the control sample.

The remainder of the acidic fraction was subjected to preparative TLC in the same solvent system and the plate was sectioned into four areas corresponding to mono-, di-, tri- and less polar bile acids, respectively. The material was eluted with acetone-ethanol (1:1) and a small portion of the material from the lithocholic acid region was rechromatographed and sprayed, and again a spot was seen with the same R_f as authentic lithocholic acid. The remainder of the monohydroxy bile acid region was methylated with BF_3-methanol reagent. TLC of a portion of the methylated material from the multiple sclerosis sample in a system of trimethyl pentane-ethyl acetate-acetic acid

(60:20:0.4) on development gave a spot with the same R_f as methyl lithocholate.

Gas liquid chromatography (GLC) of this material showed several peaks, one of which had the same retention time as methyl lithocholate in two systems (3% QF-1 and 1.5% SE-52 on Gas Chrom Q); relative retention times of 2.06 (relative to cholesterol-5.2 min) and 1.10 (relative to cholesterol-11.4 min) respectively. GLC conditions were: for 3% QF-1, column temperature 240 C, for 1.5% SE-52, column temperature 220 C; for both columns, detector 270 C, and flash heater 270 C. The carrier gas was nitrogen at a flow rate of 40 ml/min. The columns were 2 m in length with 4 mm i.d. It has previously been shown that lithocholic acid methyl ester can be separated from the related isomers on QF-1 and on a phenyl-containing phase (5). Examination of the methylated fraction by GLC mass spectrometry revealed that the peak with a retention time equivalent to a methyl lithocholate standard (13.3 min) had a spectrum with major peaks at M/e 372 (M-18), 357 (M-[18 + 15]), 318, 257 (M- [18 + 115]) and 215. GLC-mass spectrometry conditions on LKB 9000 were: column temperature 223 C, flash heater, 268 C, molecular separator 258 C, and helium flow 30 ml/min. The column was a 1.5% SE-52 column 2 m in length with 4 mm i.d. The filament temperature was 270 C and the ionizing beam was at 70 ev. Spectra of the compound from EAE guinea pig brains (1), a lithocholic acid standard, and the human MS brain are shown in Figure 1. The spectra of the MS material is similar to that of the others shown and also to other published spectra (6).

The identification of lithocholic acid in the EAE brains of guinea pigs and human multiple sclerosis brain raises the question of its origin. Experiments are underway in our laboratory to determine whether normal or EAE brain tissue possesses the capacity to form this or similar bile acids from cholesterol. It is also possible that lithocholic acid and other related acids are present in normal brain tissue, but in concentrations much smaller than those found in diseased tissue. Further work will also be directed toward establishing the precise location of the acid within the tissue. It has not been possible to extimate the quantity of lithocholic acid present in the specimen examined.

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[Received February 26, 1970]

Pancreatic Lipolysis of Enantiomeric Triglycerides[1]

ABSTRACT

Pancreatic lipase hydrolyzed fatty acids in equimolar quantities from the *sn*-1- and 3-positions of three synthetic enantiomeric triglycerides, two of which could make a racemic pair. The monoglycerides from digestions of five enantiomeric triglycerides were at least 99% representative of the 2-position. The data confirm that pancreatic lipase did not distinguish between the *sn*-1- and 3-positions and that with these triglycerides pancreatic lipolysis can be used to help establish structure.

[1]Scientific contribution No. 419 Agricultural Experiment Station, University of Connecticut, Storrs.

As part of the preparation of some cryptoactive triglycerides for investigating a method

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TABLE I

Fatty Acid Composition of Intact Triglycerides and of Monoglycerides and Free Fatty Acids Derived From Pancreatic Lipolysis of Enantiomeric Triglycerides

	Intact triglyceride			Monoglyceride			Free fatty acids		
Triglyceride	16:0	18:0	18:1	16:0	18:0	18:1	16:0	18:0	18:1
	Mole %								
18:1-18:1-16:0[a]	33.0	---	67.0	Trace	---	99+	48.3	---	51.7
18:1-16:0-16:0	67.6	---	32.4	99+	---	Trace	48.0	---	52.0
16:0-16:0-18:1	68.0	---	32.0	99+	---	Trace	49.4	---	50.6
16:0-18:0-18:1	33.1	33.3	33.6	0.3	99.4	0.3	---	b	---
18:1-18:0-16:0	33.1	34.2	32.7	Trace	99.5	0.5	---	b	---

[a]Triglycerides are numbered 1,2 and 3 from left to right in *sn* nomenclature; 18:1-18:1-16:0 would be *sn*-glycerol-1,2-dioleate-3-palmitate.

[b]Not done.

of stereospecific analysis (1), positional integrity was determined by pancreatic lipolysis. Although pancreatic lipase is known not to be stereospecific (2,3), very few data are available on the pancreatic lipolysis of synthetic enantiomeric triglycerides.

The triglycerides used as substrates were *sn*-glycerol-1,2-dioleate-3-palmitate (18:1-18:1-16:0), *sn*-glycerol-1-oleate-2,3-dipalmitate (18:1-16:0-16:0), *sn*-glycerol-1,2-dipalmitate-3-oleate (16:0-16:0-18:1), *sn*-glycerol-1-palmitate-2-stearate-3-oleate (16:0-18:0-18:1) and *sn*-glycerol-1-oleate-2-stearate-3-palmitate (18:1-18:0-16:0). These were made by standard procedures (4) from *sn*-3-acetone glycerol (5). The purity of the intermediate 1,3-diglycerides, determined as described by Sampugna and Jensen (1) was close to 99%. The pancreatic lipolysis procedure and recovery and analysis of the lipolysis products have been described (1).

Compositional data for the monoglycerides and in three cases, the free fatty acids are given in Table I. The free fatty acids were hydrolyzed from positions *sn*-1- and 3- in close to equimolar quantities, therefore pancreatic lipase did not differentiate between the two positions and earlier findings are confirmed (2,3). Since this was the case with both enantiomers of a racemic pair (18:1-16:0-16:0 and 16:0-16:0-18:1) composition of the fatty acids was not involved. The monoglycerides were more than 99% representative of the 2-position. The presence of monoglycerides, is, in itself, proof that pancreatic lipase is not stereospecific because a stereospecific lipase would hydrolyze either the *sn*-1- or 3-position only, leaving a diglyceride and no monoglyceride.

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Errata

Please note that the Short Communication "Pyrolysis–Gas Chromatography of Phosphoglycerides: A Mass Spectral Study of the Products," by E. G. Perkins and Patricia V. Johnston, was not listed in the Table of Contents of Lipids 4:301-303 (1969), nor in the Annual Index for 1969.

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Inhibition of Desaturation of Stearic Acid in Livers of Rats Fed Ethionine[1]

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ABSTRACT

The effect of ethionine on the conversion of stearic acid to oleic acid was studied. Rats were fed essential fatty acid (EFA) deficient diet for three weeks, after which time half the animals were fed 0.25% DL-ethionine for nine additional days. Seventeen hours prior to killing, they were fed a slurry of the diet containing 18-^{14}C-stearic acid. Liver triglycerides and phospholipids were extracted and separated and their fatty acid composition and the distribution of radioactivity between stearic and oleic acid was determined. In the tissues studied, oleic acid was maintained at control levels in ethionine-fed rats, but eicosatrienoic acid was significantly depressed. Distribution of radioactivity and specific activity of oleic acid in the triglycerides and phospholipids were significantly reduced by the analogue. In vitro studies of desaturation and chain elongation reactions, with liver microsomes, using 18-^{14}C-stearic and 1-^{14}C-linoleic acids as substrates, showed that ethionine depressed the synthesis of oleic acid from stearic and γ-linolenic from linoleic acid. Elongation of linoleic adie to a 20:2 fatty acid was unaffected by ethionine. Therefore, the results showed that ethionine inhibited desaturation of stearic to oleic acid in vivo and in vitro and probably also impaired the desaturation of oleic to octadeca-6, 9-dienoic acid. Maintenance of control levels of oleic acid in the tissues of ethionine-fed, EFA deficient rats suggested the presence of synthetic pathways for oleic acid other than via desaturation of stearic acid.

INTRODUCTION

A previous report (1) showed that male or female rats, fed a low protein diet supplemented with 0.25% DL-ethionine had altered fatty acid patterns in their liver phospholipids when compared with animals fed the same diet without the ethionine. The major changes noted were increased proportions of linoleic acid and decreased percentages of arachidonic and C_{22}-pentaenoic acids. Most of the differences were prevented when methionine (0.5%) was added to the diet. Further investigations (2) with 1-^{14}C-linoleic acid established that the conversion of linoleic acid to arachidonic and C_{22}-pentaenoic acids was impaired in these animals. The present study is an extension of this work designed to show if ethionine also affected the conversion of stearic acid to oleic acid and if desaturation or chain elongation was the reaction primarily inhibited.

[1]Presented in part at the AOCS Meeting, San Francisco, April 1969.

METHODS

In order to insure the synthesis of oleic acid and its unsaturated fatty acid derivatives, male Sprague-Dawley rats, weighing about 100 g, were fed a 9% casein diet containing 5% hydrogenated coconut oil and deficient in essential fatty acid (EFA) for three weeks (Table I). This length of time on an EFA deficient diet had previously been shown sufficient to raise significantly the oleic and eicosa-

TABLE I

Composition of Diets

Diet	Basal, %	0.25 DL-ethionine, %
Casein	9.0	9.0
Sucrose	79.85	79.6
Hydrogenated coconut oil (HCO)	4.0	4.0
DL-ethionine[a]	---	0.25
Salts, USP XIV[a]	5.0	5.0
Fat-soluble vitamins[b]	1.0	1.0
Vitamin mix[c]	1.0	1.0
Choline chloride	0.15	0.15

[a]Obtained from Nutritional Biochemicals Corporation, Cleveland.

[b]HCO fortified so that 1 g provided, per 100 g diet; vitamin A acetate, 400 IU; vitamin D_2, 200 IU; α-tocopherol, 10.0 mg.

[c]Mixed in sucrose so that 1 g mix provided, in mg/100 g diet; thiamine-HCl, 0.5; riboflavin, 0.5; nicotinic acid, 2.5; Ca pantothenate, 2.0; pyridoxine-HCl, 0.25; menadione, 0.05; folic acid, 0.02; biotin, 0.01; and vitamin B_{12} (as 0.1% mannitol triturate), 0.002.

TABLE II

Body Weight and Liver Lipids of Rats Fed an EFA Deficient Diet With and Without Ethionine

Animal group	Body Wt, g	Liver Wt, g	Liver lipid, %	Liver phospholipid, mg/g	Liver triglyceride, mg/g
EFA deficient control	150 ± 3.8[a]	6.8 ± 0.2	5.2 ± 0.7	25.3 ± 1.1	24.4 ± 4.8
EFA deficient +0.25% DL-ethionine	122 ± 11	4.9 ± 0.2	6.5 ± 0.7	20.8 ± 2.9	23.0 ± 10.8

[a]Mean ± SE of five rats in each group.

trienoic acid levels in the liver lipids (3). Following the three week period, all rats remained on the same basic diet for nine additional days, but half of them were supplemented with 0.25% DL-ethionine. In those experiments in which 18-^{14}C-stearic acid was administered, the rats were fasted overnight and 10 μc of the labeled fatty acid per 100 g of body weight was given by stomach tube with about 3 g of slurried diet. The animals were allowed free access to food and water until killed by decapitation 17 hr after feeding the labeled fatty acid. Livers were removed, blotted, weighed and quickly frozen and lyophilized. They were then pulverized and extracted with chloroform-methanol (2:1 v/v) as previously described (2). The entire carcass, minus head and internal organs, was put directly into a separate flask and digested with ethanolic potassium hydroxide. Saponification, extraction and analysis of the fatty acids have been described (3).

Total liver lipid and lipid phosphorus were determined by the methods of Bragdon (4) and Sumner (5). Separation of liver triglycerides and phospholipids was by silicic acid column chromatography (6). Recoveries of lipid and phosphorus averaged over 95%.

Methyl esters of the fatty acids of the lipid fractions were prepared in H_2SO_4-methanol by transesterification (7) and analyzed by gas liquid chromatography. The chromatograph (F and M, Model 810) was fitted with an effluent stream splitter (7:1 split ratio as determined from collection of 1-^{14}C-methyl linoleate) and connected to a manually operated fraction collector (Packard Instrument Co., Model 830). The fatty acid methyl esters were separated on a 1/4 x 90 in. stainless steel column packed with silanized chromosorb W coated with poly-

TABLE III

Fatty Acid Composition of Carcass Lipid and Liver Triglycerides and Phospholipids of Rats Fed an EFA Deficient Diet With and Without Ethionine

Animal group	Fatty acid (Wt %)									
	12:0	14:0	16:0	16:1	18:0	18:1	18:2	20:3	20:4	>20:4
Carcass										
EFA deficient control	3.5[a]	4.8	28.3	11.2	5.3	40.1	3.0	---	1.1	---
EFA deficient + 0.25% DL-ethionine	3.4	4.3	26.0	8.7	7.0	41.5	4.2	---	1.3	---
Liver triglyceride										
EFA deficient control	---	---	35.9 ±3.4	9.0 ±0.4	3.5 ±0.2	45.3 ±1.9	0.3 ±0.1	---	Trace	---
EFA deficient + 0.25 DL-ethionine	---	---	20.4[b] ±0.6	3.3[b] ±0.8	5.2[c] ±0.6	60.1[b] ±0.8	2.1[b] ±0.3	---	Trace	---
Liver phospholipids										
EFA deficient control	---	---	18.3	---	18.0	18.8	4.8 ±0.7	11.8 ±1.6	13.6	4.7
EFA deficient + 0.25% DL-ethionine	---	---	16.5	---	16.7	22.3	9.3[b] ±0.7	5.3[b] ±0.5	16.0	5.9

[a]Means from four control and five DL-ethionine-fed rats. Standard errors have been included only if differences between groups appeared significant.

[b]Significantly different from control, $p < .01$.

[c]Significantly different from control, $p < .05$.

TABLE IV

Distribution of Radioactivity in Stearic and Oleic Acid of Liver Lipids From Rats Fed EFA Deficient Diets With and Without Ethionine

Animal group	Distribution of radioactivity (%) 18:0	18:1	Sp. act. 18:0/Sp. act. 18:1
Liver triglycerides			
EFA deficient control	19.5 ± 4.3[a]	64.9 ± 4.7	---
EFA deficient + 0.25% DL-ethionine	37.3 ± 5.8[b]	41.3 ± 8.7[b]	---
Liver phospholipids			
EFA deficient control	57.8 ± 4.0	27.0 ± 4.0	2.5 ± 0.5
EFA deficient + 0.25% DL-ethionine	76.3 ± 3.9[b]	8.3 ± 1.1[c]	15.6 ± 4.6[b]

[a]Means ± SE of four control and five ethionine-fed animals.

[b]Significantly different from control $p < .05$.

[c]Significantly different from control $p < .01$.

diethylene glycol succinate. Calibration of the instrument was made daily using a standard mixture of methyl esters of the appropriate fatty acids. Collection of the methyl esters was accomplished with glass wool filled Pyrex tubes as described by Bennett and Coon (8).

Quantitation of the fatty acids was by means of an internal standard (heptadecanoic acid) (9). Recovery of radioactivity was between 87%-92%. The entire effluent from a sample was collected, but only those fatty acids of a particular interest were collected separately in individual tubes. For measurement of radioactivity, the entire tube was immersed directly into scintillation solution and counted in a Beckman liquid scintillation counter (Model LS 100).

For in vitro studies, Sprague-Dawley rats weighing about 150 g were fed in the same manner as previously described, except that they did not receive any radioactive fatty acid and were allowed access to food and water until killed by decapitation.

Microsomal preparation and incubation procedures for measuring desaturation and elongation reactions were those of Marcel et al. (10) except that all incubations were carried out in Erlenmeyer flasks stoppered with serum

TABLE V

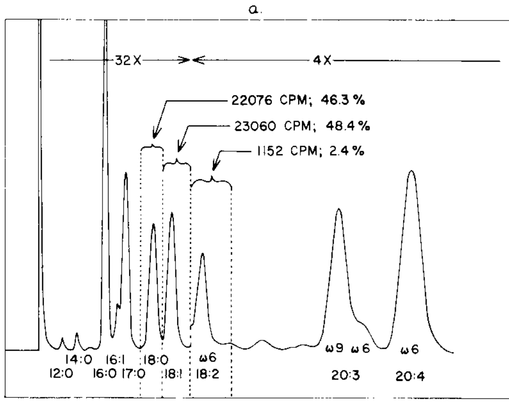
In Vitro Desaturation and Elongation of 18-^{14}C-Stearic Acid by Liver Microsomes From EFA Deficient Rats Fed Diets With and Without Ethionine

Fatty acid	Distribution of radioactivity, % EFA deficient control	EFA deficient + 0.25% DL-ethionine
Desaturation (aerobic)		
18:0	57.6 ± 10.2[a]	88.1 ± 2.9[b]
18:1ω9[c]	35.7 ± 10.5	6.8 ± 3.1[b]
20:0	1.3 ± 0.1	1.5 ± 0.6
Elongation (anaerobic)		
18:0	88.5 ± 1.5	88.2 ± 3.0
18:1ω9	5.9 ± 1.4	3.2 ± 0.4
20:0	0.7 ± 0.1	1.5 ± 0.6

[a]Means ± SE of duplicate incubations from five individual control and three individual rats fed ethionine. Per cent of radioactivity in the fatty acids was determined from the ratio of the radioactivity in the particular fatty acid to the total radioactivity collected from the gas liquid chromatograph.

[b]Significantly different from control, $p < .05$.

[c]Number following the ω denotes the position of the first double bond counting from the terminal methyl group.



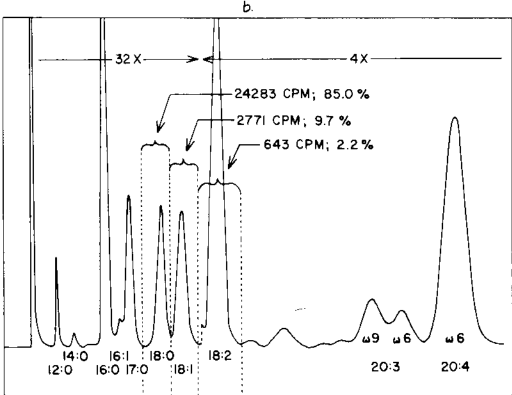


FIG. 1. Gas liquid chromatograms of liver microsomal lipids from each of 2 rats fed an EFA deficient diet, without ethionine (A) and with ethionine (B). Microsomes were incubated aerobically 25 min at 37 C (see text for details) with 18-^{14}C-stearic acid and total radioactivity of lipid extract and of stearic and oleic acid determined. Omega (ω) followed by a number is the position of the first double bond, when counted from the terminal methyl group. The numbers at the top of the Figure are the relative attenuations.

caps. Substrates for the reactions were either 100 mμmoles 18-^{14}C-stearic acid or 100 mμmoles 1-^{14}C-linoleic acid. After 30 min incubation, the contents of each flask were poured into a pre-cooled (-70 C) 50 ml screw top tube, rinsed and lyophilized. Transmethylation of the dry residues in the tubes was carried out with 2.5% H_2SO_4-methanol (freshly prepared) for 2 hr in a 65 C water bath. The tubes were capped with polyethylene cone-closure (Poly-seal) caps during the transmethylation. Methylation procedures used for pure lipid extract proved inadequate for these samples which contained organic matter other than lipid.

Statistical comparisons were made by T-test. Differences were considered as being significant if $p < .05$.

RESULTS

Body weight and liver lipids from rats fed the EFA deficient diet with and without ethionine are shown in Table II. Aside from their reduced weight gain, hence smaller liver, rats fed ethionine did not differ significantly from controls in their proportions of liver phospholipids and triglycerides. These results were comparable to those obtained previously (1,2), in which male rats fed the same level of ethionine with diets of similar protein content showed no consistent hepatic triglyceride accumulation.

Table III shows the fatty acid composition of carcass, liver triglyceride and liver phospholipid. Ethionine had no effect on the carcass lipid fatty acid composition and the proportion of oleic acid, the most prominent fatty acid, was unaffected. Liver triglycerides of animals fed the methionine analogue, however, did have reduced proportions of palmitic and palmitoleic acids accompanied by increased percentages of oleic acid. The small amount of linoleic acid in this lipid was also increased by the ethionine feeding. The major changes in hepatic phospholipids induced by ethionine were the increased linoleic acid and the reduced levels of eicosatrienoic acid, even though proportions of oleic acid were similar for both groups.

Although these results suggested that ethionine might be affecting the conversion of oleic to eicosatrienoic acid rather than the desaturation of stearic to oleic acid, the latter reaction is significantly depressed as shown in Table IV. When 18-^{14}C-stearic acid was fed to control animals, oleic acid of the liver triglycerides had over three times the stearic acid activity within 17 hr after feeding. In animals fed ethionine the distribution of the radioactivity in the two fatty acids was nearly equal. Inhibition of the conversion of stearic to oleic acid by ethionine was even more pronounced in the phospholipids. In this lipid, oleic acid accounted for 27% of the radioactivity in controls, but constituted only about 8% in the animals fed ethionine. The oleic acid from these animals also had a lower specific activity than did the controls, so their stearic acid specific activity-oleic acid specific activity ratios are at least six times that of the controls.

It appeared, therefore, that conversion of stearic acid to oleic acid is depressed in rats fed ethionine just as is the conversion of linoleic acid to arachidonic acid. The animals, however, were able to maintain tissue levels of oleic acid comparable to control rats. Even though the oleic acid was maintained, the proportion of eicosatrienoic acid, which under the conditions of this experiment is derived from oleic acid, is

TABLE VI

In Vitro Desaturation and Elongation of 1-^{14}C Linoleic Acid by Liver Microsomes From EFA Deficient Rats Fed Diets With and Without Ethionine

	Distribution of radioactivity, %			
	EFA deficient control		EFA deficient + 0.25% DL-ethionine	
Fatty acid	HCO	Fat free	HCO	Fat free
Desaturation (aerobic)				
18:2ω6[a]	84.3 ± 0.7[b]	76.5 ± 2.9	88.2 ± 0.9[c]	80.2 ± 1.4
18:3ω6	4.1 ± 0.6	9.9 ± 1.6	1.4 ± 0.1[c]	2.4 ± 0.1[c]
20:2ω6	0.8 ± 0.1	1.0 ± 0.2	0.7 ± 0.0	1.0 ± 0.1
Elongation (anaerobic)				
18:2ω6	70.9 ± 2.5	---	67.5 ± 2.0	---
18:3ω6	1.0 ± 0.1	---	1.0 ± 0.1	---
20:2ω6	12.5 ± 1.7	---	14.9 ± 1.4	---

[a]Number following the ω denotes the position of the first double bond counting from the terminal methyl group.

[b]Mean ± SE of duplicate incubations from eight individual rats in control and experimental groups fed the hydrogenated coconut oil (HCO) and from four individual rats in control and experimental groups fed the fat-free diets. Per cent of radioactivity in the fatty acids was determined from the ratio of the radioactivity in the particular fatty acid to the total radioactivity collected from the gas liquid chromatograph.

[c]Significantly different from control $p < .01$.

significantly decreased, suggesting that elongation or further desaturation of oleic acid could not occur normally.

In an effort to learn whether the defect caused by ethionine was one affecting desaturation primarily or whether elongation was also involved, we turned to in vitro systems using liver microsome preparations (10). Gas liquid chromatograms and the distribution of the radioactivity typically obtained from liver microsomal lipids from control and ethionine-fed rats after aerobic incubation of the microsomes with 18-^{14}C-stearic acid are shown in Figure 1. The increased proportions of linoleic acid and decreased proportion of eicosatrienoic acid induced by the ethionine are evident in the fatty acid patterns. About equal amounts of radioactivity were in the peaks representing stearic and oleic acids with only about 5% of the counts appearing in the other fatty acids when control microsomes were incubated. However, only 10% of the stearic acid was converted into oleic acid in those microsomes from ethionine treated rats. Averaged results from additional animals are summarized in Table V. Although the system readily demonstrated desaturase activity and its inhibition when ethionine had been fed, no significant chain elongation took place in either control or ethionine-fed animals. Results from a similar type of experiment, but with 1-^{14}C-linoleic acid as substrate are shown in Table VI. Initial experiments used microsomes from rats fed EFA-deficient diets containing hydrogenated coconut oil. Although significant depression of desaturation of linoleic acid to γ-linolenic acid by feeding ethionine could be demonstrated, the amount of conversion in control animals was much lower than that obtained by others (10) using fat free diets. We, therefore, repeated the experiments with microsomes from animals fed a fat free diet during the nine days they were fed ethionine. Under these conditions about 10% of the linoleate was desaturated to γ-linolenic acid in controls, but only 2.4% γ-linolenic acid was produced with microsomes from ethionine-fed rats. Chain elongation of the linoleic acid appeared to take place to about the same extent in controls as in the rats fed ethionine.

DISCUSSION

In vivo studies had shown previously that dietary ethionine impaired the normal transformation of linoleic to arachidonic acid (2). The present experiments, carried out in vivo as well as in vitro, have demonstrated that the analogue also interferes with desaturation of stearic acid in the synthesis of oleic acid.

Nugteren (11) had reported that only slight chain elongation of stearic acid could be demonstrated in microsomal systems which was also our experience. However, linoleic acid chain elongation of the order reported by Marcel et al. (10) for rats receiving fat occurred in control and ethionine-fed animals to about the same extent (Table VI). This result suggests

that the primary effect of ethionine is on the desaturases rather than on the enzymes involved in elongation reactions. In as much as the principal first step in arachidonic acid synthesis is desaturation of linoleic acid to γ-linolenic acid (12), it becomes apparent why linoleic acid, supplied by the diet, accumulated and arachidonic acid decreased with no evidence of accumulation of abnormal long chain intermediate fatty acids (2).

In rats fed the EFA deficient diet, no dietary oleic acid was provided, yet the animals fed ethionine exhibited levels of oleic acid in their tissue lipids comparable to control rats. Levels of eicosatrienoic acid, derived from oleic acid, were significantly depressed, however, when compared with controls (Table III). Since the desaturation of stearic acid in the synthesis of oleic acid was partially inhibited by ethionine (Tables IV and V), these results suggest that oleic acid may have been synthesized by a pathway other than from stearic acid. In the conversion of oleic acid to eicosatrienoic acid, however, the preferred initial step is a desaturation to an octadeca-6, 9-dienoic acid (13). It appears, therefore, that ethionine blocks this desaturation of oleic acid, also, and since no other pathway for the synthesis of the triene exists, its tissue level decreases. Little or no elongation of the oleic acid previous to this desaturation seems to occur, as no accumulation of $20{:}1\omega 9$ fatty acid was evident in any of the lipids studied (Table III).

Evidence of pathways for oleic acid synthesis in higher organisms other than through desaturation of stearic acid has been reported for the chicken (14) and for the rat (15). Recently, Raju and Reiser (16) have shown that when *Sterculia foetida* seed oil is fed, rats may use lauric acid for oleic acid synthesis, presumably by a reaction involving a β,γ-desaturation of the lauric acid followed by chain elongation to oleic acid. In all of our experiments in which tissue fatty acids were measured, and in which oleic acid was found, we had fed hydrogenated coconut oil, containing about 50% lauric acid. It has been estimated (16) that the alternate oleic acid synthetic pathway may normally account for at least one fourth of the oleic acid synthesized. It is possible, therefore, that when desaturation of stearic acid, the major pathway, is impaired, the alternate synthetic route is enhanced.

Unfortunately the maintenance of normal oleic acid tissue levels in the present experiment cannot, however, be unambigously interpreted as an increase in some alternate synthetic pathway, because the animals had three weeks, prior to the ethionine feeding, in which to accumulate tissue oleic acid. Following ethionine feeding there was a decrease in the utilization of oleic acid for eicosatrienoic acid synthesis and also probably a decrease in its oxidation (17). If the decreased utilization essentially balanced the depressed synthesis, no difference from control values would be seen in the short time the ethionine was fed. The question of whether an alternate pathway of oleic acid synthesis is present and contributes significantly in these animals is being investigated further.

How ethionine acts to inhibit, rather specifically, the acyl desaturases and not the elongation reactions remains unanswered. Mohrhauer et al. (18) have reported that quite different mechanisms functioned in the inhibition of chain lengthening, than those involved in desaturation. While a wide range of fatty acids of differing unsaturation and chain lengths inhibited elongation, only oleic, linolenic and docosahexaenoic acids interfered with linoleic to γ-linolenic acid conversions. A greater substrate specificity must exist, therefore, for the desaturases than for the elongation enzymes. Ethionine, a rather nonspecific inhibitor of a number of enzyme mediated reactions (19), was fairly selective for inhibiting desaturase activity. Kircher (20) and Raju and Reiser (21) have suggested that the stearoyl desaturase enzyme activity is dependent on thiol groups and that the active principle of *Sterculia foetida* oil, sterculic acid, inhibits the enzyme by irreversibly binding the thiols. Ethionine is not known as a thiol binding agent, so it is doubtful it affects desaturase activity by such a mechanism. More likely, the inhibition is induced through an effect on protein synthesis (22,23,24), in which enzyme synthesis is depressed or else nonfunctioning ethionine-containing enzymes may be produced.

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Fatty Acid Composition of the Neutral Lipids and Individual Phospholipids of Muscle of Cold-Stressed Arctic Mice

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ABSTRACT

Fatty acids of the individual phospholipids and total neutral lipid fractions in skeletal muscle of three species of Arctic mice were identified and quantitated after the mice had been classified as control, cold-sensitive or cold-resistant. The results indicate that some species increase the percentage of unsaturated fatty acids as an apparent result of cold exposure and some species do not. A common finding for all cold-sensitive mice was a significant increase in 14:0 in phosphatidic acid when compared to cold-resistant and control animals. Hypotheses are presented in an attempt to explain this finding.

INTRODUCTION

It has been determined (1) that the lipid pattern of skeletal muscle observed in some native Arctic mice varies with the animal's ability to withstand cold stress. It was considered essential to determine whether these variations were dependent upon or associated with a difference in the fatty acid composition of the lipid classes of the various classifications of animals within each species. It was also deemed important to determine whether specific fatty acids were utilized preferentially in the maintenance of body heat. This paper reports the findings of this study.

METHODS

Animal Classification

Animal classification was performed according to our standard procedure (1). In brief, the method is as follows: Three species of native Arctic mice were trapped and their offspring raised at 5 C (cold-acclimated) or 18 C (control). The cold-acclimated animals were stressed at -40 C for a 2 hr period. The animals' temperature was taken rectally every 30 min. If the temperature fell below 36 C, the animal was removed from the stress chamber and classified cold-sensitive (CS). If the animals' temperature was equal to or greater than 36 C for the full 2 hr period, the animal was considered cold-resistant (CR).

Extraction and Separation of Lipid Classes

These methods have been described in more detail elsewhere (3,4). Eight hours after stressing, the animals were decapitated, the total skeletal muscle mass of the hind legs was quickly excised, all visible adipose tissue removed and the remaining muscle frozen in liquid nitrogen. The muscle was then crushed and extracted under nitrogen three times with chloroform-methanol (C/M) solvents containing butylated hydroxytoluene (BHT) (1 mg/l) as an antioxidant. The crude lipid extract was purified on a Sephadex column (4), an aliquot of fraction one was weighed, a known amount (approximately 250 μg of phospholipid) was spotted on thin layer plates and the plates were developed two-dimensionally in chloroform-methanol-concentrated ammonium hydroxide (65:25:5 v/v/v) and chloroform-acetone-methanol-acetic acid-water (5:2:1:1:0.5 v/v/v/v/v). After development in the second dimension the plates were air dried for 5 min, the spots detected by spraying with distilled water, circled and the water evaporated under a stream of nitrogen. After drying, the spots were scraped from the plate into teflon-lined screw-capped tubes. Areas of the plate containing no lipid were used for blanks. The uncapped tubes were dried overnight in a vacuum desiccator over KOH pellets.

Transesterification and Isolation of Fatty Acid Esters

To each tube containing the dry sample was added 1.5 ml of benzene, a known amount of internal standard (methyl heneicosanoate) and 2.5 ml of freshly prepared 14% BF_3 in methanol. Each tube was flushed with nitrogen, tightly capped and heated at 100 C for 10 min. For sphingomyelin a 30 min heating period was used. After heating, the tubes were cooled, opened and 1.5 ml of water added. The tubes were shaken vigorously, centrifuged (500 x *g* for 10 min), the benzene layer transferred to 5 ml centrifuge tubes and the benzene evaporated under nitrogen. The sample was dissolved in carbon disulfide and an aliquot injected into the gas chromatograph (Barber-Colman, Series

5000, Rockford, Ill.) equipped with a flame ionization detector.

Separation and Identification of Fatty Acid Esters

The fatty acids were separated and quantitated on a 6 ft x 1/4 in. glass column packed with 10% DEGS (diethylene glycol succinate) on 80/100 mesh Gas Pak (Chemical Res. Services, Inc., Addison, Ill.). The following conditions were used for the column: on column injection; column temperature, 175 C; nitrogen carrier gas, 76 ml/min.

Fatty acids were identified by comparison of retention times with known standards on the DEGS column. Some samples were randomly selected and the identification of the fatty acids checked in a 6 ft x 1/4 in. nonpolar column (5% Apiezon L on 80/100 mesh Gas Pak, column temperature, 210 C; nitrogen carrier gas, 85 ml/min).

Identification was verified further by rerunning the fatty acids after hydrogenation. Hydrogenation was achieved as follows. An aliquot of the methyl ester mixture was added to a screw cap vial and the solvent removed. Methanol (1 ml) and a small amount of palladium black were added. Hydrogen was slowly bubbled into the methanol for 3 min, the vial sealed and heated at 75 C for 10 min. To the cooled vial were added 1 ml of water and 1 ml of hexane. The vial was vigorously shaken, the hexane removed, evaporated, the sample dissolved in carbon disulfide and reinjected into the gas chromatograph. Both the DEGS and Apiezon L columns were used for this procedure.

Fatty Acid Quantitation

Quantitation of each fatty acid was achieved by multiplying peak height by retention time (14). Each individual product was divided by the sum of the products.

Transesterification was found to be complete by thin layer chromatography of the hexane extract and the methanol-water residue using the two-dimensional solvent pair employed in phospholipid class isolation or unidimensionally using hexane-diethyl ether-acetic acid (70:30:1 v/v/v).

Identification of Aldehydes

An aliquot of the methyl ester mixture was placed in a glass tube with 3 N HCl, flushed with N_2, sealed and heated at 100 C for 2 hr. The tube was cooled, opened and made alkaline with KOH. The alkaline solution was extracted with ether, an aliquot injected into the gas chromatograph and the peak compared with known aldehyde standards. The remaining material was oxidized with acetic chromium trioxide to the corresponding fatty acid and the identity of the methyl ester also confirmed by comparison with known standards (2).

TABLE I

Fatty Acid Composition of a Selected Mixture Obtained Before and After Hydrogenation

Fatty acid	Per cent composition before hydrogenation	Per cent composition after hydrogenation
16:0	0.00	24.96
16:1	25.00	0.00
18:0	0.00	25.08
18:1	24.99	0.00
20:0	0.00	24.91
20:1	25.01	0.00
22:0	0.00	25.05
22:1	25.00	0.00

[a]See Methods section for details of procedure.

RESULTS

Table I shows the results from hydrogenation obtained by the method described and clearly demonstrates that the procedure is quantitative.

The data tabulated in Table II present the results of the fatty acid analysis for the native Alaskan mouse *Microtus oeconomus*. The results for individual fatty acids are expressed as per cent of the total fatty acid for the lipid classes of any one species. All of the phospholipids except one share a predominance of saturated fatty acids with 16:0 and 18:0 accounting for a large percentage of this total. Diphosphatidyl glycerol is the exception and it shows a high degree of unsaturation (65-75%) with 18:2 plus 18:1 accounting for over 50% of the total fatty acid.

The cold-sensitive animals have greater than a sixfold increase of 14:0 in the phosphatidic acid fraction. The cold-resistant animal, however, resembles very closely the 18 C control in fatty acid composition.

The neutral lipid contains primarily unsaturated fatty acids with 18:1 being present in greatest abundance followed by 18:2. Of the saturated fatty acids in the neutral lipid fractions, 16:0 is the most abundant. The cold-sensitive mice show a higher content of saturated fatty acids than do the cold-resistant mice for the phospholipid fractions. However, the cold-sensitive mice show a higher degree of monoenes than do the cold-resistant or control animals. When polyenes are considered, the 18 C-acclimated mice contain the largest

TABLE II

Fatty Acid Composition of Various Lipid Classes for *Microtus oeconomus*

Fatty acid	PC[a]			PE			PI			PS			DPG			PA			SPH			NL		
	CR	CS	18 C	CR	CS	18 C	CR	CS	18 C	CR	CS	18 C	CR	CS	18 C	CR	CS	18 C	CR	CS	18 C	CR	CS	18 C
9:0	1.0	0.8	Trace	0.7	—	—	0.5	0.9	0.3	1.9	2.7	2.8	—	3.3	0.2	Trace	Trace	5.8	—	4.7	4.9	0.1	0.4	0.2
10:0	1.1	2.2	0.3	1.0	1.6	1.1	Trace	1.4	0.8	3.6	4.7	2.3	2.4	3.3	0.5	Trace	7.9	1.3	—	5.6	3.4	0.1	0.2	0.1
11:0	0.2	Trace	0.2	—	0.1	Trace	Trace	0.2	Trace	0.7	1.4	0.9	0.4	0.4	0.7	2.3	2.6	2.3	—	2.8	2.4	0.1	0.1	0.1
12:0	0.2	0.4	0.2	0.3	0.4	0.4	0.2	0.5	0.4	0.9	1.4	1.3	2.4	2.8	0.3	3.9	4.3	3.4	1.8	1.7	2.6	0.2	0.3	0.2
13:0	0.4	0.4	—	0.6	1.3	0.9	0.5	1.3	0.1	1.7	0.3	0.3	0.6	1.2	0.4	4.9	0.7	0.6	3.3	2.7	0.8	0.1	Trace	—
14:0	0.4	0.2	0.2	0.6	0.9	0.7	0.4	1.4	0.5	1.4	0.7	0.4	1.6	1.4	1.8	4.7	29.2	4.1	3.6	5.4	1.5	1.4	1.2	1.6
14:0A	0.2	0.3	0.2	0.3	0.3	0.4	—	—	0.2	—	—	0.4	—	—	—	3.5	—	1.1	0.7	—	—	—	—	—
14:1	0.4	0.7	0.1	0.6	1.0	0.7	0.5	Trace	—	1.5	1.0	0.9	0.4	0.8	Trace	5.1	1.5	1.5	3.0	Trace	1.2	—	—	—
15:0	0.6	0.6	0.3	7.0	5.6	2.8	0.2	1.3	0.3	1.3	0.5	0.3	0.9	0.7	0.8	1.9	0.7	0.9	1.8	4.2	Trace	0.3	0.3	0.3
16:0	45.0	45.9	45.3	8.8	6.9	5.5	3.4	5.4	4.6	2.3	3.4	3.0	11.9	9.2	11.3	13.7	7.3	15.9	12.4	14.2	12.5	21.1	15.9	24.8
16:1	0.8	1.1	0.5	0.1	0.1	0.3	0.8	0.5	0.5	1.2	1.9	1.1	2.0	2.1	1.1	5.1	3.2	3.2	2.6	2.2	1.8	3.7	2.8	3.0
17:0	1.1	1.7	1.2	0.7	1.0	—	0.4	1.9	0.9	0.9	1.3	0.8	0.9	0.6	0.5	2.9	2.7	3.1	1.1	5.0	2.4	0.4	0.3	0.4
17:1	Trace	Trace	0.2	5.0	5.0	1.4	Trace	0.3	Trace	1.6	0.7	0.6	1.2	2.4	0.6	2.5	1.0	1.5	1.3	—	0.6	—	Trace	Trace
18:0	10.8	12.0	12.8	38.0	39.9	20.6	45.6	46.6	56.3	56.2	49.7	51.3	6.6	7.4	7.1	10.1	6.2	13.5	21.6	21:9	18.5	3.1	3.0	4.7
18:1	15.3	17.7	12.8	19.1	21.3	12.1	6.1	6.9	7.2	11.5	14.3	13.0	15.0	19.6	13.2	14.4	7.7	19.2	10.5	4.1	7.7	45.6	54.3	40.0
18:2	12.8	9.2	17.8	6.3	6.0	5.5	19.4	15.3	9.5	5.7	3.3	5.2	39.7	34.3	56.6	10.2	7.9	8.8	13.7	7.5	19.5	21.4	19.4	21.9
18:3	Trace	—	1.0	0.7	0.4	7.3	0.3	0.8	0.7	Trace	2.0	2.0	1.2	1.6	1.5	2.0	3.3	2.6	2.6	Trace	3.1	1.0	1.0	1.4
19:0	—	—	—	—	—	—	0.9	1.9	0.9	3.8	2.4	2.4	—	—	—	—	1.3	—	Trace	Trace	Trace	—	—	—
20:0	—	—	—	Trace	0.4	7.0	0.1	0.3	0.5	Trace	1.8	2.0	1.0	0.5	0.6	1.9	4.6	2.4	4.8	3.3	2.0	Trace	—	Trace
20:4	8.9	4.8	6.5	8.8	6.1	12.3	16.1	11.0	12.8	2.4	3.1	5.6	5.5	2.9	1.2	2.3	1.9	Trace	8.7	9.1	9.9	0.3	Trace	Trace
22:0	—	—	—	—	—	19.5	1.2	1.1	1.7	—	—	—	1.2	—	—	—	—	—	—	—	—	—	—	—

[a]Values expressed as per cent of total for each classification. A, aldehyde; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PI, phosphatidyl inositol; PS, phosphatidyl serine; DPG, diphosphatidyl glycerol (cardiolipin); PA, phosphatidic acid; SPH, sphingomyelin; NL, neutral lipid; CR, cold-resistant; CS, cold-sensitive; Trace component, less than 0.1%; —, not detected. Each column may not add up to 100%. Unless a fatty acid was present 1% for at least one of the categories it was not listed.

TABLE III

Fatty Acid Composition of Various Lipid Classes for *Microtus pennsylvanicus*[a]

Fatty acid	PC[b]			PE			PI			PS			DPG			PA			SPH			NL		
	CR	CS	18 C	CR	CS	18 C	CR	CS	18 C	CR	CS	18 C	CR	CS	18 C	CR	CS	18 C	CR	CS	18 C	CR	CS	18 C
9:0	Trace	Trace	Trace	Trace	Trace	1.3	—	—	—	Trace	Trace	1.3	—	—	0.6	Trace	0.8	2.2	—	—	4.7	0.7	0.8	0.9
10:0	0.5	0.8	0.4	2.4	0.9	0.6	1.3	1.1	0.5	1.2	1.1	1.6	1.3	0.9	0.5	1.4	1.7	3.3	1.0	1.4	3.2	0.7	0.9	0.7
11:0	4.6	6.2	0.4	7.2	6.1	0.5	6.1	5.9	0.3	5.3	6.2	0.7	8.2	5.9	0.5	7.0	8.5	2.9	6.6	5.2	2.1	0.2	0.3	0.2
12:0	0.5	0.4	0.4	0.7	0.7	0.8	0.4	0.7	0.3	0.6	0.6	0.7	0.7	0.6	3.6	0.4	0.8	4.9	0.7	0.4	1.5	0.3	0.7	0.5
12:0	0.4	0.2	0.4	0.4	0.2	0.6	1.1	0.3	—	0.2	0.4	—	0.5	0.4	0.2	0.1	0.1	2.9	0.2	0.1	2.8	0.1	0.2	Trace
14:0	4.5	4.1	0.8	5.0	5.1	0.9	5.3	5.0	0.2	5.0	5.8	0.8	6.9	6.4	2.2	6.5	6.8	4.8	6.6	8.8	1.7	1.6	1.6	2.4
14:0A	—	—	—	—	—	—	—	—	0.1	4.0	3.7	0.7	1.4	2.2	0.2	—	5.9	2.6	—	—	1.0	0.2	0.3	0.2
14:1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	4.8	—	0.4	—	0.3	0.5	0.5
15:1	—	—	0.3	1.2	0.8	6.5	0.2	—	0.2	—	—	1.0	Trace	0.2	1.9	—	0.1	3.3	0.1	0.1	1.7	0.1	0.2	0.3
16:0	22.3	19.1	42.7	15.4	15.5	9.3	13.8	14.1	2.9	12.2	13.8	4.0	15.0	14.2	11.9	16.6	16.0	11.5	16.1	10.1	8.0	18.0	15.8	20.6
16:1	0.7	0.3	1.2	0.8	0.2	0.3	0.3	0.3	0.5	0.4	0.4	1.1	0.3	0.5	1.4	0.4	0.2	6.3	0.2	0.5	1.3	8.4	8.3	8.5
17:0	1.5	0.9	1.1	1.1	—	1.2	1.2	1.0	0.6	0.9	1.1	0.9	1.3	0.9	0.7	1.3	1.1	2.6	1.2	0.5	1.7	0.2	0.3	0.5
17:0A	0.4	6.2	—	1.4	0.2	0.7	0.4	0.4	—	0.6	0.4	—	0.4	0.5	0.6	0.3	0.4	—	0.2	—	—	—	—	0.2
17:1	0.2	0.3	—	1.1	1.3	4.5	0.8	0.3	—	0.2	0.4	0.7	0.5	0.5	1.7	0.3	Trace	2.5	—	0.4	0.6	0.3	0.1	Trace
18:0	37.8	32.1	11.7	46.5	47.0	40.1	43.3	48.5	35.1	38.0	47.7	42.9	40.6	42.3	21.7	46.6	45.7	6.4	44.7	26.7	12.6	2.0	2.2	2.2
18:1	4.9	4.7	13.1	3.2	2.9	13.0	1.7	2.1	5.4	1.0	2.2	14.6	2.6	1.9	12.8	1.6	1.0	11.9	1.3	0.9	6.7	46.9	45.9	42.4
18:2	6.9	4.9	14.0	2.2	3.5	5.3	5.0	6.9	16.4	0.9	1.2	2.6	10.7	6.9	20.7	2.5	1.6	5.5	1.7	1.2	4.0	15.9	18.2	17.1
18:3	0.3	Trace	—	Trace	1.1	—	0.9	0.4	4.7	1.4	0.5	15.0	Trace	0.4	0.7	0.7	1.2	3.1	Trace	Trace	1.7	1.1	1.1	1.4
18:4	4.7	14.7	—	4.5	6.3	—	—	—	—	11.1	3.7	—	2.7	7.6	2.1	5.3	Trace	—	8.2	42.2	5.8	—	—	—
20:0	0.9	0.9	—	1.6	1.2	—	1.1	1.2	—	1.1	1.1	—	1.2	1.5	0.5	1.8	1.7	1.1	5.5	3.4	1.5	0.4	0.1	—
20:2	3.0	3.8	—	0.5	1.4	—	6.9	2.0	0.6	11.4	5.0	3.9	1.2	1.2	—	1.8	0.9	—	1.6	0.7	—	—	—	—
20:4	1.6	2.2	12.5	0.6	1.5	11.0	3.9	4.0	17.2	—	—	6.0	—	—	12.2	1.0	—	3.8	—	—	11.1	—	—	—
20:5	1.9	2.1	—	3.0	3.3	—	2.2	3.1	—	2.3	2.6	—	1.3	2.5	—	3.3	3.9	—	3.0	0.8	—	—	—	—
22:0	—	—	—	—	—	—	—	—	13.9	—	—	—	—	—	—	—	—	4.1	—	—	20.4	—	—	—

[a]Values expressed as per cent of total for each classification.

[b]See Table II for abbreviations.

TABLE IV

Fatty Acid Composition of Various Lipid Classes for *Clethrionomus rutilus*[a]

Fatty acid	PC[b]			PE			PI			PS			DPG			PA			SPH			NL		
	CR	CS	18 C	CR	CS	18 C	CR	CS	18 C	CR	CS	18 C	CR	CS	18 C	CR	CS	18 C	CR	CS	18 C	CR	CS	18 C
9:0	Trace	Trace	Trace	0.9	Trace	1.0	—	0.9	0.9	2.2	0.8	10.3	5.0	1.4	6.1	3.2	1.0	3.0	—	—	—	0.3	0.4	—
10:0	2.6	0.6	1.6	1.5	1.3	0.8	3.2	0.7	0.6	2.9	1.1	Trace	Trace	Trace	Trace	2.4	1.2	3.1	—	—	—	Trace	0.8	0.3
12:0	Trace	Trace	Trace	0.4	0.3	0.4	Trace	Trace	0.4	0.9	0.4	1.4	0.8	1.6	2.9	3.2	0.3	1.6	1.6	0.7	0.8	0.4	0.5	0.4
13:0	1.3	Trace	1.0	—	1.0	—	1.8	—	Trace	0.7	0.7	3.3	2.6	1.7	2.2	2.9	2.3	1.0	1.9	2.6	3.5	0.2	0.4	0.1
13:0A	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	4.1	0.9	4.2	—	—	—	—	—	—
14:0	1.0	Trace	0.8	—	0.7	—	1.6	Trace	Trace	0.6	1.0	2.5	2.5	1.7	1.5	3.7	13.7	1.4	3.5	3.8	4.6	1.6	2.2	1.4
14:0A	—	—	—	—	—	—	—	—	Trace	Trace	0.1	2.9	Trace	0.5	1.3	1.8	4.6	2.4	1.3	1.4	0.6	Trace	—	—
14:1	0.6	Trace	0.5	—	0.9	0.3	1.4	Trace	Trace	1.4	—	0.4	—	1.6	1.7	4.9	0.3	2.0	3.5	3.4	4.3	0.1	0.2	0.3
15:0	Trace	Trace	0.2	2.0	6.0	4.6	0.5	Trace	Trace	0.4	0.2	1.3	1.5	1.0	0.6	0.6	3.3	2.5	1.1	1.0	0.9	0.3	0.2	0.2
15:0A	—	—	—	—	—	—	Trace	—	Trace	Trace	0.3	—	1.0	0.8	1.0	4.9	0.3	2.0	0.5	0.5	0.7	0.4	0.9	—
15:1	0.6	Trace	0.5	Trace	0.7	0.6	1.2	0.3	Trace	1.1	Trace	2.6	2.1	1.5	1.3	3.9	0.2	1.5	3.4	3.0	0.4	—	—	—
16:0	39.3	33.2	42.3	9.5	9.7	9.2	3.5	2.4	3.6	3.3	—	4.4	2.9	4.0	7.0	11.5	5.6	9.6	9.6	10.2	14.1	18.2	15.1	19.1
16:1	1.0	0.6	0.8	Trace	Trace	0.2	0.8	0.3	0.3	1.2	0.2	1.5	1.4	1.5	1.3	3.9	0.6	2.1	2.0	2.4	2.6	2.2	2.3	2.0
17:0	0.9	0.6	1.0	0.4	0.6	0.4	0.6	0.2	0.3	1.2	0.7	0.7	2.6	1.6	1.8	1.7	4.2	3.9	2.2	1.6	2.0	0.3	0.5	0.3
17:1	2.6	—	0.6	2.4	5.5	4.1	0.5	0.2	2.3	1.6	0.3	1.2	1.3	0.8	1.2	1.8	0.1	1.0	0.8	1.3	1.1	0.2	—	0.1
18:0	11.0	10.8	9.3	30.6	31.1	26.7	45.3	40.7	55.9	34.5	21.4	42.1	1.9	2.9	4.3	9.3	5.6	5.6	28.5	21.2	22.6	3.5	4.0	2.7
18:1	9.6	10.4	10.5	19.1	20.8	20.4	3.3	3.7	3.5	11.3	5.8	13.6	5.6	7.1	9.4	10.8	2.4	6.3	4.2	6.7	6.3	38.4	35.3	44.0
18:2	17.0	18.5	17.3	6.7	8.1	6.3	3.7	21.4	3.4	3.6	2.5	2.3	59.4	58.8	51.0	5.1	2.2	3.3	3.0	15.6	3.9	21.9	23.0	23.1
18:3	1.9	—	2.4	Trace	—	Trace	—	—	—	Trace	Trace	Trace	6.1	1.4	1.0	2.9	1.7	2.2	1.4	2.8	3.0	2.2	1.0	0.8
18:4	—	—	—	—	—	—	1.8	—	—	—	—	—	—	—	—	Trace	2.4	1.7	—	—	—	—	—	—
18:0	—	—	—	Trace	—	Trace	0.6	Trace	0.4	0.9	0.6	1.2	Trace	2.1	1.1	Trace	2.7	3.1	0.8	—	2.1	—	—	—
20:0	2.6	—	1.6	Trace	—	Trace	—	—	Trace	1.3	0.5	—	Trace	1.2	0.3	Trace	2.2	4.8	2.1	1.0	2.5	1.4	1.5	0.5
20:4	8.3	10.0	9.6	26.0	13.6	17.6	28.7	26.9	28.0	4.3	12.8	3.0	—	—	—	1.1	3.0	Trace	4.3	7.4	7.7	—	—	—
20:5	—	15.3	—	—	—	—	—	—	—	24.6	49.8	4.3	—	—	—	4.4	33.7	22.7	21.9	9.4	11.1	2.6	4.2	2.3

[a]Values expressed as per cent of total for each classification.

[b]See Table II for abbreviations.

amount and the cold-sensitive mice the least.

When the neutral lipid fraction (tri-, di- and monoglycerides) of *M. oeconomus* is considered for the three categories, it is observed that the 18 C-acclimated mice contain the highest degree of saturation and the largest amount of polyenes. The cold-sensitive mice contain the greatest amount of unsaturates due to their greater amount of monoenes.

The results of the individual phospholipid and total neutral lipid fatty acid analyses for *Microtus pennsylvanicus* are shown in Table III. Without any exceptions, all of the phospholipid fractions share a predominance of saturated fatty acids with 18:0 and 16:0 accounting for a large percentage of this total. The phosphatidic acid fraction shows less of a variety than did this fraction for *M. oeconomus*. Again, however, the cold-sensitive mice show a predominance of the shorter chain fatty acids, especially 14:0 and of 14:0 aldehyde.

It appears that the saturates are accounted for in almost equal proportions by both the cold-sensitive and cold-resistant mice for *M. pennsylvanicus*, while the 18 C-acclimated mice contain the largest amount of saturated fatty acids, as was the case for *M. oeconomus*. The cold-sensitive mice contain the largest amount of unsaturated fatty acids. The 18 C-acclimated mice for the species *M. pennsylvanicus* also show a high polyene content in their neutral lipid fraction as was found for *M. oeconomus*.

The results for the individual phospholipid and total neutral lipid fatty acid analyses for *Clethrionomus rutilus* are shown in Table IV. This species shows more variation than the previous two in the amount of saturation of its fatty acids for the individual phospholipid fractions. The saturated fatty acids are accounted for mainly by 16:0 and 18:0 while the unsaturated fatty acids show a predominance of 18:1, 18:2, 20:4 and 20:5. The diphosphatidyl glycerol fraction shows a high degree of unsaturation (65-75%) as was the case for *M. oeconomus*. Again the phosphatidic acid fraction of the cold-sensitive mice shows an elevation of 14:0.

The neutral lipid fraction for *C. rutilus* shows a high degree of unsaturation in its fatty acids as was the case for the other two species. A predominance of 18:1 and 18:2 accounts for most of the total unsaturation.

The 18 C-acclimated mice show a high degree of saturation in their fatty acids of the phospholipid fractions while the cold-sensitive mice show the highest degree of unsaturation, due to the high polyene content.

In the neutral lipid fraction of *C. rutilus*, the cold-sensitive mice contain the greatest amount of saturated fatty acids while the 18 C-acclimated mice contain the greatest amount of unsaturated fatty acids due to the high monoene content. The cold-sensitive mice, however, contain the greatest amount of polyenes in their neutral lipid fraction compared to the other two categories.

DISCUSSION

It has been suggested (5,6) that the degree of unsaturation of certain body fat increases during cold exposure. Irving (7) examined the skeletal muscle in the Alaskan Caribou and found that with progression to the distal portion of the leg, the percentage of unsaturated fatty acids increased. Williams and Platner (8) examined the fat content of the liver, white adipose tissue, and brown adipose tissue in cold-exposed animals. Their results indicate that cold-acclimation increases the relative level of unsaturation in white adipose tissue of both the rat and hamster and that this level of unsaturation is maintained during hibernation in the hamster. Brown adipose tissue showed no increase in unsaturation under these same conditions. These investigators also found that during the process of cold-acclimation, a relatively higher level of unsaturation occurs in the liver of the hamster but not in that of the rat. However, the increased level of unsaturation was not seen during hibernation in the hamster.

Simon (1) has examined the phospholipid distribution pattern in muscle of *M. oeconomus* and *C. rutilus* using the same criteria for animal stressing and categorization reported in this study. In a preliminary report he suggested that there is a species difference in response to cold exposure and that following acute cold stress the cold-resistant animals of some species (e.g., *C. rutilus*) show a higher diphosphatidyl glycerol content than the cold-sensitive animals of the same species. It has also been reported that following cold stress there was a decrease in most species in the total amount of phospholipid in cold-sensitive mice but that cold-resistant mice maintain or increase their muscle phospholipid content.

The results, therefore, are inconclusive as to whether cold-exposure increases the unsaturation of skeletal muscle neutral lipid. It is possible that what is being observed here is principally a species difference in response to cold-exposure. There is very little evidence to indicate a decreased capacity of the skeletal muscle to oxidize short-chain fatty acids as previously found in liver (9-11). It is interesting to note, however, that the phosphatidic acid fraction of all cold-sensitive mice showed a marked ele-

vation of 14:0.

The results of the fatty acid analyses for the phospholipid fractions show even more variance than the neutral lipid fatty acid analyses for the different species. In *M. oeconomus* and *C. rutilus*, the diphosphatidyl glycerol is from 65-75% unsaturated, with 18:2 accounting for 40-60% of this unsaturation. Similar results have been found for rat liver mitochondria (12) and beef heart mitochondria (13). On the other hand, *M. pennsylvanicus* shows a predominance of saturated fatty acids (50-80%, mostly 18:0) in its diphosphatidyl glycerol fraction.

It might be expected that the large increase in the 14:0 content of phosphatidic acid would be reflected in the 14:0 content of other glycerophospholipids. This is not the case however, and there are two possible explanations for this phenomenon: (a) there could be a block in utilization of 14:0-containing phosphatidic acid which might indicate an enzyme specificity limitation, or (b) the time of stressing being relatively short, the increase in 14:0 content of other phosphoglycerides is masked by the fatty acid composition of those glycerides that are present prior to stressing.

Either of two basic mechanisms may be responsible for the apparent increase in the 14:0 content of phosphatidic acid of all cold-sensitive mice: (a) the cold-sensitive animal has difficulty in metabolizing 14:0, and the animal returns this fatty acid to the fatty acid pool for resynthesis into glycerol lipids, or (b) the fatty acid synthesizing system tends to release more 14:0 under cold stress conditions instead of the usual 16:0 or 18:0 fatty acid. Further work is needed for clarification.

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Effect of Culture Temperature on Fatty Acid Composition of *Chlorella sorokiniana*[1]

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ABSTRACT

Chlorella sorokiniana was grown for extraction of fatty acids at seven temperatures ranging from 14 C to 38 C. The predominant fatty acids in *C. sorokiniana* grown at 38 C were saturated (46% of total); at 22 C, triunsaturated (40% of total); and at 14 C diunsaturated (47% of total). Increasing temperature resulted in an increase in the degree of unsaturation from 14 C to 22 C, but further increases in temperature always resulted in a decrease in unsaturation. At any point in the temperature range used, an increased temperature always resulted in fatty acids with a lower average chain length. Total fatty acid production was greatest at the extremes of temperature and lowest at 26 C. The chain length and degree of saturation of fatty acids increased at temperatures lower than 22 C. Therefore, the fatty acids of *C. sorokiniana* do not have an increasingly lower melting point when the culture temperature is reduced at temperatures 22 C or below.

INTRODUCTION

Environmental temperature is an important factor in the life of plants and animals. Several authors (1-3) have reported that animals respond to a lower environmental temperature by the accumulation of an increased percentage of unsaturated fatty acids in their body lipid. This is true in animals in controlled-diet experiments (4,5) as well as in those in which lipid content of the diet was not reported. The phenomenon of lower environmental temperature resulting in the presence of a greater percentage of unsaturated fatty acids is also apparent in microorganisms such as *Anacystis nidulans* (6), *Candida* sp. (7) and *E. coli* (8), although sometimes an opposite temperature effect is noted (9). Marr and Ingraham (8) studied the effect of temperature on fatty acid content at eight temperatures ranging from 10 C to 43 C, but most authors have chosen to compare data from only two temperatures. The value of such data is thus limited by the lack of more points for comparison. It is quite possible that the results could depend upon the level of the two temperatures chosen for study. Lewis (3) suggested that an increase in unsaturated fatty acids, in response to lower temperatures, could act to preserve protoplasmic viscosity in colder habitats. To determine whether a lipid mixture more resistant to solidification at low temperatures is being synthesized, we need to examine both the molecular weight of the fatty acids as well as the degree of unsaturation and not just the percentage of unsaturated fatty acids. To meet these requirements, *Chlorella sorokiniana* was chosen as a plant which would grow over a wide range of temperature. This paper reports the content of fatty acids in *C. sorokiniana* at seven temperatures ranging from 14 C to 38 C.

MATERIALS AND METHODS

Cells of *C. sorokiniana* (Shihira and Krauss) were grown in the dark in large test tubes containing 400 ml of growth medium which was bubbled with 1% CO_2 in air. The medium was composed of 1 g/liter KNO_3, 0.5 g/liter KH_2PO_4, 0.5 g/liter K_2HPO_4, 0.25 g/liter $MgSO_4 \cdot 7H_2O$, and the micronutrients described for *Chlorella* culture by Thomas and Krauss (10). Glucose at 0.5% provided the carbon source for heterotrophic growth. Temperature was controlled by a Forma controlled temperature bath and did not differ more than 0.5 C from the set temperature. *C. sorokiniana* was cultured at increasingly lower temperatures until growth ceased. The lowest temperature at which consistent and measurable growth occured was 14 C. Cells were grown at 18 C for extraction by taking an inoculum from the 14 C culture, and then making three or more transfers at 18 C. An inoculum from 18 C, was then used for inoculation at 22 C, etc. Cells were grown for extraction at seven temperatures at four-degree intervals from 14 C to 38 C. A culture was always harvested when its optical density reached 0.6 on a Coleman Junior Spectrophotometer (or a dry wt of 0.8-0.9 g/liter), since preliminary work showed some variation in fatty acid content with age of culture. At harvest, an

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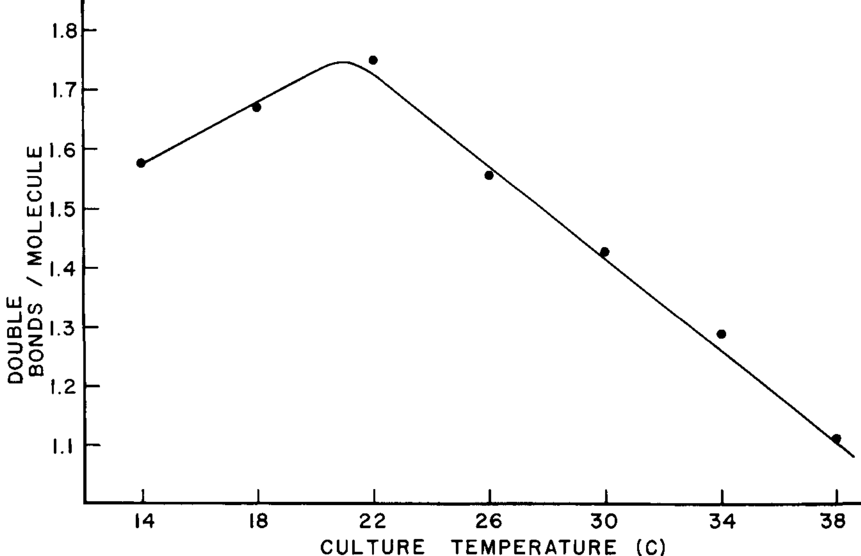

FIG. 1. Effect of culture temperature on the degree of unsaturation (double bonds per molecule) of *C. sorokiniana* fatty acids.

aliquot of cells was removed for dry weight determination. The remainder was centrifuged, resuspended in methanol, transferred with a pipette to an extraction thimble and extracted overnight with chloroform-methanol (2:1) in a Soxhlet apparatus.

Total lipid was determined by evaporating the solvent, resuspending the lipid in chloroform, and filtering the lipid into a weighted beaker to remove nonlipid particulate material. After evaporation of the chloroform, the total lipid weight was obtained. The lipid was saponified with a 100% excess of KOH in 80% ethanol, diluted threefold with water, acidified with HCl, and extracted overnight with diethyl ether in a liquid-liquid extraction apparatus. After evaporation of ether to dryness, the fatty acids were methylated by heating for 3 min with BCl_3-methanol reagent (Applied Science Labs). Fatty acid methyl esters were partitioned into hexane, and the hexane was evaporated to dryness under N_2. The sample was redissolved in hexane and transferred to a vial, leaving behind the last traces of BCl_3.

The fatty acid esters were analyzed by a Glowall model A-110 gas chromatograph. The operating conditions were: (a) column 1.8 m x 3.4 mm i.d., 15% HiEff lBP on Gas Chrom P, (Applied Science Labs) 20 psi, and 165 C; (b) argon ionization detector, 900 v, 200 C; and (c) flash heater, 205 C. The fatty acids were identified by thin layer chromatography on Silica Gel G, developed with 20% ether in hexane, and followed by gas chromatography using known fatty acids as standards. Quantitative data were obtained by measuring peak areas using a disc integrator.

RESULTS AND DISCUSSION

The major fatty acids of *C. sorokiniana* were identified as 16:0, 16:1, 16:2, 16:3, 18:0,

TABLE I

Effect of Culture Temperature on the Relative Proportions of Various Fatty Acids in *C. sorokiniana*

Culture temperature, C	Individual fatty acids as percentage of total fatty acids							
	16:0	16:1	16:2	16:3	18:0	18:1	18:2	18:3
14	21.7	3.3	10.8	10.3	0.5	4.7	36.4	11.6
18	26.1	3.1	7.9	12.8	0.3	4.9	29.5	15.2
22	29.3	2.4	4.2	16.9	0.3	3.8	19.7	23.3
26	33.1	2.6	6.0	13.4	1.0	4.0	20.9	18.3
30	37.5	2.7	6.8	11.5	1.2	4.3	20.1	15.8
34	39.2	3.4	7.7	9.6	2.6	4.4	21.0	11.8
38	40.7	4.4	10.2	5.4	5.3	4.7	21.3	7.5

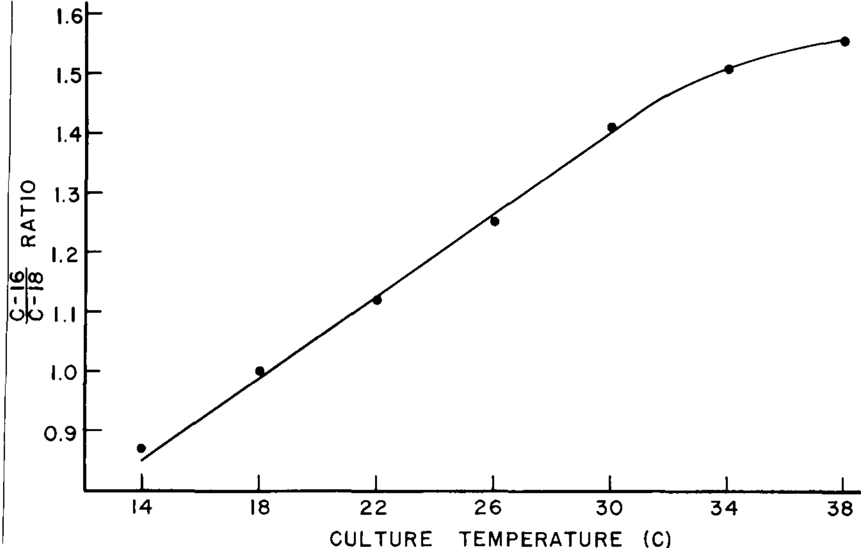

FIG. 2. Effect of culture temperature on the C_{16}/C_{18} ratio of *C. sorokiniana* fatty acids.

18:1, 18:2 and 18:3. Traces of 14:0 and other unidentified acids were detected, but they made up less than 1% of the total fatty acids and were not studied further.

Table I indicates that as the culture temperature increased, the saturated fatty acids made up an increased percentage of the total fatty acids. At the lower temperatures the percentage of triunsaturated acids also increased. These increases were balanced at the lower temperatures by a significant decrease in the proportion of diunsaturated acids. At temperatures above 22 C, the relative proportion of saturates increased, that of triunsaturates decreased and that of diunsaturates remained relatively constant. The proportion of monounsaturates remained essentially constant regardless of temperature.

It can be seen, then, that the predominant acids at 14 C were diunsaturated (47.2% of total), at 22 C were triunsaturated (40.2% of total) and at 38 C were saturated (46.0% of total). Figure 1 shows how the degree of unsaturation of *Chlorella* fatty acids changed with culture temperature. The degree of unsaturation was greatest at about 22 C. From this point there was apparently a linear decrease in unsaturation with either an increase or decrease in temperature. From this graph it can be understood how confusion in the literature could have arisen on the effect of temperature on the degree of unsaturation of fatty acids. If 14 C and 22 C were the temperatures selected for study, it would appear that increased temperature increased the degree of unsaturation. If 22 C and 30 C were selected, it would appear that increased temperature decreased the degree of unsaturation. Unfortunately, only two temperatures have been selected for study in many published works of this kind. The conclusions

TABLE II

The Effect of Culture Temperature on the Absolute Concentrations of Fatty Acids and Total Lipid in *C. sorokiniana*

Culture temperature, C	Per cent dry weight									
	Individual fatty acids								Total fatty acids	Total lipid
	16:0	16:1	16:2	16:3	18:0	18:1	18:2	18:2		
14	1.26	0.19	0.63	0.60	0.03	0.27	2.11	0.67	5.8	10.5
18	1.59	0.19	0.48	0.78	0.02	0.30	1.80	0.93	6.1	10.2
22	0.76	0.06	0.11	0.44	0.01	0.10	0.51	0.61	2.6	9.1
26	0.43	0.03	0.08	0.17	0.01	0.05	0.27	0.24	1.3	10.2
30	0.75	0.05	0.14	0.23	0.02	0.09	0.40	0.32	2.0	10.5
34	1.42	0.12	0.28	0.35	0.09	0.16	0.76	0.42	3.6	10.8
38	2.46	0.27	0.62	0.33	0.32	0.29	1.30	0.46	6.1	11.9

that can be drawn from these studies are thus limited.

It is apparent from Table I that the ratio of 16-carbon acids to 18-carbon acids must also be increasing with increasing temperature. Figure 2 shows that this increase was linear up to 30 C, at which temperature it began to level off.

Although data on relative proportions of various fatty acids are helpful, in order to get a true picture of the changes in fatty acid composition with changing temperature, one must also calculate the data on a dry weight basis to obtain absolute concentrations of fatty acids. Table II shows that when one goes from 38 C to lower temperatures, all fatty acids decrease in absolute concentrations. Changes in relative proportions of fatty acids are seen in Table I, because various fatty acid concentrations were decreasing at different rates. At 26 C all fatty acid concentrations reached their lowest point, and further decreases in temperature generally resulted in an increase in the content of all fatty acids.

The total fatty acids made up from 1.3% to 6.1% of the dry weight of the algal cell and the higher values were obtained at the extremes of temperature. The growth rate of *C. sorokiniana* is highest (about six doublings per day under conditions given) at 38 C and declines with lower temperatures. The total fatty acids made up only 13% of the total lipid at 26 C, but were 50% to 60% of the total lipid at the extremes of temperature. Since the total lipid was relatively constant at about 10% of the dry weight, some other component of the lipid fraction must be changing too. Since chlorophyll makes up a large portion of *Chlorella* lipid, changes in chlorophyll concentration are suspected.

It is clear that culture temperature is an important environmental factor affecting fatty acid content of *Chlorella*. The maximum degree of unsaturation of fatty acids was attained at 22 C with less unsaturation at lower or higher temperatures, although the proportion of total unsaturated fatty acids was always increased at lower temperatures (Table I). Chain length of fatty acids always decreased with increasing temperature within the limits used in this study. From the data presented here, it appears that fatty acid composition does change in a way which would tend to reduce protoplasmic viscosity as temperatures are reduced to 22 C. If temperatures are reduced below 22 C, the fatty acid composition changes in a direction which would tend to increase protoplasmic viscosity. However, these changes in viscosity could also be counterbalanced by changes in chlorophyll content or content of other lipids.

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Quantitative Determination of Glycerol, Mono- and Diglycerides by Gas Liquid Chromatography[1]

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ABSTRACT

Standard chemical procedures for the determination of glycerol, mono- and diglycerides in food products are lengthy, particularly when more than one component has to be determined in the same sample. Gas chromatographic techniques are applicable to these compounds when they are converted into their trimethylsilyl derivatives. A simple, rapid and quantitative gas chromatographic procedure has now been developed which is based on the addition of an internal standard (cholesteryl acetate) to samples which are subsequently silylated. This procedure has been applied to the direct determination of glycerol, mono- and diglycerides in emulsifiers, shortenings and other samples.

INTRODUCTION

Mono- and diglycerides (and mixtures thereof) are common emulsifiers used in shortenings, margarines and other foods. One of the most common procedures for the determination of monoglycerides in these mixtures is based on periodic acid titration (1) which determines the alpha monoglycerides by oxidation of adjacent hydroxyl groups. Beta monoglycerides are not oxidized by periodic acid because the hydroxyl groups are not adjacent. This procedure is an official AOCS Method of Analysis (2). It is quite lengthy and it is further lengthened when glycerol and monoglycerides are to be determined in the same sample.

On occasion, silicic acid column chromatography (3,4) is employed, followed by the gravimetric determination of the fractions eluted. This procedure is even lengthier than the periodate titration and considerable difficulties can be encountered in determining completeness of elution from the column and the weighing of small residues from large volumes of eluates. Both procedures are time consuming and neither satisfied our goal for a quick, simple and quantitative procedure that would determine glycerol, mono- and diglycerides simultaneously in the same sample.

Since 1965, a number of papers on the analysis of monoglycerides as trimethylsilyl (TMS) derivatives by gas liquid chromatography (GLC) have been published (5-14). Most of these reports have dealt with the determination of chain lengths and isomer distributions of fractions first separated by silicic acid column chromatography. Apart from a preliminary reference in a recent paper by Distler and Baur (15), the direct gas chromatographic analyses of mixtures such as the commercial products we will discuss have not been reported. We have now developed a simple, rapid and quantitative procedure for the direct GLC analysis of glycerol, mono- and diglycerides in emulsifiers, shortenings and other samples.

EXPERIMENTAL PROCEDURES

Materials

Hexamethyldisilazane (HMDS) and trimethylchlorosilane (TCS) were obtained from Supelco and glycerol, 99.45% pure, from Lever Brothers Company. Mono-, di- and triglycerides, 99+% pure, were obtained from the Hormel Institute and Supelco. Monoglycerides (Hormel) consisted of about 90% α-isomer; diglycerices (Hormel) consisted of a mixture of 1,3- and 1,2-isomers.

Pure 1,2- and 1,3-dimyristin (Supelco) were

TABLE I

Area-Weight Correction Factors (f) Relative to Cholesteryl Acetate

Sample	(f)	Standard deviation (N=25)
Glycerol	0.50	0.020
Monomyristin	0.84	0.022
Monopalmitin	0.90	0.017
Monostearin	0.85	0.020
Monoolein	0.87	0.043
Cholesteryl acetate	1.00	---
Dimyristin	1.01	0.018
Dipalmitin	1.06	0.022
Distearin	1.06	0.028
Diolein	1.06	0.031
Trimyristin	1.27	---
Tripalmitin	1.31	---

[1]Presented at the AOCS Spring Meeting, San Francisco, California, April 1969.

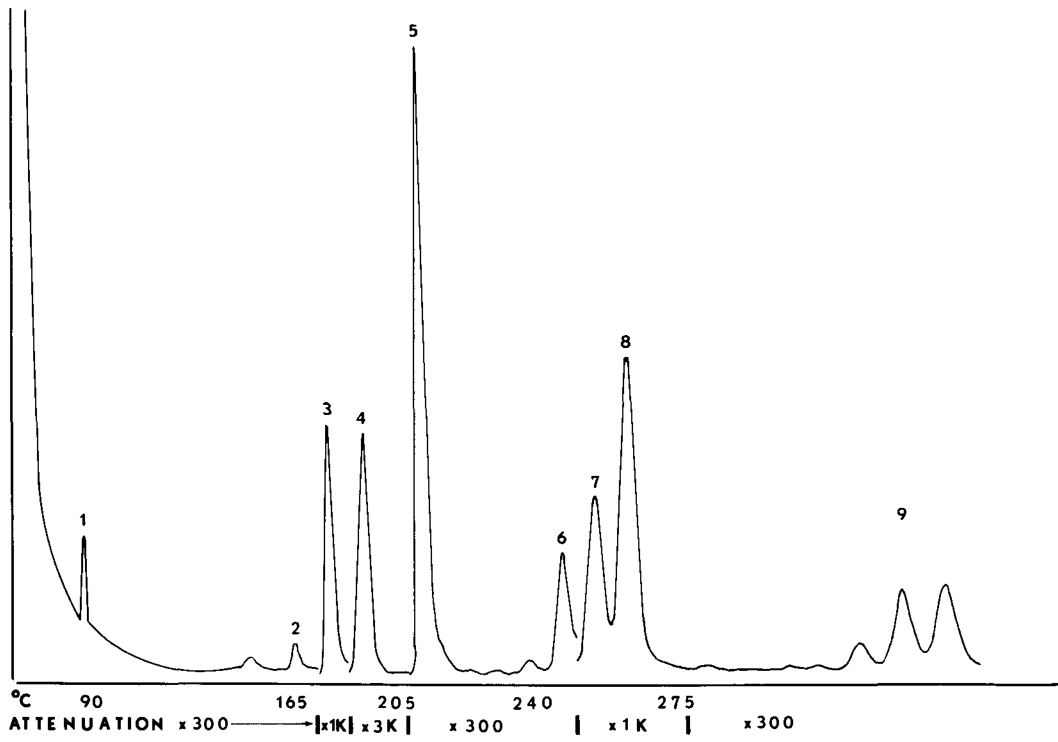

FIG. 1. Chromatogram of emulsifier A after silylation. 1: Glycerol; 2-4: C_{14}, C_{16} and C_{18} monoglycerides; 5: cholesteryl acetate; 6-8: C_{32}, C_{34} and C_{36} diglycerides; 9: triglycerides.

also run independently to determine their retention temperatures. Cholesteryl acetate, 99+% pure was obtained from Hormel. All these standards were used as received.

Gas Chromatography

A Barber Colman Model 5000 gas chromatograph, equipped with hydrogen flame detector and temperature programmer was used in this study. We used a glass column 1 ft x 4 mm i.d. (Glenco) packed with 3% OV-1 on Chromosorb W, 80-100 mesh. The columns were first cured overnight at 350 C before placing them in the chromatograph. These columns had an efficiency of 13,400 plates, as calculated from the cholesteryl acetate peak. The flash heater temperature was about 325 C; the detector was 300 C. Carrier flow rate was measured at 75 ml/min. The temperature program began at 50 C with a simultaneous sample injection: temperature rate, 12 C/min to 300 C. On reaching the end of the temperature program, the column was kept at this temperature until the triglycerides were eluted. Glycerol was eluted at 90 C; monoglycerides between 165 and 205 C; cholesteryl acetate, the internal standard, at about 210 C; and diglycerides between 240 and 275 C.

TABLE II

Analysis of a Known Mixture[a]

Sample	Amount taken mg	Peak area percent	(f)	Amount found mg
Glycerol	0.35	2.3	0.50	0.27
Monostearin	7.5	35.7	0.85	7.2
Cholesteryl acetate	7.0	30.2	1.00	(7.00)[b]
Dimyristin	7.5	31.8	1.01	7.4

[a]Milligrams found = f (area of component/area of internal standard) mg I.S.
[b]Assumed.

TABLE III

Precision of Analysis of Three Replicate Samples of Emulsifier A (Wt %)

Sample	GLC Procedure 1	2	3	Average	Silicic acid	Periodic acid
Glycerol	0.15	0.16	0.15	0.15	1.5	0.12
Monoglycerides	41.1	40.7	41.4	41.0	44.2	46.9
Diglycerides	41.6	41.2	42.2	41.6	43.7	NA[a]
Triglycerides	6.5	4.4	NA[a]	5.5	10.8	---

[a]Not available.

The silicone rubber septa used in our columns had a tendency to bleed. The material which bled from the septa in the high temperature flash heater area apparently condensed on the upper part of the column which is generally kept at lower temperatures. We noticed that the first run of the day, after the column had been standing overnight at 50 C, gave rise to a group of peaks resembling a homologous series of compounds, particularly between 170 and 230 C. These peaks were eluted when a column was taken through the temperature cycle even when there was no injection. Therefore at the beginning, we always began the workday with a blank run. We attempted to overcome this problem by using septa claimed by a supplier to be specially prepared to reduce bleeding. Use of these septa complicated the situation; they did not hold well to the glass wall of the column due apparently to their composition, and unexpectedly popped up when runs were performed, resulting in a shower of packing and the loss of the column. Finally we totally eliminated the problem by heating the septa overnight in a 280 C muffle furnace.

Detector Response Factors (f)

Calibration mixtures containing different ratios of glycerol, mono-, di and triglycerides vs. cholesteryl acetate were prepared by weight, silylated and chromatographed. For each component, the weight ratio (weight of component/weight of cholesteryl acetate) and area ratio (peak area of component/peak area of cholesteryl acetate) were calculated. The Detector Response Factors (f) were determined by dividing each weight ratio by the corresponding area ratio.

Procedure

The sample is accurately weighed in a 1 dr (approximately 4 cc) vial and 1 ml of internal standard solution, as defined later, is added. For emulsifiers, about 60-80 mg are weighed; for shortenings, between 180 and 200 mg. In both cases, a solution of 200 mg of cholesteryl acetate in 25 ml of pyridine is used as internal standard. For margarines and other products where the level of monoglycerides is expected to be low, about 350 mg of sample are weighed and a solution of 20 mg of cholesteryl acetate in 25 ml of pyridine is used as internal standard. A micromagnetic stirrer is placed in the vial and the sample dissolved with stirring. This process can be sped by placing the vial in a 30 ml beaker containing a few milliliters of lukewarm water. In a matter of seconds the sample is dissolved. Then, and in this order, 0.2 ml of HMDS and 0.1 ml of TCS are added with continuous stirring for 5 extra minutes. The

TABLE IV

Analysis of Emulsifiers by Two Procedures

Emulsifier	Glycerol GLC	Glycerol Periodic acid[a] Lab. 1	Glycerol Periodic acid[a] Lab. 2	Monoglycerides GLC	Monoglycerides Periodic acid[a] Lab. 1	Monoglycerides Periodic acid[a] Lab. 2
08[b]	0.80	0.73	---	90	93	---
18	0.10	0.06	0.12	42	47	45
20	0.13	0.08	0.11	48	47	44
25	0.22	0.22	0.34	43	42	47
26	0.10	0.08	0.12	41	46	44

[a]See Reference 2.

[b]This emulsifier was run in triplicate: glycerol 0.79, 0.81, 0.85; monoglycerides 91.0, 90.6, 89.3.

TABLE V

Recoveries Obtained on Adding Pure Glycerides and an Emulsifier to a Shortening

Sample	Added, mg	Found, mg	Recovery, percent
Pure standards			
Monostearin	7.9	8.1	102
Dimyristin	9.3	8.8	95
Commercial emulsifier			
Monoglycerides	9.5	9.0	95
Diglycerides	9.6	9.5	99
Average			97

micromagnetic stirrer is retrieved and a portion of the supernatant is injected into the gas chromatograph.

RESULTS

Nine calibration mixtures, each composed of four or five components, were prepared by weight, and Detector Response Factors (f) were determined. Table I shows the combined results obtained from all the runs for each compound assayed and the standard deviation of each value. These factors increase essentially with molecular weight or, better, extent of esterification.

To test the accuracy of the procedure, a sample containing glycerol, monostearin, cholesteryl acetate and dimyristin was prepared by weight. The results and calculations are shown in Table II. The first column shows the actual amounts of the four components taken. The next column gives the peak areas obtained and the last column, the amount found.

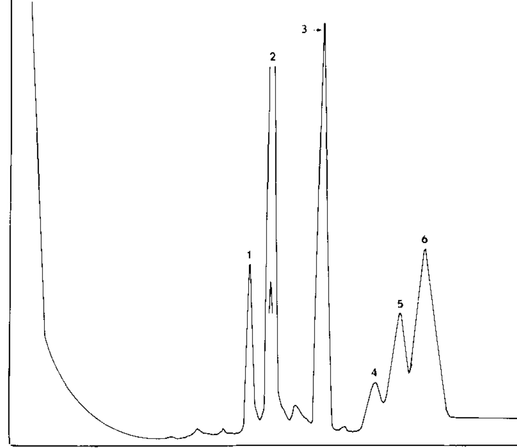

FIG. 2. Chromatogram of a commercial shortening after silylation. 1-2: C_{16} and C_{18} monoglycerides; 3: cholesteryl acetate; 4-6: C_{32}, C_{34} and C_{36} diglycerides.

Figure 1 is a chromatogram obtained from a commercial emulsifier A. All peaks were identified by comparison of their retention temperatures with those of known pure standards. For the middle peak in the diglyceride area (7), we did not have a standard with the same retention temperature. However, the diglyceride fraction of this emulsifier was isolated by silicic acid chromatography. Based on fatty acid analyses of this fraction, we assigned a C_{16}-C_{18} diglyceride structure (C_{34}) to the middle peak.

In our columns, resolution occurred according to chain length and not according to position isomers. This point is illustrated for instance in the peak of C_{18} monoglycerides which represents monostearin, monoolein and monolinolein, with the fatty acids in positions 1 or 2. The resolution of the 1- and 2-monoglyceride isomers, or the 1,2- from the 1,3-diglycerides, known to take place in longer columns, does not take place in our 1 ft columns. The particular column and conditions used in this investigation were selected to provide this separation according to chain length only. Separations other than these were not desirable since they would give rise to GLC curves more complicated and difficult to interpret.

The retention temperatures indicate that cholesteryl acetate is a very good internal standard. Its elution temperature is such that it is eluted immediately after the monoglycerides and a few degrees ahead of the diglycerides. Other internal standards could be used if other emulsifier mixtures are to be quantitated. For instance methyl stearate or stearyl alcohol could be used to advantage if only glycerol and monoglycerides are to be quantitated. Their retention temperatures are such that they peak at about 150-160 C, between the glycerol and monoglyceride peaks.

Emulsifier A was assayed in triplicate in order to obtain an estimate of the precision of the method. Table III shows the results obtained by our procedure. The individual values obtained are listed along with the

TABLE VI

Level of Monoglycerides (%) in Other Samples as Determined by Two Procedures

Sample	GLC	Periodic acid[a] Lab. 1	Periodic acid[a] Lab. 2	Literature
Commercial shortening	2.60[b]	2.8	2.9	
Refined soybean oil	0.13	NA[d]	NA[d]	0.12-0.47[c]
Margarine	0.19	NA[d]	NA[d]	

[a]See Reference 2.
[b]Average of three determinations.
[c]See Reference 16.
[d]Not available.

average. Also included in the table are the results obtained on the same emulsifier when silicic acid chromatography (3) and periodic acid titration (2) procedures were used.

The procedure was applied to five other commercial emulsifiers. The results obtained are shown in Table IV. All these emulsifiers were analyzed for glycerol and monoglycerides by the GLC and the periodic acid procedures. Two operators performed the periodic acid titrations at two different locations.

Figure 2 shows the chromatograms obtained when the procedure was applied to a commercial shortening. In addition to the C_{16} and C_{18} monoglycerides and the corresponding diglycerides, a number of small unidentified components are observed. Some of these overlap into the internal standard region but that can be corrected by making runs without internal standard. In this case, the correction was so small that it could be ignored.

Table V shows the results obtained when a recovery study was carried out. Known weights of pure glycerides were added to a blank shortening, containing very low levels of mono- and diglycerides. The level of addition was approximate to that found in commercial shortenings. In this experiment, recoveries were of the order of 95 to 102%. In another recovery experiment, a known weight of an emulsifier, previously analyzed, was added to the blank shortening. Again, the recoveries were good running from 95% to 99%.

Table VI shows the results obtained with shortenings when analyzed by the GLC and the periodic acid titration method. A refined soybean oil and a soybean oil-based margarine are also included in this table. No periodate results were available for the soybean oil or margarine samples but both results reported here are in the expected range of values.

Figure 3 is the chromatogram of the monoglyceride region of the soybean oil sample. A higher sensitivity was used in the instrument which accounts partially, for the higher baseline. The dotted outline indicates where the internal standard is eluted. A very similar chromatogram was obtained from a soybean oil-based margarine. The high level of other (unidentified) components eluted allows for only semiquantitative estimates of monoglycerides in such samples.

DISCUSSION

Two approaches can be used to obtain quantitative data by gas chromatography on samples such as we have described. The first is to quantitatively isolate a fraction by some

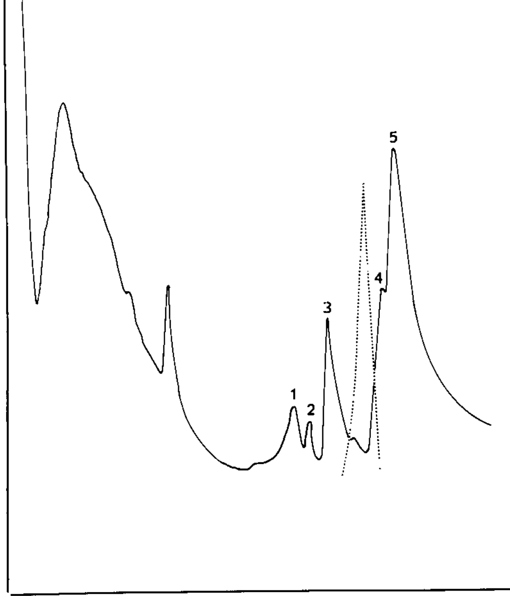

FIG. 3. Chromatogram of soybean oil after silylation. 1-3: monoglycerides; 4-5: diglycerides; dotted line: internal standard.

method such as silicic acid chromatography or thin layer chromatography (TLC) and then perform qualitative analysis on this fraction by gas chromatography. This approach has been used by other workers (6,7,9,11,13,14). It is, however, too lengthy for routine determinations of mono- and diglycerides in industrial samples.

The second approach, which we have followed, is to add a known quantity of an internal standard to the sample and compare the peak areas of the components of interest with that of the internal standard. Consequently, the flame detector response factors, relative to cholesteryl acetate, of glycerol, mono- and diglycerides were determined.

Their absolute values will have little value to other laboratories since it is generally recognized that calibration factors are dependent upon the sensitivity of the instrument and operating conditions and will, therefore, have to be calculated under the conditions prevailing in each laboratory.

The response factors obtained in our experimental conditions showed an increase in value according to the degree of substitution of the hydroxyl groups. Glycerol has the lowest value, indicating that the detector had a high response to this compound. The monoglycerides showed values in between glycerol and that of cholesteryl acetate. The slight intragroup differences according to chain length and unsaturation are probably not significant.

Excellent precision and recovery of glycerol, mono- and diglycerides were achieved. However, it will be noted that the GLC analysis of Emulsifier A only accounted for 88% of the sample. We believe that most of this discrepancy is due to errors in measuring the triglyceride levels, caused in part by volatilization problems on injection and in part by excessive column bleed at the relatively high temperatures required to elute these components. We therefore do not recommend this procedure for determining triglycerides levels.

In our work with margarines and oils, where the monoglyceride level is quite low, large injections were attempted to obtain a readable chromatogram. These large injection volumes and weights caused overloading of our short columns and the peaks were slanted and nonsymmetrical. We consequently reduced the volume of injection in order to avoid this tailing and increased the sensitivity of the instrument. Another difficulty with these samples was that a number of unknown peaks were noticed in the area where the internal peak should be eluted. Other components overlapped into the monoglyceride region. Consequently, under these circumstances only semiquantitative estimates of monoglycerides or emulsifier levels can be performed with these samples.

A simple and rapid procedure for the determination of glycerol, mono- and diglycerides in emulsifiers and shortenings has been developed. The data obtained showed good accuracy for samples where the monoglyceride level was at least 2%. Values obtained when the procedure was applied to oils and margarines do not have such a good accuracy. However, even in these cases, the procedure can be used for rough estimates of monoglycerides levels in these products.

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Cerebrosides of Rabbit and Pigeon Aorta: Isolation and Fatty Acid Distribution

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ABSTRACT

Quantitative determinations have been made on the cerebrosides isolated from aortas of cholesterol-fed and normal rabbits, and mature and young pigeons. The fatty acid distributions of these cerebrosides were determined. Cerebroside concentration was higher in pigeons than in rabbits and higher in the animals expected to have atherosclerosis. Comparing supposedly atherosclerotic animals to normal animals, the atherosclerotic animals generally had more unsaturated fatty acids and more 18:0 and 18:1 as compared to 16:0 and 16:1. These trends are consistent with fatty acid data from human aorta. Pigeon data were more similar to human data than were the rabbit data.

INTRODUCTION

Cerebrosides have recently been isolated from human aorta (1) and partially characterized. The cerebrosides from a diseased area of an aorta contained more unsaturated fatty acid than cerebrosides from a normal area of the same aorta. This difference was mostly expressed in the C_{18} acids as shown by the larger 18:0/18:1 for normal tissue and generally less 18:2 in the normal area.

It seemed desirable to see if cerebroside concentration or fatty acid distribution varied as an experimental animal developed atherosclerosis so that these variables might be investigated under more controlled conditions than possible using human tissue. This paper reports studies to this end using rabbits and pigeons.

MATERIALS AND METHODS

All reagents not specifically noted were analytical reagent grade. All solvents were distilled before use. Solutions are expressed by volume.

New Zealand *albino* male rabbits, weighing approximately 900 g each, were obtained from the Kuiper Rabbit Ranch (Gary, Ind.). The rabbits were divided into two groups, experimental and control, of 12 each. The dietary regimen used by Newman and Zilversmit (2), containing 1 g of cholesterol suspended in 2.8 g cottonseed oil per 100 g Wayne rabbit chow, was adopted for the experimental rabbits. The control rabbits received plain rabbit chow. All animals were fed water and chow ad lib. Six rabbits of each group were killed after two months. The entire aorta was removed and cleaned of adhering fat. Three aortas from one group were analyzed together, thus providing duplicate analyses for each diet at two months. The remaining animals were killed after three months and the aortas removed. Since only four control animals survived, the aortas were pooled and only one analysis was done. Five experimental animals survived and were divided in two groups (two and three animals).

The pigeons were all White Carneau. Young pigeons were six weeks old and were obtained from the Palmetto Pigeon Plant, Sumter, S.C. Each set of young pigeons consisted of six birds. The two sets were obtained at different times. Mature pigeons were a gift from The Upjohn Company, Kalamazoo, Mich. and were retired breeders from their colony. Each set of mature pigeons consisted of four birds. Sets 1 and 2 were obtained together and Set 3 at a later time. Plaques were very evident in the mature aortas. Pigeons were not separated into experimental and control groups, but were analyzed as young and mature animals. This strain of pigeon developed atherosclerosis spontaneously.

Lipids were extracted from the pooled whole aortas and cerebrosides isolated as reported previously (1). Preliminary experiments indicated that the cerebrosides of both the normal and cholesterol-fed rabbits contained no appreciable amount of fatty acid 22:1 while the pigeon preparations had no 20:0. Hence, methyl erucate and methyl arachidate (Applied Science Laboratories, State College, Pa.) were correspondingly employed as internal standards. A quantity of internal standard equivalent to approximately 8% of the estimated cerebroside weight was added to the purified cerebrosides. The amounts of cerebrosides were then calculated from the weight of the methyl esters.

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TABLE I

Concentration of Aortic Total Lipids and Cerebrosides From Rabbits and Pigeons

Animal	Aortas wet weight, g	Total lipid, % wet tissue	Cerebrosides, % lipid
Rabbit			
Two month			
Control 1	3.0	11.7	0.04
2	3.5	15.9	0.05
Cholesterol-fed 1	3.0	10.4	0.07
2	3.2	11.8	0.14
Three month			
Control	4.7	12.5	0.02
Cholesterol-fed 1	2.2	12.0	0.05
2	3.9	11.0	0.03
Pigeon			
Young 1	1.4	5.6	0.15
2	2.0	4.2	0.14
Mature 1	2.6	3.3	0.28
2	2.7	3.4	0.53
3	3.3	3.0	0.12

Methyl esters were prepared and analyzed by gas liquid chromatography as before (1). Duplicate chromatograms of esters had a relative error of 5% or less for major components (>5%), except for 22:0 of the first two-month rabbit control where the relative error was 14%. Standards KD and KF (Applied Science Laboratories, State College, Pa.) were chromatographed periodically. The composition of the standards corresponded to the stated value with a relative error of 5% or less. Plots of relative retention time versus carbon number were utilized in the identification of substances not represented in the standards. Area calculations were based on measurements of peak height and width at half the peak height.

RESULTS AND DISCUSSION

No fatty deposits were seen by gross examination in any of the aortas from the two month rabbits, the three month control rabbits or the young pigeons. The three month cholesterol-fed rabbit aortas had very few small whitish yellow deposits raised from the intima. The mature pigeon aortas had several small round yellow deposits in each aorta. These covered approximately 5% of the inner surface in each case.

The relative amounts of total lipid and the per cent of cerebrosides are given in Table I. There were no significant differences in the rabbit total lipid either with time or diet. The mature pigeons actually had slightly less total lipid per gram of aorta than did the young pigeons. Pigeons in general had only one third to one half as much lipid as the rabbits.

Cerebrosides were in greater concentration for the two-month cholesterol-fed rabbits than for the two-month control rabbits. At three months the cerebroside concentration was still greater in the cholesterol-fed group, but both groups showed a decrease and the difference was small. It appears that the normal trend for aortic cerebrosides is to decrease, at this age, and the cholesterol diet may cause a resistance to this decrease. In mature pigeons, which are naturally atherosclerotic, the cerebroside levels were in general higher than in rabbits. Also, two of the mature groups had much more cerebroside than the young pigeons. In general then, aortic cerebroside concentration is higher in animals with atherosclerosis. This appears to be in contrast to human aorta where the more atherosclerotic tissues had lower concentrations of cerebrosides (1). The apparent disagreement may be another feature of animals developing a different atherosclerosis than humans, or it may reflect the kinetics of plaque development. The human tissue was all fairly well advanced with atherosclerosis while the animals studied showed much less plaque formation, and the rabbit plaques were in a very early stage of development. The more advanced human plaques may have simply gone beyond the stages of the animals used and had deposited large quantities of the lipids which diluted the cerebrosides.

The normal fatty acid distributions of the rabbit aortic cerebrosides (Table II) were quite similar to those reported from human aortic cerebrosides (1). The fatty acid patterns have several characteristic features. There were

TABLE II

Percentage Composition of Normal Fatty Acids in Rabbit Aorta Cerebrosides

	Two months				Three months		
	Control		Experimental		Control	Experimental	
Fatty acid[a]	1	2	1	2	1	1	2
14:0	2.1	3.1	3.2	2.7	2.2	2.1	2.2
15:0	1.2	1.4	1.2	1.1	1.2	1.0	1.1
16:0	32.6	33.0	24.8	32.5	25.7	26.5	24.5
16:1	3.3	4.4	4.4	5.0	3.6	3.2	2.4
17:0	1.2	1.4	0.8	1.3	1.2	1.1	1.1
18:0	18.8	18.0	12.4	13.4	13.7	17.9	13.4
18:1	14.5	17.8	24.8	27.1	16.4	16.9	8.0
18:2	3.0	7.5	4.0	7.9	2.8	2.3	1.4
20:0	2.4	1.7	1.7	---	4.1	2.8	4.4
20:1	---	---	1.0	1.3	1.4	1.2	1.2
21:1	---	---	1.2	0.6	---	---	---
22:0	5.5	3.5	4.0	1.1	4.8	5.2	7.2
22:1	---	---	---	---	4.7	6.9	8.0
23:0	5.7	2.0	4.0	1.1	2.0	1.5	4.7
24:0	5.9	3.8	5.0	1.1	7.9	4.0	9.9
24:1	3.8	2.3	4.6	1.2	3.7	3.0	6.7
25:0	---	---	---	---	4.6	4.6	3.7
Total unsat.	24.6	32.0	42.9	45.8	32.6	33.5	27.7
18:0/18:1	1.30	1.01	0.50	0.49	0.84	1.06	1.68
$\frac{16:0+16:1}{18:0+18:1}$	1.08	1.04	0.78	0.93	0.97	0.85	1.26

[a]Traces ($<$1%) of 14:1, 15:1, 17:1 and 21:0 were present in the two-month experimental samples.

almost no hydroxy fatty acids. Of the normal acids, 16:0, 18:0 and 18:1 generally predominated in all samples and together constituted about 60% of the total normal acids. Most samples had little variation between the duplicates for the major acids. The three-month experimental Group 2 displayed a lower 18:1 value, and also had a rather high percentage of longer chain fatty acids (22:0, 22:1, 24:0 and 24:1). Pigeon aortic cerebrosides (Table III) had fatty acid distributions similar to the rabbit with somewhat greater variability among like

TABLE III

Percentage Composition of Normal Fatty Acids in Pigeon Aorta Cerebrosides

	Young		Mature		
Fatty acid	1	2	1	2	3
14:0	3.0	1.9	3.0	3.5	2.5
15:0	2.5	1.3	1.6	1.8	1.0
16:0	24.6	25.9	28.6	21.7	20.2
16:1	5.0	3.2	3.7	6.9	3.3
18:0	18.3	23.4	21.2	13.4	19.1
18:1	8.6	10.0	20.9	25.4	12.4
18:2	1.5	5.1	4.4	16.1	10.7
20:1	---	---	2.6	2.0	---
22:0	9.3	6.8	---	---	5.2
22:1	6.0	3.7	5.6	4.6	4.6
23:0	7.5	2.8	---	---	4.1
24:0	7.2	7.2	3.8	1.7	6.6
24:1	6.4	8.7	4.6	2.9	10.3
Total unsat.	27.5	30.7	41.8	57.9	41.3
18:0/18:1	2.13	2.34	1.01	0.53	1.54
$\frac{16:0+16:1}{18:0+18:1}$	1.10	0.87	0.77	0.74	0.75

samples.

Considering the pigeons and the two-month rabbits, two features of the fatty acid data are consistent when comparing a supposedly atherosclerotic animal with a normal. These two features are the total per cent of unsaturated fatty acids and the relative amounts of 16 carbon acids versus 18 carbon acids. The first of these features has been mentioned for human tissue (1). The second feature was not calculated previously, but is also valid for the human data.

Pigeons and two-month rabbits agree very well with respect to total unsaturation. Using averages, the mature pigeons had 160% as much and the experimental rabbits 157% as much unsaturated fatty acid as the young or controls, respectively. As with the human tissue, the greatest increase in unsaturation was present in the 18 carbon acids. Again the greater concentrations of 18:1 and, with some irregularity, 18:2 are seen in mature pigeons and two-month experimental rabbits.

A calculation of the ratio 16:0 + 16:1/18:0 + 18:1 (Tables II and III) shows that the mature or two-month experimental animals have more 18 carbon acids relative to 16 carbon acids than do the corresponding control animals. Calculations using data from human tissue (1) reveal the same relationship. In fact the lowered ratio was even more pronounced in the human tissue. While the ratio for the atherosclerotic animals was about 80% of the ratio for the control or young animals, the human tissue had ratios from diseased areas of aorta of only about 50% of the normal areas. The greater concentration of 18:0 and 18:1 acids in diseased tissue probably reflects an increased synthesis of fatty acids by the chain elongation mechanism (3).

In general, the fatty acid composition in cerebrosides from the three-month experimental rabbits appeared to be similar to those rabbits fed the normal diet, in contrast to the differences observed with the younger rabbits. The amount of cerebroside was still higher after three months of atherogenic diet, but the total concentration of unsaturated fatty acids was at the level of rabbits fed the normal diet. Group 1 experimental rabbits had a somewhat low (16:0 + 16:1)/(18:0 + 18:1) ratio, but the Group 2 ratio was high. However, Group 2 had considerably more fatty acids of 20 or more carbons than did either of the other three-month groups. Chain elongation may have been accelerated in these rabbits but continued past 18 carbons to a greater extent than in other animals studied.

The similarities of the pigeon data to the human data are consistent with earlier observations which found that the lesions of pigeon atherosclerosis resemble rather closely those of human atherosclerosis (4).

Since the rabbit is known to be resistant to atherosclerosis, the data suggest that a rabbit has a significant capability to adjust its metabolism and thus, after some delay, partially return to normal cerebroside quantity and fatty acid distribution.

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Desaturation of Saturated Fatty Acids by Rat Liver Microsomes

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ABSTRACT

A method was developed for the rapid determination of the initial velocity of the desaturation of saturated fatty acids. In the reaction, DPNH was a more efficient electron donor than TPNH. Fat-deficient rats have a 2.5-fold greater level of acyl desaturase per milligram of liver microsomal protein than did animals fed lab chow. Increasing the chain length of the acyl substrate from 10:0 to 18:0 increases the rate of monoene formation, but 19:0 is desaturated at a rate lower than that for 15:0. The energy of activation (Ea) for the overall desaturation reaction has been determined for 12:0 through 19:0. The Ea values for desaturation of 13:0 and 16:0 are markedly lower than for the other acids. An interaction between the alkyl chain of the substrate and polyunsaturated acids of the microsomal membrane-bound phospholipids is postulated to explain the recurring 3-carbon pattern of the relative reaction rates of the various acyl substrates.

INTRODUCTION

Studies of acyl desaturase in normal rat liver microsomes have focused on the desaturation of stearate and stearyl CoA. Oshino et al. (1) and Jones et al. (2) have reported methods for the study of the initial velocity of desaturase in liver microsomal systems using stearyl CoA. Other workers have investigated the desaturation of free stearate (3,4) and the saturated series 12:0-20:0 (5), apparently at equilibrium. Conclusions concerning the specificity and specific activity of acyl desaturase from non-first order reaction conditions (3-5) are open to question. Incorporation of substrate into lipids lowers the concentration of available substrate when low concentrations such as 10 to 20 μmolar are employed. Inhibition of the substrate desaturation by endogenous microsomal fatty acids has been demonstrated (4). Dilution of substrate by endogenous fatty acids of the same type would lead to false values. These problems can be best corrected by selecting a system in which the quantity of microsomes is low so as to provide a minimal contribution of endogenous fatty acid. The substrate concentration should be high enough to saturate the enzyme system, and the reaction rate should be proportional to protein concentration.

For this reason we developed an assay system for the measurement of the initial velocities of acyl desaturase from rat liver microsomes. The assay is linear with respect to time, directly proportional to protein concentration, and independent of substrate concentration over a threefold range above the point of saturation of the enzyme by substrate. Using this method, specific activities, substrate specificities, Arrhenius plots, and the energies of activation of the acyl desaturase reaction were determined. A novel hypothesis to explain the relative rates of desaturation of 10:0 through 18:0 is proposed.

Chemicals

The carboxyl-labeled acids, 12:0, 14:0, 16:0 and 18:0 were purchased from New England Nuclear Corp., Boston. The acids 15:0, 17:0 and 19:0, were synthesized in this laboratory by the method of Baumann and Mangold (6). Final purification of these odd-chain fatty acids was carried out according to the preparative GLC procedure of Schlenk and Sand (7). The acids 11:0 and 13:0, were purchased from Mallinckrodt Nuclear, St. Louis. The radio and chemical purity of all acids was found to be >98% by thin layer (TLC) and gas liquid chromatography (GLC). Nonlabeled fatty acids used as diluents and starting materials for syntheses were purchased from the Lipids Preparation Laboratory of The Hormel Institute, Austin, Minn. DPNH, TPNH, ATP and CoA were purchased from both Sigma Chemical Co., St. Louis, and P & L Biochemicals, Milwaukee, Wis. All other chemicals were of reagent grade quality and all solvents were redistilled.

Preparation of Microsomes

Male albino rats were maintained on either commercial lab chow or a synthetic diet containing no fat (8). The rats were killed under ether anesthesia by exsanguination; the livers were removed and placed in an ice cold solution of 250 mM sucrose and 5 mM $MgCl_2$. After weighing, the livers were minced and gently homogenized in 2 vol of sucrose-$MgCl_2$ (w/v).

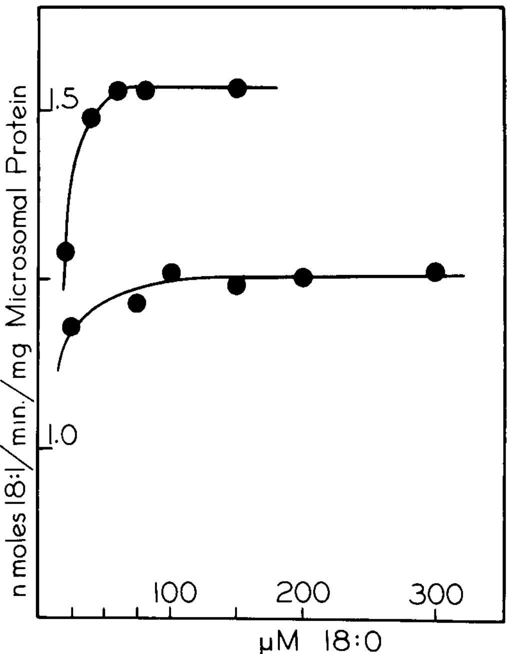


FIG. 1. The relationship of initial velocity to substrate concentration. The assay was performed at 40 C using two different microsomal preparations obtained from two EFA-deficient rat livers.

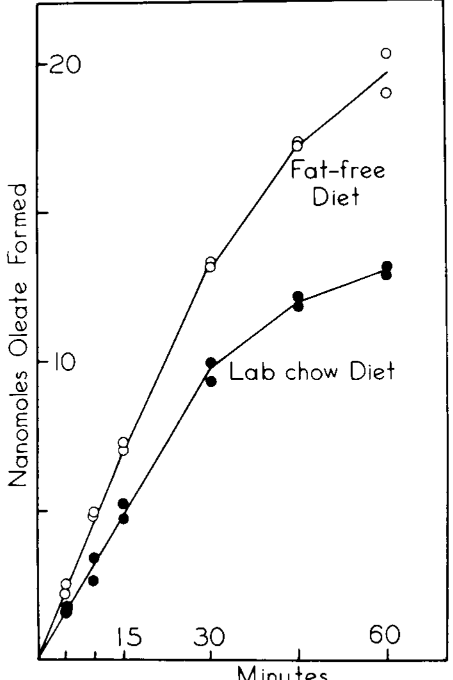


FIG. 2. The desaturation of stearate as a function of time by microsomes from rats fed fat-free diet and lab chow diet.

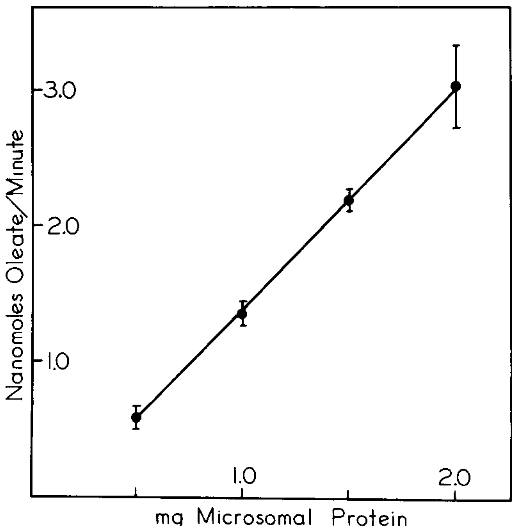


FIG. 3. The desaturation of stearate as a function of microsomal protein concentration. Microsomes were obtained from EFA-deficient rat livers. The bars represent the range of four determinations.

The homogenate was centrifuged for 30 min at 15,000 x *g* at 0 C. The supernatant fluid was decanted into another set of 50 ml tubes and centrifuged at 50,000 x *g* for 2 hr. The resulting microsomal pellet was suspended in sucrose-$MgCl_2$ solution in a volume equivalent to the initial liver weight. Aliquots of the microsomal suspension were placed in screw cap tubes, flushed with N_2, capped and quick-frozen in dry ice-acetone. The preparations were stored at -20 C and were found to be stable for at least one month. Neither the supernatant nor the pellet of material sedimenting between 50,000 and 100,000 x *g* contained any desaturase activity according to the assay described below. Repetitive freezing and thawing, up to four times, had no significant effect on the enzymatic activity.

Enzyme Assay

The stored microsomes were thawed in an ice cold water bath as needed. Protein levels were measured by the biuret procedure (9). Appropriate dilutions of the microsomes were made with sucrose-$MgCl_2$ to yield 2.5 mg/ml of which 0.4 ml was used for each assay. In a total volume of 1 ml, the final incubation mixture contained 1.0 mg microsomal protein, 2.5 mM $MgCl_2$, 5 mM ATP, 0.25 mM CoA, 1 mM DPNH, 0.1 M sodium phosphate, 0.125 M sucrose, and 0.15 mM 1-^{14}C-labeled fatty acid (1.0 mc/mmole) prepared as previously described (10) at a pH of 7.0. The incubations were performed in 5 ml test tubes in a thermo-

TABLE I

Specificity Toward Reduced Pyridine Nucleotide

DPNH[a]	TPNH[a,b]
1.12	0.78
1.15	0.79
1.04	0.77
0.98	0.73
1.07[c] ± 0.077[d]	0.77 ± 0.026

[a]Nanomoles of oleate/min/mg microsomal protein.

[b]TPNH was added in the same molar quantity as DPNH.

[c]The means are significantly different ($p < 0.05$).

[d]Mean ± S.D. of four determinations from pooled liver microsomes from four EFA-deficient rats.

TABLE II

Desaturase Activity

Fat free diet[a]	Lab chow diet[a]
1.69	0.37
1.04	0.28
0.89	0.44
0.90	0.72
1.51	0.20
0.84	0.40
0.78	
1.09[b] ± 0.33[c]	0.40 ± 0.16

[a]Nanomoles of oleate/min/mg microsomal protein. Each value is the average of triplicate determinations from a single rat liver microsomal preparation.

[b]Means are significantly different ($p < 0.01$).

[c]Mean ± S.D.

regulated shaking water bath. The temperature of the bath was 40 C unless otherwise indicated since this was found to be very near the optimum for all the acids of the series. The reaction was terminated by the addition of 1.0 ml of 5% methanolic HCl. One half milligram of an equal mixture of 16:0 and 16:1 was added to serve as carrier. The total lipids were extracted by the method of Bligh and Dyer (11). The lipid extract was dried under a stream of N_2 with slight warming and then dissolved in 0.1 ml of $CHCl_3$-methanol (2:1 v/v) and esterified according to the method of Glass and Christopherson (12). An aliquot of the methyl esters in $CHCl_3$ was separated on glass fiber sheets impregnated with silicic acid (ITLC Type SA, Gelman Instrument Co., Ann Arbor) previously dipped in a 1% solution of $AgNO_3$ in methanol according to the method of Graff et al. (13). With the aid of 2,7-dichlorofluorescein, the bands of the saturated and monounsaturated esters were made visible, marked, cut from the paper and placed in scintillation vials. The vials were filled with 15 ml of a toluene-based scintillation solution containing 5.5 g of Permablend III (Packard Instruments Co., Downers Grove, Ill.) per liter. The radioactivity was determined in a Packard liquid scintillation spectrometer. The values for desaturation were first calculated as percentages and then converted to nmoles/min/mg of protein by use of appropriate factors.

RESULTS AND DISCUSSION

The assay described above is coupled to acyl activation of the free acid to the CoA derivative. It was necessary, therefore, to demonstrate that desaturation rather than acyl activation was the rate limiting step. In our system, we found the value for acyl desaturation by normal rat liver microsomes to be 0.40 nmoles/min/mg which compares favorably with the value of 0.35-0.40 nmoles/min/mg for the desaturation of stearyl CoA (1). This indicates that the CoA derivative was generated in situ at a rate sufficiently rapid not to be the limiting factor. Indeed, the data of Pande and Mead (14) and of Graff and Holman (unpublished results) for the activation of 10:0, 12:0, 14:0, 16:0 and 18:0 to the CoA derivative suggest that the rate of acyl activation is 10 to 80 times that of desaturation. Nakagawa and Uchiyama (15) found the rates of acyl CoA formation from 16:0 and 18:0 to be nearly identical and therefore not responsible for the difference in the rates of desaturation of the two acids. From these data we conclude that acyl desaturation, not activation, is the rate limiting step in our assay, and we assume that the same holds true for the odd-chain length fatty acids tested.

Figure 1 reveals the relationship of initial velocity to substrate concentration. The rate is independent of substrate concentration from 100 to 300 μM 18:0. With minor variation this was found to be true for the other acids. The assays of initial rates at low substrate concentrations were not studied because of an unknown and uncontrollable level of endogenous free fatty acids. If the concentration of endogenous fatty acids were large in comparison to the added substrate, Michaelis constants derived from 1/[V] vs. 1/[S] would give anomalous results.

Figure 2 demonstrates the linearity of the assay of desaturation of 18:0 to 18:1 by microsomes from rats fed fat-free and lab chow diets. Although the rates were linear for all acids for 30 min, the data presented in the balance of this work were obtained from incubation times

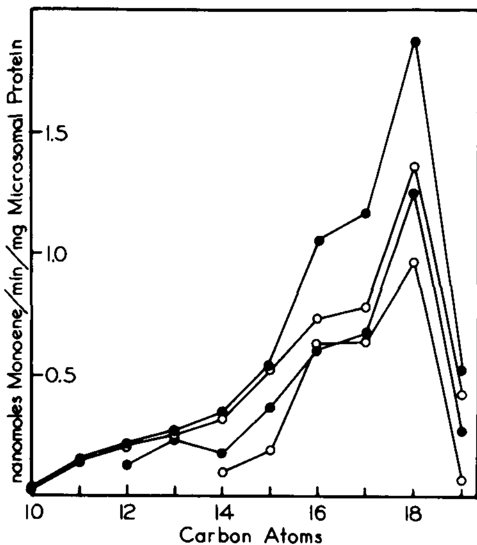


FIG. 4. The substrate specificity of desaturation as measured at 40 C in four separate microsomal preparations.

of 15 min for acids of 14 to 19 carbon atoms. For the acids 10:0 to 13:0, the reaction was linear to at least 1 hr. Longer incubation times were used when studying these acids in order to have sufficient monoene to be accurately determined. For the same reason, i.e., higher monoene production, EFA deficient rats were used for the balance of the work reported in this paper.

The rate of desaturation of 18:0 is directly proportional to the amount of added microsomal protein over the range of 0.5 to 2.0 mg shown in Figure 3. These data, together with those in Figures 1 and 2, clearly show that the assay described here gives a true measure of initial velocity under pseudo first order reaction conditions.

Oshino et al. (1) found DPNH to be more efficient than TPNH as a hydrogen (electron) donor for acyl desaturation. Table I shows that the same is true in our assay system.

The influence of diet on the level of acyl desaturase in liver microsomes is shown in Figure 2 and Table II. The maintenance of rats on a fat-free diet results in a significant increase in this enzymatic activity compared with normals maintained on lab chow. The activity does not vary significantly with the length of time on the fat-free diet from seven days to 18 months. Starvation of the fat deficient rats for 24 hr reduced the level of acyl desaturase below that of the controls. This is in agreement with the data of Uchiyama et al. (4).

Figure 4 presents the findings for the

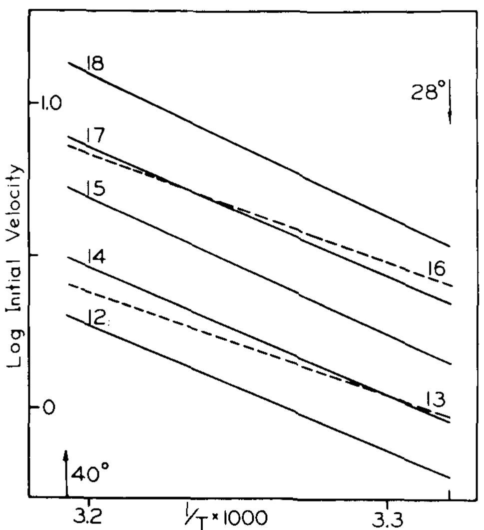
TABLE III

Energies of Activation for the Desaturation of 12:0 Through 19:0

Fatty Acid	Kcal/mole
12:0	18.8
13:0	15.5
14:0	19.2
15:0	20.8
16:0	16.3
17:0	19.8
18:0	21.8
19:0	24.1

specificity of acyl desaturase toward the saturated fatty acid series, 10:0 to 19:0. Each of the curves represents a different microsomal preparation obtained from the livers of EFA-deficient rats. It is clear that desaturase activity increases with increasing carbon number from 10:0 through 18:0. Desaturation of 19:0 occurs to a lesser extent than 15:0 in all cases. Possibly 19:0 is too long for the enzymatic site. Of the acids tested, 18:0 is clearly the best substrate for desaturation. This is consistent with the fact that 18:1ω9 is the predominant monoene in the rat. In fact, the mass per cent of 18:1 exceeds that of 18:0 in triglycerides and phospholipids. This is not the case for 16:1/16:0 (16). The results for desaturation of 18:0 and 16:0 are in disagreement with the data of Nakagawa and Uchiyama (15). The difference in results may be due to the non-first order assay conditions which they used. Their substrate concentration (12 μM) is well below the optimum for our system (>100 μM). The system described by Johnson et al. (5) used hen liver as the source of microsomes. In their system, the desaturation of 14:0 was greater than or nearly equal to that of 18:0 and greater than all other acids tested. Their system employed very little substrate and an undefined level of protein, and no evidence was given that their system was pseudo first order. It is not clear whether their data represents the rate of desaturation or the equilibrium attained for each acid. This uncertainty precludes making a decision as to whether their is a true phylogenetic difference in acyl desaturase specificity.

Rate studies performed as a function of temperature (28 to 40 C) were graphed in the form of Arrhenius plots ($\log_{10}$ velocity versus 1/T absolute). The slopes of the lines were determined by the formula of the least squares using 30 to 60 data points for each acid. Figure 5 presents the results of these determinations. According to the Arrhenius equation in which $\log_{10}$ has been substituted for ln, the product of the slope and 2.303 R when the value of R is



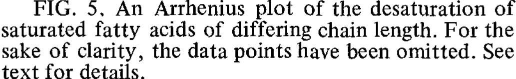


FIG. 5. An Arrhenius plot of the desaturation of saturated fatty acids of differing chain length. For the sake of clarity, the data points have been omitted. See text for details.

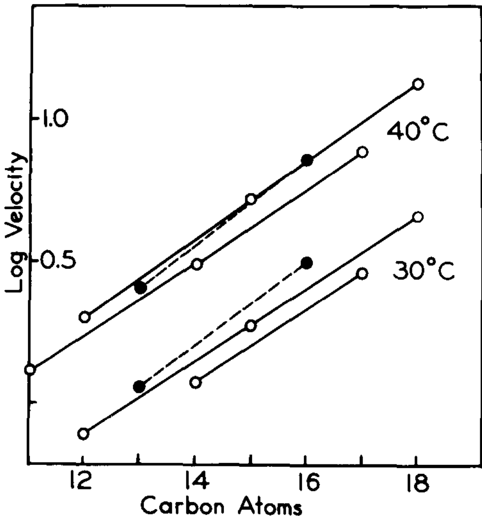


FIG. 6. The logarithm of velocity of desaturation versus substrate chain length as determined at two temperatures.

1.986 kcal/mole gives the energy of activation (Ea) for the overall reaction. Table III contains the results of these calculations. Despite the large differences in the rates of desaturation of the various acids, the energies of activation for the desaturase reaction are similar for 12:0, 14:0, 15:0, 17:0 and 18:0. The Ea values may be similar because the reaction produces $\Delta 9$ double bonds regardless of the acyl chain length (5). This implies that the rate of the overall reaction is dependent on some event other than the catalytic event leading to the formation of the double bond, assuming that the catalytic event has the highest energy of activation. The rate of desaturation may be controlled by the rate of substrate binding or of product release.

Fatty acids of chain length 13, 16 and 19 carbon atoms have greatly different Ea values. The Ea value for 19:0 → 19:1, which is much higher than that for other acids tested, is accompanied by a sharply lower rate of desaturation. On the other hand, the Ea values for the reactions 16:0 → 16:1 and 13:0 → 13:1 are lower than the other acids in the series but are not accompanied by sharply higher rates of desaturation. That is, the increments of one carbon atom beyond 12 do not yield simple proportional increments in Ea values. Chain lengths of 13:0, 16:0 and 19:0 seem to possess some unique characteristic in the reaction.

Figure 6 is a plot of the logarithm of velocity of desaturation versus the substrate carbon number for two different temperatures. For each temperature, the acids 12:0, 15:0 and 18:0 fall on a straight line; whereas, the values for 11:0, 14:0 and 17:0 form a line slightly below that of the former. As would be expected from their Ea values (Fig. 5 and Table III), the points for 13:0 and 16:0 shift their positions with respect to the other acids as a function of temperature. The same phenomenon was observed consistently for three preparations of microsomes from animals of different ages. These data suggest that the saturated fatty acids fall into three groups, and that the members of each group differ by three carbon atoms. The Arrhenius plots for desaturation of 13:0 and 16:0 likewise indicate that these acids are quite different from the other acids.

When the data of Abou-Issa and Cleland (17) for the acylation of α-glycerolphosphate by saturated acyl CoA thioesters are plotted as the logarithm of velocity versus carbon number of the substrate, a simple straight line relationship is found. This is in contrast to the 3-carbon rhythm of specificity in the desaturation of saturated acids. We speculate that this rhythm in reactivity shown by 11, 14, 17 vs. 12, 15, 18 vs. 13, 16 is the result of interaction of the alkyl chain with a binding site in a highly directed fashion, and that the binding site should itself possess a repeating 3 atom sequence. Binding an alkyl chain to the relatively polar peptide sequence which has a repeating C-C-N sequence, seems thermodynamically unlikely. Interaction of alkyl chains with nonpolar side chains of amino acids

is possible, but no 3 atom sequence is readily apparent. The desaturation of stearyl CoA is known to cease when the lipid components of the microsomes are partially removed, suggesting the role of lipid as a cofactor (2). Most of the microsomal lipid is phospholipid which contains a high proportion of polyunsaturated acids with a repeating 3 carbon unit:

$$\overset{2}{-CH_2}-\overset{3}{CH}=CH-\overset{1}{CH_2}-\overset{2}{CH}=\overset{3}{CH}-\overset{1}{CH_2}-\overset{2}{CH}=\overset{3}{CH}-CH_2-$$

The activated methylene groups (labeled 1) could explain a repeating 3 carbon rhythm of reactivities, if substrates were bound to a site containing one or more such systems. In contrast, the linear pattern in the reaction of saturated acyl CoA thioesters with α-glycerolphosphate suggests that in this case interaction of substrate is with saturated acyl groups in lipids of microsomes. Because microsomes carry such a large proportion of phospholipids, the association of the acyl CoA with the microsomal surface may be in the one case with the position 2 fatty acids, and in the other with the position 1 fatty acids of the phospholipids.

ACKNOWLEDGMENT

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Fatty Acid Metabolism in *Drosophila melanogaster*: II. Metabolic Origin of Monoenes

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ABSTRACT

Evidence is presented that *Drosophila* larvae produce monounsaturated fatty acids by two independent pathways. One of these pathways, involving the direct desaturation of long chain precursors, is sensitive to inhibition by linoleate. The other pathway is resistant to linoleate inhibition and probably has tetradecenoate-Δ^5 *cis* and tetradecenoate-Δ^7 *cis* as intermediates in the synthesis of palmitoleate and oleate, respectively. The use of radioactive precursors of varying chain length and labeled at different positions in the carbon chains provided evidence for the isolation and structure of intermediates involved in the linoleate resistant pathway.

INTRODUCTION

It is well-known that *E. coli* and many other bacteria synthesize unsaturated fatty acids by forming an intermediate chain length unsaturate and subsequently chain elongating that intermediate (1,2). Yeast (3), insects (4-6) and mammals, on the other hand, form monoenoic fatty acids by the direct desaturation of the saturated homologues. Yeast and other fungi can probably form monoenes by no other route. However, in other eukaryoates, Donaldson (7-9) employing quail homogenates, Raju and Reiser (10,11) using material from rats and Keith (12) with axenic cultures of *Drosophila melanogaster* larvae demonstrated that an alternate pathway was responsible for oleate synthesis when the direct desaturation of stearate was blocked. Raju and Reiser (11) have further shown that 1-^{14}C-laurate or some other intermediate chain length component was probably desaturated and then elongated to oleate. A route of this type would be similar to the bacterial mechanism for monoene synthesis.

The present report attempts to establish the nature of intermediates involved in forming palmitoleate and oleate in axenic cultures of *Drosophila* larvae when the direct desaturation of the saturated homologues (i.e., palmitate and stearate, respectively) is inhibited by high concentrations of dietary linoleate.

MATERIALS AND METHODS

The methods and culture conditions employed for the study of in vivo fatty acid synthesis in *Drosophila* larvae have been detailed in an earlier report (12). An isogenic strain (alpha-i) of Oregon-R *Drosophila melanogaster* was cultured on semisynthetic medium under axenic conditions and 84-hour-old larvae (third instar) were collected for fatty acid analyses. Dietary linoleate used as an inhibitor of direct desaturation was purified to greater than 98% purity as determined by analytical gas liquid chromatography (GLC).

Lipid extractions followed the Folch procedure (13). Saponification was carried out in dilute methanolic KOH and methylation in dry methanolic HCl as described by Bottcher et al. (14). Fatty acid methyl esters were analyzed by analytical GLC or collected by preparative GLC for isotope analysis by scintillation spectroscopy. Analytical GLC was conducted on a Perkin-Elmer Model 800 gas chromatograph equipped with a stainless steel 8 ft x 1/8 in. analytical column packed with acid washed 60/80 mesh Chromosorb W coated with 15% diethyleneglycol succinate (DEGS) at a column temperature of 160 C. Preparative GLC was carried out on a Varian Aerograph Model 700 preparative gas chromatograph equipped with a 20 ft x 1/4 in. copper column packed with acid washed 60/80 mesh Chromosorb W and coated with 18% DEGS at a column temperature of 150 C.

Radiotracers 9,10-^{3}H-palmitate, 9,10-^{3}H-oleate, 2-^{14}C-malonate, 1-^{14}C-butyrate, 1-^{14}C-laurate, 1-^{14}C-myristate, 1-^{14}C-stearate, 1-^{14}C-caproate, 1-^{14}C-capryolate, 1-^{14}C-caprate were purchased from New England Nuclear, Boston, Mass., and ^{3}H-acetate and 1-^{14}C-palmitate were purchased from Nuclear Chicago, Des Plaines, Ill. All radiochemicals were verified to be at least 98% pure on analysis. Simultaneous ^{3}H and ^{14}C analyses were conducted on a Beckman liquid scintillation spectrometer equipped with an external standard for quench correction. Simultaneous ^{3}H and ^{14}C analyses were resolved when necessary according to the method of Klein and Eisler (15) or simply by adjusting the gain so that negligible ^{3}H counts appeared in the ^{14}C channel. A carrier methyl ester mixture pre-

TABLE I

Distribution of ^{3}H From Dietary 9,10-^{3}H-Palmitate and 9,10-^{3}H-Oleate Into the Fatty Acids of *Drosophila* Larvae

Radioactivity recovered in:	Radioactive fatty acid supplement			
	9,10-^{3}H-Palmitate		9,10-^{3}H-Oleate	
	Counts (dis/min)	Per cent	Counts (dis/min)	Per cent
14:0	35,213.2	6.6	5,520.2	0.6
14:1a	2,806.0	0.5	65,273.8	7.1
14:1b	6,685.8	1.3	2,980.7	0.3
14:1c	411.0	0.1	117.0	0.01
16:0	200,108.6	37.7	4,816.0	0.5
16:1	145,966.3	27.5	44,338.6	4.8
18:0	5,493.6	1.0	1,321.7	0.1
18:1	134,587.4	25.3	796,197.1	86.5

pared from *Drosophila* larvae was collected after each GLC analysis and served as the standard background count for each tracer-containing component.

Highly purified 14:1 Δ^9 *cis* (Applied Science Laboratories, State College, Pa.) and 14:1 Δ^5 *cis* (Hormel Institute, Austin, Minn.) were used as standard retention markers in analytical GLC.

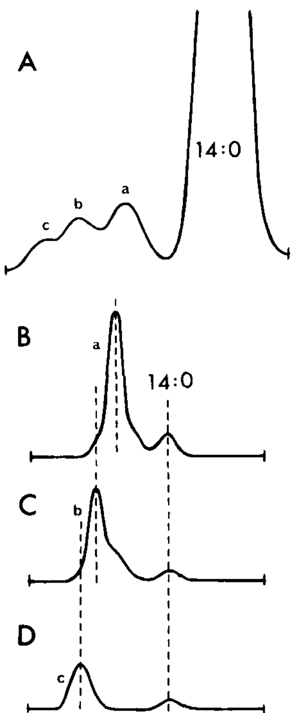

FIG. 1. Separation of the complex 14:1 peaks. A, tracing from a preparative GLC analysis at reduced temperature (150 C); B, C and D, isolation of 14:1 peaks, designated a, b and c, after repeated preparative collections and reinjection into a Perkin-Elmer, Model 800, gas chromatograph equipped with a stainless steel analytical column 8 ft x 1/8 in. packed with Chromosorb W and coated with 15% diethyleneglycol succinate at a temperature of 160 C. A small amount of myristate was added as a retention marker.

RESULTS

For some years we have observed that the GLC spectra of *Drosophila* fatty acids sometimes yielded an asymmetric 14-carbon monoene peak. Under normal dietary conditions (no fatty acid supplement), a fatty acid analysis at reduced temperature (150 C) and employing a 20 ft GLC column produced the pattern shown in Figure 1A. These three peaks (a,b,c) were repeatedly collected individually and reinjected into an analytical GLC instrument which yielded the spectra shown in Figure 1B, C and D. Hydrogenation revealed that the three peaks disappeared and added to myristate. A highly purified 14:1 Δ^9 *cis* reinforced peak c, and a highly purified 14:1 Δ^5 *cis* reinforced peak a when each was added as methyl esters to mixtures of the three peaks.

The origin of components in the asymmetric 14-carbon monoene peak was indicated by the use of radioactive precursors. When larvae were grown on 9,10-^{3}H-oleate, radioactivity was found to predominate in peak a but not in peaks b or c (Table I). However, in the presence of 9,10-^{3}H-palmitate, radioactive counts increased in peak b (Table I). No such specific labeling is observed when oleate or palmitate are labeled in the 1-C position. These data employing labeled palmitate and oleate demonstrate that oleate is chain shortened to 14:1 Δ^5 and that the desaturated product of palmitate, palmitoleate, is chain shortened to 14:1 Δ^7. Observe that since the labeled palmitate is 9,10-^{3}H-16:0, half of the ^{3}H is lost in forming palmitoleate. Thus the radioactive counts detected in palmitoleate and 14:1 Δ^7*cis* (but not 14:1 Δ^5) fractions reflect only half the quantity converted into these two components.

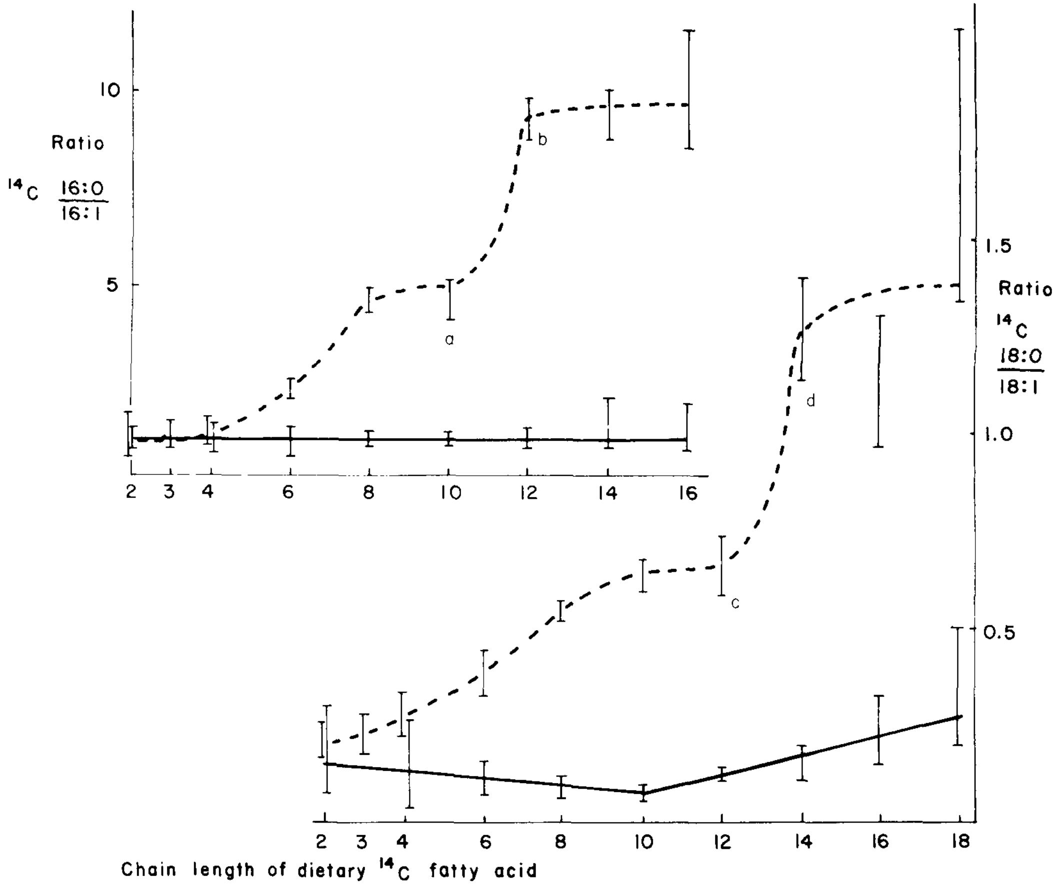

FIG. 2. Ratio of radioactivity of saturated 16:0- and 18:0-carbon fatty acids to that of monounsaturated 16:0- and 18:0-carbon fatty acids in extracts of *Drosophila* larvae reared in the presence of ^{14}C-fatty acids of increasing chain length: ---, linoleate (9 mg/ml) added to growth medium (vertical lines represent range of three independent determinations) as an inhibitor of direct desaturation; ——, no added linoleate (vertical lines represent range of two independent determinations); a, b, c, d, each represent five determinations.

It was previously demonstrated that dietary linoleate could be used to impair the ability of *Drosophila* larvae to directly desaturate palmitate and stearate to their corresponding monoenes (12). This property of dietary linoleate was taken advantage of in tests to elucidate an alternate mechanism for synthesizing palmitoleate and oleate. Utilizing all even numbered fatty acids from 2 to 18-carbons and malonic acid (each ^{14}C-labeled except acetate), it was possible to determine the ability of the different fatty acids to serve as precursors of palmitoleate and oleate when synthesis by direct desaturation was inhibited with linoleate. The results graphed in Figure 2 reveal little change in ^{14}C distribution with respect to saturates and unsaturates (i.e., ratio of ^{14}C counts in the saturate to ^{14}C counts in the unsaturated homolog) in the absence of dietary linoleate. However when linoleate is included in the diet as an inhibitor of direct desaturation, there is a several-fold change in the ^{14}C distribution with respect to saturates and unsaturates. Observe that the saturate to unsaturate ratio (16:0/16:1 and 18:0/18:1), depending upon the precursor, goes up when linoleate is added to the diet. An increasing ratio implies that monoenes are not being synthesized from their saturated homologs. Thus under these conditions, the results show that decanoate (10:0) and laurate (12:0) are the longest chain components utilized to synthesize palmitoleate and oleate, respectively.

DISCUSSION

The data obtained from axenic cultures of *Drosophila* larvae are consistent with the existence of a monoene synthetic pathway

similar to that reported for *E. coli* by the groups of Bloch (2,16), Wakil (17) and others. If such a mechanism involving β-,γ-desaturation and subsequent chain elongation exists in *Drosophila*, then a 10-carbon acid desaturated at the Δ^3 position and then elongated by two carbon units would represent the longest chain component capable of entering into palmitoleate (16:1 Δ^9) when direct desaturation is inhibited. Likewise, a 12-carbon acid also desaturated at the Δ^3 position and chain elongated by two carbon units would lead to the formation of oleate (18:1 Δ^9).

The scheme postulated above predicts the existence of several intermediates. The isolation of three different 14-carbon monoenes (with a double bond at either the Δ^5, Δ^7 or Δ^9 position) may suggest a role for the 14:1 Δ^5 and 14:1 Δ^7 forms in the pathway described above. Thus if 10:1 Δ^3 serves as the precursor for 16:1 Δ^9 and elongation proceeds by 2-carbon units, the sequence of intermediates would be 12:1 Δ^5 and 14:1 Δ^7. In the same manner, if 12:1 Δ^3 serves as the precursor for 18:1 Δ^9, the sequence of intermediates would be 14:1 Δ^5 and 16:1 Δ^7. Eventual proof for this synthetic route will involve the isolation of other intermediates and the enzymes involved in the processes.

Results obtained to date indicate that *Drosophila* is capable of producing monoenes by at least two different routes. Further research will characterize the nature, metabolic importance and possible significance of a bacterial and yeast pathway for synthesizing monoenes in animals.

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Effect of Fructose on Rat Lipids

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ABSTRACT

Young and mature albino rats were fed Purina chow with and without fructose in the drinking water, and the lipid contents in the serum and livers were determined. Fructose elevated the serum triglyceride and caused an accumulation after 24 hr of liver triglyceride in fed but not fasted mature rats. In young male rats, the liver triglyceride was increased initially but was not found after 10 days. Serum phospholipids were increased in young and mature rats; the content and specific activity of the high density (HD)-lipoproteins being increased in young rats. In vivo incorporation of labeled acetate into liver cholesterol was reduced. Results suggest that fructose or triglyceride metabolism, or both, in rats differ with age.

INTRODUCTION

It has been reported that fructose administration increases the serum triglyceride in man (1,2) and in experimental animals (3,4) and may increase serum cholesterol in man (5), rats (6,7) and rabbits (8). Uric acid production is also increased by fructose both in man (9) and rats (10). Since elevation of rat serum triglyceride occurs after 48 hr feeding (7), it was of interest to determine whether the increased serum level was maintained and in view of the reported changes in serum cholesterol to determine whether the fructose affected hepatic cholesterol synthesis. The effect on serum lipoproteins was also investigated.

MATERIALS AND METHODS

Young rats, 120 g body weight or mature albino rats 180-250 g body weight of both sexes, were fed Purina chow with and without fructose, as 10% (w/w) in the drinking water for periods up to 21 days. In one experiment, male rats were fasted for 18 hr prior to being killed but allowed froctose in the drinking water. In all experiments, food was withdrawn 4 hr before killing. Labeled precursors were injected intraperitoneally 1 hr before killing. The serum lipoproteins were separated into dextran sulfate soluble high density (HD) lipoproteins and dextran sulfate insoluble low density (LD) lipoproteins (11). A part of the livers were homogenized in ethanol-ether (3:1 v/v) and extracted twice at 60 C for 2 hr.

Initially, the triglyceride glycerol was determined manually, by a modification of the method of Moore (12) (Method A) and subsequently by an automated modification (13) of the method of Laurell (14) (Method B). A sample of serum was added to 30 vol isopropyl ether-ethanol mixture and 500 mg silicic acid was added. The sample was shaken, centrifuged and 4 ml of the supernatant saponified with ethanolic KOH. After acidification, samples of the aqueous fraction were taken for determination of glycerol and radioactive content.

A sample of the liver extract was evaporated to dryness under nitrogen, 15 g of silicic acid added and the triglyceride extracted with three successive washes of isopropyl ether and the glycerol and radioactive content determined. Under the experimental conditions, ^{3}H-glycerol was incorporated only into triglyceride in the isolated fraction, while less than 5% of the 2-^{14}C-acetate was incorporated into the cholesterol or cholesterol esters in the fraction as shown by thin layer chromatography (15).

The phospholipids were digested and the phosphorus was determined according to the method of Kraml (16). It has been reported that labeled choline is rapidly incorporated into rat serum phospholipids (17) while Gould et al. (18) reported that 95% of labeled leucine injected into rats is present in serum lipoprotein protein. Only 6% of the labeled material can be removed from the low density lipoprotein (unpublished data). It is therefore considered that the labeled choline and leucine are present in the phospholipid and protein moieties of the lipoproteins. The nitrogen and cholesterol contents were determined on the Technicon Autoanalyzer (19) and method No. 24, respectively.

To determine hepatic cholesterol synthesis, 2-^{14}C-acetate was injected intraperitoneally 1 hr before killing, the liver was perfused with ice cold saline and 1 g of liver was homogenized in 2.5 ml of o.1 M potassium phosphate buffer, pH 7.4. An amount of 1.5 ml of the cell-free homogenate was added to tubes containing 50 mg cholesterol carrier, saponified and the neutral lipids extracted as described by Cayen and Dvornik (20). The cholesterol was isolated from the petroleum ether extract as the 5,6-

TABLE I

Effect of Fructose on Serum and Liver Lipids in Young and Mature Rats[a]

Fructose feeding	Serum (mg/100 ml)			Liver, g wet weight	Liver, mg/g wet weight		
	Triglyceride glycerol	Phospholipid	Cholesterol		Triglyceride glycerol	Phospholipid	Cholesterol
10 Days							
Young female rats[b]							
Untreated (6)	4.8 ± 0.3[c]	117 ± 3	73 ± 3	7.61 ± 0.19(12)	0.77 ± 0.07[c]	1.39 ± 0.07	2.46 ± 0.06
Treated (6)	7.2 ± 0.7[c]	143 ± 4[d]	84 ± 3[e]	7.15 ± 0.25(12)	0.77 ± 0.09[c]	1.43 ± 0.04	2.40 ± 0.06
21 Days							
Untreated (5)	5.3 ± 0.6[c]	108 ± 2	64 ± 2 (22.4 ± 1.3)[f]	8.10 ± 0.18(10)	0.68 ± 0.03[c]	1.23 ± 0.05	2.00 ± 0.11
Treated (5)	4.1 ± 0.4(4)[c]	126 ± 3[d]	77 ± 3[d] (28.6 ± 1.3)[d,f]	7.77 ± 0.12(10)	0.41 ± 0.03[c,d]	1.31 ± 0.06	2.32 ± 0.11
10 Days							
Male rats[g]							
Untreated (4)	19.3 ± 1.7	123.3 ± 0.8	41 ± 1	13.26 ± 0.60	1.31 ± 0.12	1.14 ± 0.05	1.9 ± 0.07
Treated (6)	27.0 ± 3.6[e]	151.5 ± 7.1[e]	46 ± 2	14.80 ± 0.38	3.28 ± 0.35[d]	1.25 ± 0.52	2.1 ± 0.08
Female rats[h]							
Untreated (6)	12.2 ± 1.1	133.8 ± 4.2	67 ± 2	8.27 ± 0.41	1.44 ± 0.10	1.25 ± 0.09	2.46 ± 0.22
Treated (6)	18.7 ± 1.1[d]	152.5 ± 4.0[d]	67 ± 4	9.27 ± 0.52	2.81 ± 0.26[d]	1.29 ± 0.07	2.31 ± 0.09

[a]Rats were fed Purina chow with and without fructose as 10% (w/w) of the drinking water. Number of samples is given in parenthesis. Phospholipid calculated as P x 25. Results expressed as mean ± S.E.

[b]120 ± 5 g body weight; 2 Sera, 1 ml/rat, pooled/sample.

[c]Triglyceride glycerol determined by Method A. Remainder determined by Method B. The difference found between serum triglyceride content determined by Methods A and B, results in a change in source of supply of the rats, since both methods give identical results (13).

[d]$P < 0.01$.

[e]$P < 0.05$.

[f]Cholesterol present in LD-lipoproteins.

[g]250 ± 10 g body weight; Individual Sera.

[h]180 ± 10 g body weight; Individual Sera.

TABLE II

Effect of Fructose on the Specific Activity and Content of Serum and Liver Triglycerides in Rats

	Triglyceride glycerol[a]			
	Serum		Liver	
Treatment in days	mg/100 ml	^{3}H dpm/mg	mg/g	^{3}H dpm/mg
Female rats[b]				
1 Untreated	11.5 ± 1.2	149,320 ± 17,980	0.62 ± 0.08	57,910 ± 5,340
1 Treated	13.4 ± 1.5	142,790 ± 16,920	0.41 ± 0.03[c]	130,470 ± 20,245[d]
2 Untreated	9.8 ± 0.4[e]	32,215 ± 3,240	0.91 ± 0.08	20,680 ± 8,030
2 Treated	13.0 ± 1.0	28,490 ± 2,990	1.03 ± 0.08	15,535 ± 1,580
3 Untreated	16.3 ± 1.9	12,695 ± 996	1.11 ± 0.08	7,730 ± 910
3 Treated	12.8 ± 1.0	23,930 ± 3,860[d]	1.38 ± 0.12	6,410 ± 1,040
Male rats[f]				
1 Untreated (9)[g]	10.2 ± 0.6	66,390 ± 16,480[h]	0.16 ± 0.02	45,640 ± 6,170[h]
1 Treated	10.6 ± 0.8	84,040 ± 14,590	0.34 ± 0.04[d]	34,000 ± 3,095
2 Untreated (9)	11.0 ± 0.7	78,950 ± 17,430	0.29 ± 0.02	39,110 ± 8,140
2 Treated	12.5 ± 0.7	52,600 ± 9,250	0.47 ± 0.04[d]	22,690 ± 1,810
10 Untreated	12.4 ± 1.0	57,150 ± 15,530	0.23 ± 0.03	19,770 ± 785
10 Treated	12.8 ± 0.5	43,590 ± 4,030	0.27 ± 0.04	19,230 ± 1,420

[a]Triglyceride glycerol determined by Method B.

[b]Female rats, 120 ± 5 g body weight, were fed Purina chow with or without fructose as 10% (w/w) of the drinking water for 3 days. After 24 hr fructose feeding, each rat was injected intraperitoneally with 20 μc 9.10^{3}H palmitic acid and groups of rats killed after 1, 24 and 48 hr. Ten rats per group. Mean value ± S.E.

[c]$p < 0.05$.

[d]$p < 0.01$.

[e]All rats were maintained for 1 week before use and care was taken to exclude serum from rats showing any sign of pneumonia on sacrifice in order to minimize fluctuations in triglycerides.

[f]Male rats 120 ± 5 g body weight, fed as above for 10 days and groups killed after 1, 2 and 10 days. One hour prior to killing on each day, each rat injected intraperitoneally with 10 μc ^{3}H-glycerol and 4.5 μc Me^{14}C choline. Ten rats per group except where noted in parentheses.

[g]No ^{14}C radioactivity detected in the triglyceride samples.

[h]No ^{3}H radioactivity detected in cholesterol or cholesterol esters as shown by TLC (15).

dibromocholesten-3β-ol (21) and samples taken to determine the radioactivity.

Radioactivity

Aqueous samples were added to Bray's liquifluor (60 g naphthalene, 4 g PPO, 0.2 g POPOP, 100 ml methanol and 20 ml ethylene glycol and made up to 1 liter with dioxane), while the ethanol-ether samples were added to vials, the extract evaporated and 15 ml of omnifluor added (4 g/liter of toluene).

Radioactivity was measured in a Nuclear Chicago liquid scintillation system model 720: efficiency, singly labeled ^{14}C 75%, ^{3}H 35%; doubly labeled ^{14}C 30%, ^{3}H 27%.

2-^{14}C-acetate (54.7 μc/μmole), ^{3}H-glycerol (500 μc/μmole), ^{3}H-leucine (45 μc/μmole), 9.10^{3}H-palmitic acid (100 μc/μmole), Me.^{3}H-choline (200 μc/μmole) and Me.^{14}C-choline (32 μc/μmole) were purchased from the Radiochemical Center, Amersham, England.

RESULTS

As shown in Table I, the serum triglyceride in young female rats was unaltered by administration of fructose, given as 10% (w/w) in the drinking water for periods up to 21 days, but was significantly increased in mature rats. The serum phospholipids were increased in both young and mature rats, while the cholesterol content increased only in the LD lipoproteins (Table I) in young rats.

Fructose feeding did not alter the liver weight nor the phospholipid and cholesterol contents in the liver of mature rats but decreased the triglyceride content of young female rats after 21 days treatment. A marked accumulation of liver triglyceride occurred in mature rats.

Since fructose feeding has been reported to increase the triglyceride in rat serum after short-term feeding (7), the effect of feeding fructose for 24 and 48 hr on serum triglycerides

TABLE III

Effect of Fructose on the Serum and Liver Triglycerides in Mature Male Rats[a]

Treatment in days		Serum			Liver			
			Triglyceride glycerol[b]			Triglyceride glycerol[b]		
			dpm/mg x 10^{-3}				dpm/mg x 10^{-3}	
		mg/100 ml	^{14}C	^{3}H	Wet weight g	mg/g	2.^{14}C	^{3}H
	Untreated (5)	13 ± 1	17.8 ± 3.5(3)	154.8 ± 13.2	11.90 ± 0.35	0.44 ± 0.09	8.2 ± 1.7	9.1 ± 1.2
	Treated (5)	22 ± 3[c]	82.0 ± 6.9(4)[c]	108.6 ± 20.8	12.64 ± 0.57	0.87 ± 0.21[d]	19.8 ± 4.5[c]	11.2 ± 1.6
1	Fasted							
	Untreated (5)	11 ± 1	8.2 ± 0.6	44.6 ± 5.9	8.10 ± 0.10	0.78 ± 0.16	3.9 ± 0.9	18.4 ± 2.5
	Treated (5)	15 ± 2	9.2 ± 1.7	38.0 ± 8.4	9.34 ± 0.39[d]	0.82 ± 0.13	3.7 ± 1.2	9.4 ± 0.6[c]
	Untreated (5)	18 ± 3	14.8 ± 2.1	52.2 ± 6.7	12.34 ± 0.60	0.46 ± 0.10	11.4 ± 2.4	13.4 ± 0.9
	Treated (5)	27 ± 3[d]	44.2 ± 7.3[c]	51.0 ± 4.5	12.65 ± 0.76	0.83 ± 0.23	22.8 ± 6.7	13.8 ± 2.3
2	Fasted							
	Untreated (5)	(11-5)[e]	(8.3)[e]	(43.6)[e]	7.50 ± 0.41	0.37 ± 0.16	6.1 ± 1.0	20.8 ± 1.7
	Treated (5)	16 ± 4	10.0 ± 1.4	42.1 ± 5.0	9.56 ± 0.31[d]	0.70 ± 0.23	5.5 ± 2.1	10.5 ± 2.2[c]
	Untreated (4)	17 ± 1	23.8 ± 4.1(3)	43.0 ± 2.2	14.39 ± 0.81	0.96 ± 0.11	10.0 ± 1.6	9.6 ± 2.3
	Treated (4)	22 ± 3	27.0 ± 6.8(3)	29.3 ± 3.6[d]	15.65 ± 0.66	0.84 ± 0.27	19.2 ± 3.7	11.3 ± 1.9
10	Fasted							
	Untreated (4)	12 ± 9	8.4 ± 1.0	42.6 ± 3.4	9.33 ± 1.03	0.54 ± 0.11	5.7 ± 0.11	32.1 ± 2.9
	Treated (3)	16 ± 2	25.7 ± 9.8[d]	23.9 ± 6.9[d]	12.42 ± 0.28	0.70 ± 0.06	4.5 ± 3.0	8.8 ± 0.4[c]

[a]Rats, 260 ± 10 g body weight, were fed either Purina chow with and without fructose as 10% (w/w) of the drinking water or were fasted 18 hr prior to killing but allowed fructose-water. Groups of rats were killed after 1, 2 and 10 days. In each case, each rat was injected 1 hr before killing with 10 μc 2 ^{14}C-acetate and 10 μc ^{3}H glycerol. Number of individual sera given in parentheses. Mean values ± S.E.

[b]Triglycerides determined by Method B.

[c]$p < 0.01$.

[d]$p < 0.05$.

[e]Since the untreated values of fasted rats after 1 and 10 days are similar, the average of the two values is given for the second day, as the actual determinations were lost.

TABLE IV

Liver Cholesterol Biosynthesis in Rats Fed Fructose[a]

Experiments	Liver, wet weight, g	Cholesterol mg/liver	Cholesterol[b]	
			dpm/mg N	dpm/mg sterol
Experiment I				
Female rats[c]				
Untreated (10)	8.10 ± 0.18	16.6 ± 0.8	103 ± 11	821 ± 141
Treated (10)	7.77 ± 0.12	18.0 ± 0.4	76 ± 9	481 ± 61
Experiment II				
Male rats[d]				
Untreated (5)	13.26 ± 0.60	25.2 ± 0.4	41 ± 5	805 ± 95
Treated (5)	14.80 ± 0.38	29.7 ± 0.6	24 ± 2[f]	485 ± 37[g]
Female rats[e]				
Untreated (6)	8.27 ± 0.41	20.7 ± 0.3	79 ± 16	1569 ± 281
Treated (6)	9.27 ± 0.52	21.4 ± 0.7	41 ± 6[g]	910 ± 194

[a]Rats were fed Purina chow with and without fructose as 10% of the drinking water and 1 hr prior to killing injected intraperitoneally with 10 μc 2-^{14}C-acetate (38 μc/μmole). Cholesterol was isolated as dibromocholesterol from liver homogenates (20) and purified according to Fieser (21). Samples run in duplicate. Number of samples given in parentheses and expressed as mean ± S.E.

[b]Isolated as 5,6-di-bromocholestan-3β-ol.

[c]120 ± 5 g body weight, were fed fructose for 21 days and treated as in a.

[d]250 ± 10 g body weight, were fed fructose for 10 days and treated as in a.

[e]180 ± 5 g body weight, were fed fructose for 10 days and treated as in a.

[f]$P < 0.01$.

[g]$P < 0.05$.

in young and mature rats was investigated. As indicated in Table II, fructose feeding did not alter the serum triglyceride in young rats but caused a significant increase in liver triglyceride in male rats after 24 and 48 hr, which was not found after 10 days. Initially, significantly more labeled palmitic acid was incorporated into liver triglycerides in female rats fed fructose.

The serum triglyceride was increased in fed but not fasted rats given fructose (Table III), while the liver triglyceride was increased in fed rats. In fasted rats, the incorporation of labeled acetate and glycerol into serum triglyceride was reduced and was unaffected by feeding fructose. In fasting rats, the incorporation of acetate was reduced and glycerol increased in liver triglycerides. On feeding fructose to fasting rats, the incorporation of acetate was unaltered, while the glycerol incorporation was markedly reduced. Similar results were found after 10 days of treatment.

As shown in Table IV, the in vivo incorporation of labeled acetate into liver cholesterol was reduced in young and mature rats.

The HD-lipoprotein phospholipid was increased, Table V, in young female rats fed fructose, the increase appearing after two days of treatment (serum untreated 113±13; treated 136±5 mg phospholipid/100 ml, 10 sera/group). The specific activity, dpm/mg phospholipid and dpm/mg nitrogen, of HD and LD-lipoproteins which was increased after 10 days (Table V), was unaltered after 21 days treatment. After 10 days fructose feeding, the specific activity, Me.^{14}C-choline precursor, of the liver phospholipid was increased (untreated, 131±8; treated, 449±47 dpm/g wet weight x 10^{-3} $P < 0.01$), 11 rats per group).

DISCUSSION

Since fructokinase activity is greater than glucokinase and hexokinase (7,22) and since the rate limiting phosphofructokinase step is bypassed, fructose is more readily metabolized than glucose. The increase in pyruvate kinase and malic enzyme on feeding fructose (23) may lead to excess acetyl CoA formation which in turn could stimulate pyruvic carboxylase. Although fructose metabolism might elevate α-glycerophosphate concentration and thus increase fatty acid synthesis (24), Zakim et al. (25) were unable to find any in vivo relationship between the α-glycerophosphate content and fatty acid synthesis.

This study confirms the findings of Nikkila and Ojala (3) that both the content and specific activity (labeled palmitate precursor) of serum triglyceride were increased in mature male rats fed a 10% fructose diet after 24 hr and was evident after 10 days in both male and female

TABLE V

Effect of Fructose on Rat Serum Lipoproteins[a]

Treatment	Lipoproteins							
	HDL				LDL			
	Phospholipid		Nitrogen		Phospholipid		Nitrogen	
	mg/100 ml	^{14}C dpm/mg	mg/100 ml	^{3}H dpm/mg	mg/100 ml	^{14}C dpm/mg	mg/100 ml	^{3}H dpm/mg
10% Fructose for 10 days								
Untreated (6)[b]	83 ± 3	3,330 ± 170	1090 ± 30	5,670 ± 180	34 ± 1	1,260 ± 80	47 ± 1	11,420 ± 455
Treated (6)[b]	105 ± 4[c]	10,570 ± 230[c]	1090 ± 20	13,240 ± 1020[c]	38 ± 1	6,890 ± 770[c]	43 ± 8	33,620 ± 460[c]
10% Fructose for 21 days								
Untreated (4)[e]	77 ± 00	11,903 ± 420	930 ± 30	---	23 ± 2	5,270 ± 790	37 ± 3	---
Treated (4)[e]	93 ± 2[c]	12,790 ± 1020	940 ± 10	---	37 ± 2[c]	6,690 ± 350	33 ± 1	---
		^{3}H dpm/mg				^{3}H dpm/mg		
Untreated (5)[d]	82 ± 1	46,150 ± 8280	1070 ± 50	---	26 ± 2	10,865 ± 1220	43 ± 3	---
Treated (5)[d]	96 ± 4[c]	39,700 ± 1190	1100 ± 10	---	26 ± 3	14,105 ± 1500	58 ± 3[f]	---

[a]Female rats, 120 ± g body weight, were fed Purina chow with and without fructose as 10% of the drinking water, and groups of rats were killed after 10 and 21 days. Rats were injected intraperitoneally 1 hr prior to killing with labeled precursors. Number of samples given in parentheses. Results expressed as mg/100 ml serum. Mean values ± S.E.

[b]Rats injected on 10th day with 10 μc Me.^{14}C-choline and 10 μc ^{3}H leucine. Two sera pooled per sample.

[c]$P < 0.01$.

[d]Rats injected on 21st day with 10 μc 2^{14}C-acetate and 10 μc Me.^{3}H-choline. Two sera, 1 ml/rat, pooled per sample.

[e]Rats injected on 21st day with 8 μc Me.^{14}C-choline. Three sera, 1 ml/rat, pooled per sample.

[f]$P < 0.05$.

rats. However, in immature male and female rats, the serum triglyceride was unaltered even after fructose feeding for 21 days.

In fasted mature male rats, the incorporation of glycerol into triglyceride was increased. Since the activity of triokinase and aldose (26) and phosphoenol pyruvate (27) is depressed on fasting, the increased incorporation of glycerol into liver triglyceride in fasted mature male rats, implies glycerol may be metabolized predominantly via glycerokinase (22). As fructose feeding to fasted rats increases both the triokinase and aldolase activities (26), the decreased incorporation of glycerol found suggests that fructose metabolized via di-hydroxyacetone phosphate spares the glycerol incorporation.

Although the serum and liver triglyceride was increased in mature rats fed 10% fructose, no change occured in starved or in immature male or female rats. MacDonald (28) reported fructose increased the sērum triglyceride in men and old women but failed to alter the content in young women. Sex differences in the metabolism of fructose have been reported in the baboon (29). Care should be taken when comparing data obtained from man and rats since several differences in carbohydrate metabolism occur in the two species (22).

As reported previously (6), fructose increased rat LD-lipoprotein cholesterol in young female rats but not in mature rats. Kritchevsky et al. (8) recently reported the LD-lipoprotein cholesterol in rabbits was increased by carbohydrate diets. Since the content of liver cholesterol was unaltered, although hepatic synthesis was reduced, the elevated LD-lipoprotein cholesterol may be of extrahepatic origin. However, it is unknown if the decreased incorporation of labeled acetate into cholesterol was due to an enlarged acetate pool in fructose-fed rats.

Zakim et al. (7) reported that 70% fructose-Purina chow diet given for 48 hr increased the serum phospholipid content, while Eaton (30) reported a 20% to 40% monosaccharide diet fed for four days increased the leucine incorporation into the LD-lipoproteins. Eaton (30) suggested that excessive carbohydrate stimulated hepatic protein synthesis. Although fructose is predominantly incorporated into phospholipids (31), it is unknown how fructose increased the synthesis of HD-lipoprotein phospholipid since the nitrogen content was unaltered. Recently, Spritz and Mishkel (32) reported that the addition of fat to the diet in man decreased the LD-phospholipid moiety without affecting the protein moiety. It is of interest that in young rats fructose increases serum phospholipids without elevation of triglyceride, while in mature rats hypertriglyceridemia is followed by elevation of serum phospholipid. The increase in serum and liver triglyceride in mature rats suggests a difference in the metabolism of carbohydrate or triglyceride, or both, with age.

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The Metabolism of Linoleic and Arachidonic Acids in Rat Testis[1]

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ABSTRACT

Linoleic and arachidonic acids, labeled with ^{14}C and injected intratesticularly, were used to study with time the interconversion of polyunsaturated fatty acids in rat testis and their incorporation into the major lipid classes. With both substrates ^{14}C activity was readily incorporated into longer chain, more highly unsaturated fatty acids. After the injection of 1-^{14}C-linoleic acid the major portion of the ^{14}C was found in palmitic, linoleic, 8,11,14-eicosatrienoic, 5,8,11,14-eicosatetraenoic, 7,10,13,16-docosatetraenoic and 4,7,10,13,16-docosapentaenoic acids. Hydrogenation of the total fatty acids isolated from rat testes after intratesticular injection of 1-^{14}C-linoleate revealed that the polyenoic acids hydrogenating to lignoceric acid (previously characterized as 9,12,15,18-tetracosatetraenoate and 6,9,12,15,18-tetracosapentaenoate) had a relatively high specific activity. After the injection of 1-^{14}C-arachidonate significant ^{14}C activity was found in palmitate, 7,10,13,16-docosatetraenoate, 4,7,10,13,16-docosapentaenoate, 9,12,15,18-tetracosatetraenoate and 6,9,12,15,18-tetracosapentaenoate. The biosynthesis of the $\omega 6$ polyunsaturated fatty acids in rat testis is discussed in relation to these data. Investigation of the distribution of label in the complex lipid fractions demonstrated the majority of the ^{14}C activity to be present in phosphatides and triglycerides after injection of either of these ^{14}C substrates with only small quantities being present as nonesterified acids. At the time periods studied the polyenoic acids of triglycerides had a higher specific activity than the corresponding acids of phosphatides with the exception of linoleate.

INTRODUCTION

Testicular tissue of the rat contains a relatively large amount of polyunsaturated fatty acids of the $\omega 6$ type (1-3). It has been suggested that these polyenes may have functional significance in the spermatogenic process (1,4). However, results of experiments using essential fatty acid-deficient rats indicate that these polyenoic acids may not be absolutely necessary for the maintenance of the spermatogenic process (5).

The metabolism of various fatty acid precursors and of ^{14}C fatty acids has been studied in rat testis (6-9). Davis and Coniglio (6), by direct intratesticular injection of ^{14}C fatty acids complexed with albumin, elucidated the pathway of biosynthesis of 4,7,10,13,16-docosapentaenoate and other fatty acids in rat testis. Nakamura and Privett (7,8) have studied the metabolism of 1-^{14}C-linoleic acid and of glyceryl 1-^{14}C-trilinoleate injected as emulsions into rat testes. Bridges and Coniglio (9) recently demonstrated the biosynthesis in rat testis of 9,12,15,18-tetracosatetraenoate and 6,9,12,15,18-tetracosapentaenoate by the elongation and desaturation of 1-^{14}C-linoleate or 1-^{14}C-arachidonate. In this paper we report a more complete study of metabolic interconversions of intratesticularly injected 1-^{14}C-linoleate and 1-^{14}C-arachidonate and of their incorporation into esterified lipids.

MATERIALS AND METHODS

Animals used in these experiments were Sprague-Dawley rats (10-14 weeks of age, weighing 250-400 g) which were maintained on Purina Laboratory Chow. The ^{14}C fatty acid, complexed with fatty acid-poor albumin (Pentex Corporation, Kankakee, Ill.), was injected directly into the testes of rats which had been lightly anesthetized with Nembutal (Abbott Laboratories, North Chicago, Ill.). The rats were sequentially decapitated in groups of two at 1/6, 1/2, 1, 2, 7, and 14 days after the

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TABLE I

Distribution of Radioactivity in Lipid Classes After 1-^{14}C-Linoleate Injections

Time (days)	^{14}C Recovered in total lipid (% of injected dose)	Lipid fractions[a]					
		Phospholipid	Cholesterol	Free fatty acid	Triglyceride	Glyceryl ether diester	Cholesterol ester
1/6	35.5	84.1	2.6	1.9	10.1	0.8	0.5
1/2	25.3	84.1	3.3	0.8	10.5	0.4	0.9
1	15.5	83.3	3.8	0.8	10.2	0.6	0.6
2	11.9	84.8	3.9	1.2	8.4	0.8	1.0
7	8.7	80.7	4.2	1.6	11.4	1.0	1.2
14	6.3	81.1	1.0	1.8	13.9	0.8	1.4

[a]Lipid fractions isolated by TLC from a pooled sample of testicular tissue. Per cent of total ^{14}C recovered from TLC plates.

injections. One testis from each animal was used for the extraction of total fatty acids. The other testis was combined with similar ones from the other animals of the same group and the total lipid extracted from the pooled samples.

The 1-^{14}C-linoleic acid (Volk Chemical Corporation, Burbank, Calif.) had a radiochemical purity greater than 97% by gas liquid radiochromatography (GLR) of its methyl ester and of its hydrogenated methyl ester. This diene was therefore used without further purification. Five microcuries (0.062 mg) of 1-^{14}C-linoleate in a total aqueous volume of 50 μl were injected into each testicle of the rat.

The 1-^{14}C-arachidonic acid (Hoffmann LaRoche Pharmaceutical Company, Nutley, N.J.) was purified by preparative gas liquid chromatography (GLC) so that its final purity was greater than 93% as determined by GLR. Approximately 1 μc (0.26 mg) of the purified tetraene was injected in a total aqueous volume of 100 μl into each testicle.

The tissue used for extraction of total fatty acids was immediately hydrolyzed in 10% ethanolic potassium hydroxide at reflux temperature and under an atmosphere of nitrogen. Hydroquinone (final concentration of 0.5 mg/ml) was used as antioxidant. After nonsaponifiable material was removed by extraction with petroleum ether, the total fatty acids were extracted from the acidified solution with petroleum ether and methylated according to the method of Metcalfe and Schmitz (10) with BF_3-methanol (14% w/v, Applied Science Laboratories, State College, Pa.). Fatty acid methyl esters were hydrogenated by the method of Farquhar et al. (11).

The total lipids of the pooled sample of testicular tissue were immediately extracted three times using each time 20 vol of chloroform-methanol (2:1 v/v) as previously described (1). The combined filtrates were evaporated to dryness on a rotary evaporator at room temperature, and the residue was redissolved in petroleum ether-chloroform (90:10 v/v). Fractionation of the total lipids was carried out on thin layer plates having a 1 mm layer of Silica Gel HF_{254} (Brinkman Instruments Inc., Westbury, N.Y.). The lipid-containing bands, as viewed under ultraviolet light, were scraped from the plates and the lipid isolated by four successive washings of the silica gel with 95% ethanol-chloroform-water-glacial acetic acid

TABLE II

Distribution of Radioactivity in Total Fatty Acids After 1-^{14}C-Linoleate Injections

Time (days)	Fatty acid[a]					
	16:0	18:2	20:3	20:4	22:4	22:5
1/6	6.6 ± 0.1	77.7 ± 0.7	6.8 ± 0.8	5.3 ± 0.05	0.2 ± 0.1	0.3 ± 0.2
1/2	7.0 ± 1.8	70.0 ± 0.8	7.1 ± 0.5	11.2 ± 1.2	0.7 ± 0.0	0.8 ± 0.5
1	10.4 ± 0.8	58.1 ± 0.4	8.8 ± 1.3	16.1 ± 0.6	1.2 ± 0.0	0.8 ± 0.5
2	14.6 ± 2.7	34.6 ± 13.9	10.1 ± 1.7	27.3 ± 4.8	2.4 ± 0.4	4.1 ± 1.8
7	19.7 ± 0.1	23.2 ± 2.0	7.2 ± 0.6	24.7 ± 2.4	3.0 ± 0.3	11.7 ± 1.1
14	17.8 ± 0.0	16.4 ± 1.6	6.0 ± 0.6	19.7 ± 0.3	3.2 ± 0.1	21.9 ± 1.1

[a]Mean ±the deviation from the mean of two values. Per cent of total ^{14}C recovered from GLC.

TABLE III

Relative Specific Activities of the Polyenoic Acids of the Phospholipid and Triglyceride Fractions After Injection of 1-^{14}C-Linoleate

Time	Sample	Relative specific activity[a] of the fatty acid				
		18:2	20:3	20:4	22:4	22:5
4 hr[b]	Phospholipid[c]	10.2	6.0	0.3	---	---
	Triglyceride[d]	6.4	11.2	---	---	---
12 hr	Phospholipid	7.9	7.6	0.8	0.3	0.04
	Triglyceride	3.4	14.6	1.6	2.3	0.2
24 hr	Phospholipid	7.9	2.8	0.9	0.8	0.1
	Triglyceride	5.0	8.4	1.2	1.2	0.2
48 hr	Phospholipid	6.4	7.1	1.0	1.3	0.2
	Triglyceride	2.0	9.3	1.9	2.2	0.8
1 week	Phospholipid	3.6	5.6	1.3	1.2	0.7
	Triglyceride	2.2	5.7	3.1	3.7	1.4
2 weeks	Phospholipid	3.0	3.6	1.2	2.7	0.9
	Triglyceride	1.2	8.4	1.5	2.8	1.4

[a]Relative specific activity expressed as a percentage of total counts divided by the percentage of total fatty acids of the individual polyenes.

[b]Time after the injection.

[c]Fatty acids of the phospholipid fraction isolated from a pooled sample of testicular tissue.

[d]Fatty acids of the triglyceride fraction isolated from a pooled sample of testicular tissue.

(100:30:20:2), according to the procedure of Biezenski (12). The recovery of radioactivity from the thin layer plates ranged from 85% to 95% of the amount applied to the plates. The fatty acids of the lipid fractions were isolated as previously described.

All radioactivity determinations were made in a Packard Tri Carb Scintillation Spectrometer (Model 3000 series) operating at about 78% efficiency. Aliquots of the total fatty acids and lipid fractions were evaporated to dryness and toluene containing PPO-POPOP (Liquifluor, T. M. Pilot Chemicals Inc., Watertown, Mass.) was added as solvent and fluor.

Analytical GLC was done on a Varian Aerograph Model 1520 equipped with a flame ionization detector. Standard methyl esters (Hormel Institute, Austin, Minn., and Applied Science Laboratories, State College, Pa.) were used for calibration of the detector. Fatty acids were identified by their retention time and the retention time of their hydrogenated derivatives. The stainless steel column was 8 ft x 1/8 in. o.d. and was packed with diethylene glycol succinate polyester (12.0% by weight) coated on 110/120 mesh Anakrom ABS. The column temperature was 200 C and the carrier gas (He) flow rate was 30 ml/min. The injector temperature was 265 C and the detector temperature was 235 C.

GLR was done by two methods: (a) By collection of individual methyl esters from a stream splitter in cartridges packed with silanized glass wool. The contents of the cart-

TABLE IV

Relative Specific Activities of Hydrogenated Total Fatty Acids of Rat Testis After the Injection of 1-^{14}C-Linoleate

Saturated methyl ester	1 Week		2 Weeks	
	Per cent of total methyl esters	Relative[a] specific activity	Per cent of total methyl esters	Relative[a] specific activity
16:0	23.8	0.78	24.1	0.52
18:0	27.4	0.82	27.0	0.71
20:0	18.6	1.27	18.3	1.64
22:0	23.7	1.29	24.3	0.98
24:0	3.1	1.55	3.7	1.22

[a]Relative specific activity expressed as in Table III.

TABLE V

Distribution of Radioactivity in Lipid Classes After 1-^{14}C-Arachidonate Injections

Time (days)	^{14}C Recovered in total lipid (% of injected dose)	Lipid fractions[a]					
		Phospho-lipid	Cholesterol	Free fatty acid	Triglyceride	Glyceryl ether diester	Cholesterol ester
1/6	24.3	76.9	2.5	1.5	17.6	0.7	0.8
1/2	22.2	84.5	3.0	0.6	10.3	0.7	0.8
1	20.7	82.1	2.2	0.5	13.6	0.8	0.8
2	16.9	85.1	2.5	0.2	10.5	1.0	0.6
7	10.4	75.9	4.6	1.3	14.5	2.4	1.3
14	9.8	79.1	2.0	0.1	15.3	1.9	1.6

[a]Lipid fractions isolated from a pooled sample of testicular tissue. Per cent of ^{14}C recovered from TLC plates.

ridges were emptied into counting vials, fluor solution added and radioactivity determined in a liquid scintillation spectrometer. (b) By continuous monitoring with a gas flow heated proportional detector (Nuclear Chicago Model 4998). The efficiency of the detector under our operating conditions was about 25% of ^{14}C using ^{14}C-methyl palmitate as standard.

Oxidative ozonolysis of unsaturated fatty acids was done by the method of Stoffel (13). Gas chromatography of the methylated dicarboxylic acid products was performed under the same conditions as has been described previously for fatty acid methyl esters, but with a column temperature of 165 C. The radioactivity of the individual dicarboxylic acid products was determined by collection in U-shaped tubes immersed in liquid nitrogen and counting of the collected effluent in a liquid scintillation spectrometer.

RESULTS

The incorporation of radioactivity into the total lipids and the per cent distribution of ^{14}C in the lipid classes after 1-^{14}C-linoleate injections are given in Table I. The amount of ^{14}C activity retained in the testes as per cent of the injected dose ranged from 35.5 at 4 hr to 6.3 at the two-week interval. At 4 hr after injection about 40% of the recovered ^{14}C had been metabolized to $^{14}CO_2$ and about 20% of the recovered ^{14}C had been transported to the other tissues of the body. The total recovery of the injected ^{14}C in excreta and tissues was between 60% and 70% of the injected dose. Most of the ^{14}C not recovered in these experiments may have been contained in water-soluble compounds and have been lost during the extraction of fatty acids or lipids. Injection of only one testicle in a rat resulted in less than 0.3% of the total recovered ^{14}C in the noninjected testicle. These data indicate that any observed conversion of the 1-^{14}C-linoleate in the testis actually occurred in this tissue.

The phosphatides and triglycerides contained the largest proportions of the radioactivity with less than 2% of the total ^{14}C recovered from the thin layer plate as free fatty acid 4 hr after the injection. The cholesterol (probably contaminated with diglyceride), glyceryl ether diester and cholesterol ester fractions also contained only a small proportion of the total recovered ^{14}C.

TABLE VI

Distribution of Radioactivity in Total Fatty Acids After 1-^{14}C-Arachidonate Injections

Time (days)	Fatty acid[a]						
	16:0	20:3[b]	20:4	22:4	22:5	24:4	24:5
1/6	2.1 ± 0.2	3.9 ± 0.2	64.0 ± 3.7	5.9 ± 0.2	2.5 ± 0.8	1.0 ± 0.3	1.0 ± 0.2
1/2	4.2 ± 0.2	1.7 ± 0.3	63.3 ± 0.1	9.8 ± 0.9	1.8 ± 0.1	2.8 ± 0.1	1.8 ± 0.2
1	5.5 ± 0.2	1.1 ± 0.2	56.8 ± 2.8	11.4 ± 0.8	5.5 ± 0.2	4.4 ± 0.2	2.7 ± 0.9
2	7.1 ± 0.0	1.0 ± 0.2	53.9 ± 2.6	9.7 ± 0.1	7.7 ± 2.2	4.7 ± 0.1	3.7 ± 0.0
7	9.9 ± 0.6	1.1 ± 0.2	39.6 ± 0.3	7.0 ± 0.2	18.6 ± 0.6	3.6 ± 1.0	3.4 ± 1.1
14	4.1 ± 0.8	1.6 ± 0.1	26.1 ± 3.9	5.9 ± 0.1	32.8 ± 1.1	6.4 ± 0.2	6.0 ± 0.4

[a]Mean ± the deviation from the mean of two values. Per cent of ^{14}C recovered from GLC.
[b]Possibly contaminated to a small extent with 20:4.

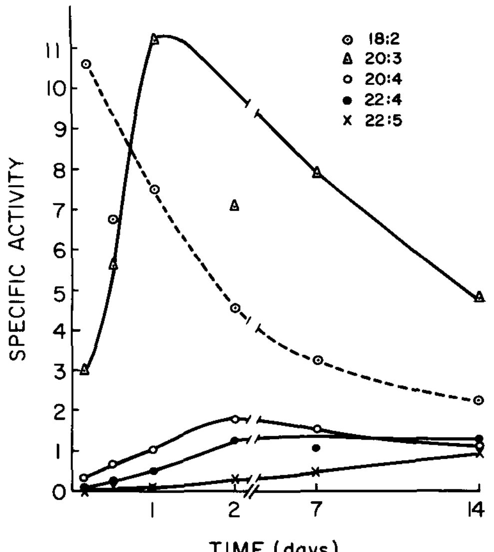

FIG. 1. Relative specific activities expressed as a percentage of total counts divided by the percentage of total fatty acids of octadecadienoic (18:2), eicosatrienoic (20:3), eicosatetraenoic (20:4), docosatetraenoic (22:4), and docosapentaenoic acid (22:5) isolated from rat testis at various times after intratesticular injection of 1-^{14}C-linoleate. Each point represents the average of values obtained on two animals.

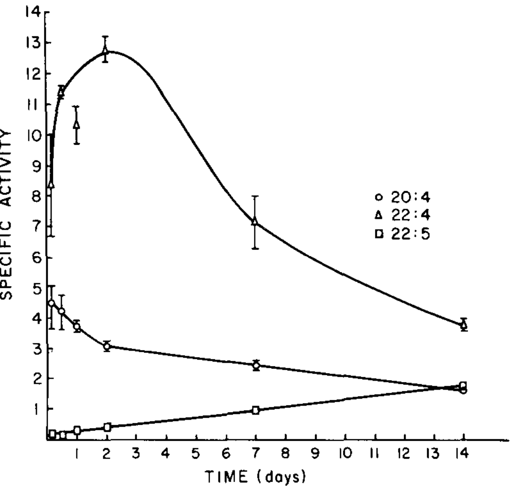

FIG. 2. Relative specific activities of testicular polyenes after intratesticular injection of 1-^{14}C-arachidonate. Specific activities are expressed as in Figure 1. Each point on the curve is the average of the two values shown by the vertical lines.

The distribution of radioactivity in the individual fatty acids after 1-^{14}C-linoleate injections is shown in Table II. Significant quantities of radioactivity were incorporated into palmitate presumably by de novo synthesis from 1-^{14}C-acetate units resulting from the degradation of the injected 1-^{14}C-linoleate. Incorporation of ^{14}C activity into the higher polyenes probably occurred by the elongation and desaturation of intact 1-^{14}C-linoleic acid. In the testes of animals killed two weeks after the injection of 1-^{14}C-linoleate a greater proportion of the radioactivity was present in arachidonate and in 4,7,10,13,16-docosapentaenoate than in the isolated dienoic acid.

Upon calculation of the relative specific activities of the polyenoic acids (Fig. 1) isolated from the total fatty acid fraction, a precursor-product relationship (18:2 > 20:3 > 20:4 > 22:5) was established at the 4 and 12 hr intervals. Twenty-four hours after the injection 8,11,14-eicosatrienoate had a higher specific activity than did linoleate. The specific activity of docosapentaenoate increased linearly throughout the two-week experiment.

GLR of the fatty acids of phosphatides and triglycerides revealed that greater proportions of the recovered ^{14}C were in linoleate and arachidonate of phosphatides than in the corresponding acids of triglycerides. Greater proportions of the recovered ^{14}C were found in eicosatrienoate, docosatetraenoate and docosapentaenoate of triglycerides than in the corresponding acids of phosphatides. At each time period the specific activities of the polyunsaturated fatty acids, other than linoleate, were higher in the triglycerides than the corresponding polyenoic acids of phosphatides (Table III).

The total fatty acids were hydrogenated and the saturated methyl esters were analyzed by GLR. A small but appreciable quantity of radioactivity was observed in the 24 carbon saturated fatty acid, lignoceric acid. At the one- and two-week intervals, lignoceric acid had a relatively high specific activity as compared to those of the other saturated fatty acids (Table IV). However, less than 4% of the total saturated fatty acids was lignoceric acid upon hydrogenation. The acids giving rise to lignocerate upon hydrogenation have been characterized as 9,12,15,18-tetracosatetraenoate and 6,9,12,15,18-tetracosapentaenoate and have been shown to be the elongation and desaturation products of linoleic and of arachidonic acids (9).

Results obtained after the intratesticular injection of 1-^{14}C-arachidonate were consistent with the data obtained after the intratesticular injection of 1-^{14}C-linoleate. The amount of radioactivity in the total lipid and its distribution into the lipid classes after 1-^{14}C-arachidonate injections are given in Table V. In

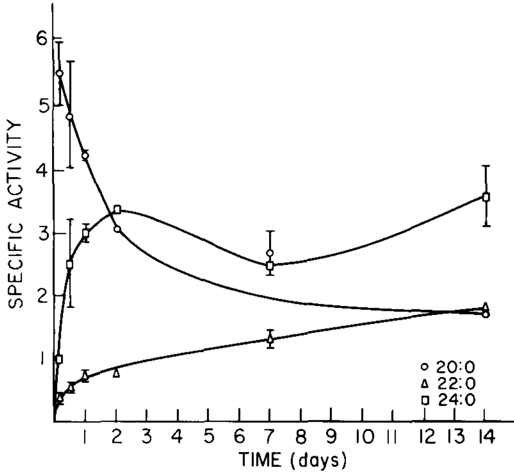

FIG. 3. Relative specific activities of hydrogenated total fatty acids of testes of rats injected intratesticularly with 1-^{14}C-arachidonate. Specific activities are expressed as in Figure 1. Each point on the curve is the average of two values shown by the vertical lines.

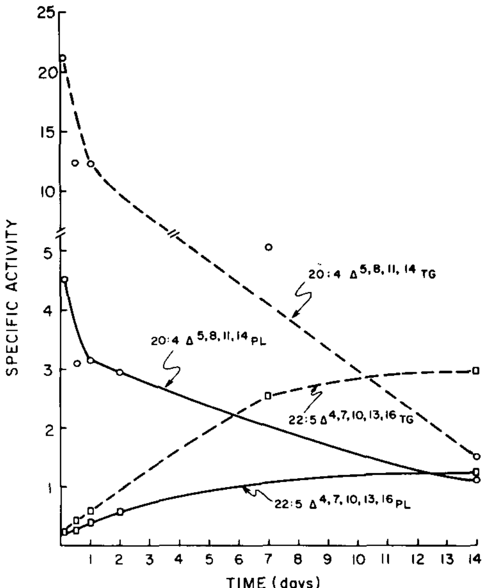

FIG. 4. Relative specific activities of polyenes of testicular phospholipid (PL) and triglyceride (TG) fractions after intratesticular injection of 1-^{14}C-arachidonate. Specific activities are expressed as in Figure 1. Each point represents a single determination from a pooled sample.

the 24 hr sample a larger proportion of the injected ^{14}C was found in total lipids after the injection of 1-^{14}C-arachidonate than after the injection of 1-^{14}C-linoleate. As in the experiment with 1-^{14}C-linoleate the phosphatides and triglycerides contained the largest proportions of the radioactivity. The free fatty acid fraction contained less than 2% of the recovered ^{14}C even as early as 4 hr after the injection of the acid. The cholesterol (probably contaminated with diglyceride), glyceryl ether diester and cholesterol ester fractions contained small but definite amounts of ^{14}C. Results obtained by fractionation of the total lipid by silicic acid column chromatography according to the method of Hirsch and Ahrens (14) and those obtained by thin layer chromatography (TLC) were similar.

The distribution of ^{14}C in the fatty acids after the injection of 1-^{14}C-arachidonate is presented in Table VI. Incorporation of ^{14}C into palmitate occurred probably by de novo synthesis using ^{14}C acetate units derived from the metabolic breakdown of the injected 1-^{14}C-arachidonate. On the other hand, incorporation of label into 22:4, 22:5, 24:4 and 24:5 presumably occurred by the direct elongation and desaturation of the intact 1-^{14}C-arachidonate. The relatively small incorporation of ^{14}C into 20:3 may be a useful index for estimating the extent to which 1-^{14}C-acetate units (derived from the metabolic oxidation of 1-^{14}C-arachidonate) were being utilized in the elongation of pre-existing long chain, unlabeled acids. Upon calculation of the relative specific activities of the polyenoic acids (Fig. 2) derived from the total lipid fraction, the specific activity of 7,10,13,16-docosatetraenoic acid was found to be always higher than its presumed precursor, arachidonate. The specific activity of 4,7,10,13,16-docosapentaenoate increased linearly throughout the two-week study. Hydrogenation of the total fatty acids (Fig. 3) revealed that the 24 carbon polyenoic acids as a group had a higher specific activity than did the 20 and 22 carbon polyenoic acids 48 hr after the injection of 1-^{14}C-arachidonate. Thus, 1-^{14}C-arachidonate appears to be a more immediate precursor of the 24 carbon tetraene and pentaene than was the 1-^{14}C-linoleate.

Calculation of specific activities of the higher polyenes of phosphatides and triglycerides (Fig. 4) revealed that the specific activities of arachidonate and docosapentaenoate of triglycerides at each time period were greater than those of the corresponding fatty acids of phosphatides. The decrease in specific activity of arachidonate and the increase in specific activity of docosapentaenoate were more rapid in the triglyceride fraction than in the phosphatide fraction.

To prove the structures of the labeled 24 carbon polyenoic acids and to locate the relative position of the ^{14}C, the labeled unsatu-

TABLE VII

Oxidative Ozonolysis of Labeled 24 Carbon Polyenoic Acids Biosynthesized From 1-^{14}C-Arachidonate in Rat Testis

Dicarboxylic acid	Per cent of total counts recovered in dicarboxylic acid fragments	
	24:4	24:5
C_3	4.7	4.8
C_4		8.6
C_5	4.8	8.4
C_6	1.2	63.1
C_7	8.7	5.9
C_8	3.9	2.5
C_9	74.2	5.3

rated fatty acids were isolated and cleaved by oxidative ozonolysis (Table VII). The isolated tetraene and pentaene biosynthesized from 1-^{14}C-arachidonic acid were shown by hydrogenation and by GLR to have a purity of 81.2% and 77.2%, respectively. Upon oxidative ozonolysis of the tetraene most of the radioactivity (74%) was present in azelaic acid, the nine carbon dicarboxylic acid from the carboxyl end. Oxidative ozonolysis of the labeled pentaenoic acid resulted in most of the ^{14}C (63%) in adipic acid, the six carbon dicarboxylic acid from the carboxyl end. These data are consistent with the chemical designation of these compounds as 9,12,15,18-tetracosatetraenoate and 6,9,12,15,18-tetracosapentaenoate and are consistent with the suggestion presented elsewhere (9) that these compounds are formed metabolically by the elongation and oxidative desaturation of linoleic acid.

DISCUSSION

The data in this paper concerning the metabolism of intratesticularly injected 1-^{14}C-linoleate are comparable in some respects to those reported recently by Nakamura and Privett (7). However, certain differences between these experiments are apparent. Incorporation into Folch-extractable lipids reported by Nakamura and Privett (7) was much higher at all time periods than the values reported in this paper. For example, Nakamura and Privett reported a recovery in testes of 30.2% of the injected radioactivity at 48 hr after injection whereas in our experiments only 11.9% of the injected radioactivity was recovered at the same time period. Nakamura and Privett further found no transport of the injected ^{14}C whereas our results indicate that transport as well as metabolic oxidation of the injected ^{14}C diene occurred.

Significant differences in distribution of radioactivity in the lipid fractions are apparent between these two experiments. Nakamura and Privett (7) reported a higher proportion of the ^{14}C in free fatty acids, diglycerides and triglycerides, and a lower proportion in phosphatides than was found in our experiment. It is probable that these differences are due to the physical state of the injected ^{14}C diene, although more subtle differences (age of rats, diet, etc.) between these experiments cannot be overruled as contributing factors. Nakamura and Privett injected the diene as an emulsion whereas the diene was injected as a water-soluble albumin complex in our experiments.

Our results indicate that the oxidation of injected ^{14}C fatty acid is the major cause of the low recovery of ^{14}C. This suggestion is supported by the appearance of nearly 40% of the recovered radioactivity in the expired CO_2. Oxidation of the injected polyenoic acid is also indicated by the presence of ^{14}C in palmitic acid. Also of importance in the disappearance of the injected radioactivity from the testis was the transport of the labeled fatty acids to other tissues with subsequent storage in or oxidation by these tissues or both.

Incorporation of the ^{14}C into the complex lipids (chiefly phosphatides and glycerides) of testicular tissue occurred very readily with less than 2% of the total recovered ^{14}C being present as free fatty acids 4 hr after the injections. The results of these experiments may indicate the suitability of the physical form of the injected labeled substrate (i.e., solution of albumin-bound fatty acid) as a physiological substrate.

The polyenoic acids of triglycerides (with the exception of linoleate) had a higher specific activity than the corresponding acids of phosphatides. This distribution of radioactivity does not define the pathway of incorporation of the labeled acids into complex lipids but probably only reflects the relative pool sizes and turnover rates of these complex lipid fractions. Davis et al. (1) have demonstrated that triglycerides are a small component of the total lipid of rat testis. Nakamura and Privett (8) recently reported a rapid turnover rate of the acyl moiety of triglycerides injected directly into the rat testis. Therefore, it is probable that the small pool size and rapid turnover rate of triglycerides in rat testis contributed to the higher specific activities of its polyenoic acids.

Conversions of 1-^{14}C-linoleate or 1-^{14}C-arachidonate to longer chain, more highly unsaturated fatty acids must have actually

occurred in the testis, since only small amounts of ^{14}C (< 0.3% of the recovered ^{14}C) was found in the noninjected testis 4 hr after the injection of the other testis of the same rat. In testes of rats killed 4 and 12 hr after the injection of 1-^{14}C-linoleate a precursor-product relationship existed in the labeled products so as to establish the metabolic pathway of docosapentaenoate synthesis as: 18:2 → 20:3 → 20:4 → 22:4 → 22:5 in confirmation of data reported by Davis and Coniglio (6). Twenty-four hours after the injection of 1-^{14}C-linoleic acid, 8,11,14-eicosatrienoic acid had a higher specific activity than did its presumed precursor linoleate. After intratesticular injection of 1-^{14}C-arachidonic acid, the specific activities of the isolated fatty acids did not conform to the required criteria of precursor-product related metabolites.

The metabolic product, 7,10,13,16-docosatetraenoate, had a higher specific activity than did arachidonic at all times after the injection of this 1-^{14}C-tetraene. These data also probably reflect the pool sizes of eicosatrienoate and docosatetraenoate and their relative turnover rates. It is probable that more than one pool of linoleic or arachidonic acids exists and that the injected ^{14}C fatty acid may not distribute uniformly in all pools during its conversion to the more highly unsaturated or longer chain polyenes, or both. Further work is obviously necessary to describe more specifically the pools which contain these polyenes and the role they play in these conversions.

1-^{14}C-arachidonate was demonstrated to be a more immediate precursor of 9,12,15,18-tetracosatetraenoate and 6,9,12,15,18-tetracosapentaenoate than 1-^{14}C-linoleate. The relatively high specific activities of the 24 carbon polyenoic acids as a group may be due to their small pool sizes in testicular tissue as well as their relatively high turnover rate. The exact pathway of biosynthesis of the 24 carbon polyenes of rat testis was not defined by these experiments, but the results support data reported elsewhere (9) that they are synthesized by the elongation and desaturation of linoleic acid. These compounds constitute a significant portion of the acyl moities of glyceryl ether diesters, cholesterol esters and triglycerides of rat testis (3). However, their biochemical or physiological significance is yet to be determined.

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Lipid Monolayers: Mechanisms of Protein Penetration With Regard to Membrane Models[1]

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ABSTRACT

The influence of lipid and protein on the properties of the air-water interface is analyzed with the view to formulate a mechanism of interaction of protein with lipid monolayers. The increase in surface pressure ($\Delta\Pi$) and the quantity of protein incorporated in the lipid film after injection of protein under lipid monolayers were studied as a function of both lipid structure and protein structure. With rabbit γ-globulin, the values of $\Delta\Pi$ were cholesterol $>$ phosphatidyl choline $>$ sphingomyelin. Similar results were obtained with ribonuclease, lysozyme and serum albumin. The quantities of protein found in films of either cholesterol or phosphatidyl choline (egg lecithin) were much larger than those calculated from a geometric model in which a protein monolayer occupies the area made available by the compressed lipid. Arguments are produced against penetration based on simple mechanisms of compressibility of the lipid film. The mechanisms operating in the incorporation of protein into lipid monolayers are grouped into three categories: (a) free penetration, typical of lecithin; (b) binding-mediated penetration, typical of cholesterol and some glycosphingolipids; and (c) binding-inhibited penetration, typical of the albumin-ganglioside system and a specific lipid hapten-antibody system. A model is described in which nonspecific protein interacts with polymeric lecithin structures (surface micelles). In the sequence of events $X \rightarrow Y \rightarrow Z$, the globular protein X is activated into the expanded or extended form Y by contact with the lipid and then restructured into a compact form Z with release of water and free energy. The resulting lipid-protein assembly has a mosaic structure in which lipid and protein polar surfaces are exposed to water. Accessibility of lecithin to phospholipase A is consistent with the model and with current views on the state of protein in biological membranes; according to such views, protein is more likely structured inside the lipid milieu and not simply denatured on the lipid-water interface.

INTRODUCTION

Interest in lipid-protein interaction stems from the experience in at least five areas of biology: cell membranes, lipoproteins, lipid hapten-antibody complex, enzyme activation by lipids and synthesis and breakdown of lipids by enzymes. A fundamental feature of all these systems is the association of lipid with protein in a noncovalent type of bonding (including enzyme-substrate complex), the nature of which is not fully understood. The interest in lipid monolayers is prompted by the observation that lipids, as amphipathic and water insoluble molecules, collect, orient and dwell at water interfaces, and thus form interfaces with water. Since such molecular organization is mandatory for lipids, and these inevitably operate by the same principle in biological systems, the monomolecular film of lipid at the air-water interface is the simplest model in which one type of lipid-protein interaction (as opposed to bulk systems) can be studied readily and with a high degree of accuracy and information. Relevance of the model to the biological systems should be assessed only after sufficient experience with monolayers is available. Hitherto however, little information has existed, and that has come from very few laboratories and few systems, such as the action of phospholipases A and B on lecithin monolayers (1-3), the nonspecific interaction of proteins with lipid monolayers (4-9), a specific lipid hapten-antibody interaction (5,10), and the interaction of the apoprotein of high density lipoprotein with lipid monolayers (10,11).

Since the cell membrane is the most obvious expression of lipid-protein association, models of membrane structure can be introduced as a convenient frame of reference in the study of lipid-protein interactions in monolayers. I shall therefore cite three models, which exemplify

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three different concepts of molecular organization in biological membranes: Danielli-Davson's of 1935, Sjostrand's of 1963 and Benson's of 1966. According to the first model, a bimolecular lipid leaflet is inserted between two monolayers of protein adsorbed onto the polar groups of the lipid (12). Sjostrand's model features a mosaic in which globular protein is alternated with globular or micellar lipid structures (13). According to Benson, the cell membrane consists of globular protein onto which individual lipid molecules are adsorbed by association of their hydrophobic chains with the nonpolar sites of protein structures (14). Whereas in the Danielli-Davson model the core of the membrane consists of lipid, which is coated with protein and presumably excluded from the water interface, in the other two models protein is also at the center of the membrane, and both protein and lipid sites are exposed to the water. Also in 1966, a fourth model was proposed by Lenard and Singer (15). According to these authors, the red blood cell membrane is probably a mosaic of lipid penetrated by protein. In this respect, the model is a direct departure from the unit membrane, as the continuous lipid bilayer of the latter (16) is replaced by a discontinuous one and part of the protein is structured in the lipid medium. This model bears an important relation to the phenomenon of penetration of lipid films by proteins. In spite of the apparent differences, an essential feature of the three alternative models is the orientation of lipid and protein molecules between two water interfaces. In these terms, the three models are thermodynamically feasible, whereas one should hasten to say that a continuous lipid interface coated with extended protein is untenable (17). That not withstanding, the bimolecular lipid leaflet is the inevitable basic unit of every membrane. Detail and variety of structure and function must then pertain to the mode of penetration, adsorption and structuring of the protein. Elucidation of the mechanism of enzyme activation by lipids can also profit from a discussion of the general problem of lipid-protein interaction in membranes and model systems.

Since in the organization of lipid and protein in membranes the basic process must be one in which the membrane components are brought together, the model which considers the penetration of protein into lipid films acquires relevance to problems of membrane structure and membrane biogenesis. Several techniques for bringing lipid and protein together in monolayers have been described (4,7,10). By far the most widely used technique is one in which

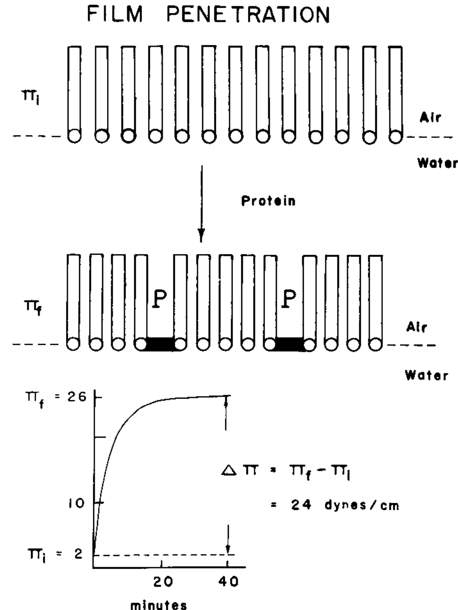


FIG. 1. Schematic representation of penetration of lipid monolayer by protein at the air-water interface. The letter P, in central panel, designates the protein, which is filling some spaces between the lipid.

(Fig. 1) a lipid film is formed at a defined initial pressure (Π_i); the protein is then injected into the subphase, and the increase in film pressure ($\Delta\Pi$) is measured as a function of time. This increase in film pressure is grossly referred to as film penetration. A detailed inquiry into the mode of penetration of protein in the lipid film was not possible as yet because of lack of data. In reference to Figure 1 the following questions must be considered: (a) How much protein enters the film? (b) Why and how does protein enter the film? (c) Once the protein has penetrated the lipid film, how is it structured relative to the lipid? In this communication I shall describe the experimentation designed to answer these questions.

Although work with specific proteins found in biological structures has begun (10,11), this communication deals with nonspecific proteins: γ-globulin, albumin, ribonuclease and lysozyme. Information about the surface behavior of nonspecific proteins is still needed to explain previous observations and to further understand differences between specific and nonspecific proteins.

MATERIALS AND METHODS

Lipids

Phosphatidyl choline (egg lecithin), sphingomyelin from beef heart and cholesterol prepara-

tions were already described (4,5,10,11); they were homogeneous on thin layer chromatography (TLC), in which 100 μg lipid were applied.

Fully saturated phosphatidyl choline (HPC) was obtained by catalytic hydrogenation of egg lecithin over 5% Pd on charcoal in absolute ethanol; 25 mg catalyst and 25 mg lipid were used in 10 ml ethanol. Under these conditions no breakdown of lecithin took place, which however was extensive when PtO_2 in absolute ethanol was used as a catalyst. Completion of hydrogenation was monitored by I_2 staining on thin layer chromatograms, by gas liquid chromatography and force area curves; absence of I_2 uptake by titration with thiosulfate revealed also completion of hydrogenation.

Ganglioside was provided by Maurice M. Rapport as "very highly purified mixed gangliosides from ox brain gray matter"; the major component was a disialo-ganglioside. TLC on silicic acid plate showed the presence of several components typical of mixed gangliosides; each component gave a positive resorcinol test for sialic acid (18).

A solution of 0.5 μg/μl lipid in chloroform-methanol 85:15 was used to spread the lipid film. Such solutions were stored for not longer than three days at 0 C. The organic solvents, reagent grade, were redistilled before use.

Proteins

Rabbit γ-globulin (Lot 42P) and 2x crystallized rabbit serum albumin (Lot 9), used in previous studies (4,5), were Pentex products (Pentex Inc., Kankakee, Ill.). Lysozyme, a salt free preparation from egg white (Lot LYF-6JA), ribonuclease, bovine, 2x crystallized (Lot 6RLA) and trypsin, 2x crystallized, 50% $MgSO_4$ (Lot TR-359) were purchased from Worthington Biochemical Corp., Freehold, N.J. Pronase, 45,000 PUK units/g, (Lot 71879) was obtained from Calbiochem, Los Angeles, Calif.

Rattle snake (*Crotalus atrox*) and cobra (*Naja naja*) venoms were products of Ross Allen Reptile Institute, Silver Springs, Fla; they were used without further purification, and their solutions were prepared as already described (1). When so indicated, destruction of proteolytic activity was effected by heating the acidic stock solution for 10 min at 100 C (19,20).

Hypophase

Unless otherwise specified, the subphase for monolayer studies consisted of 0.04 M potassium phosphate buffer containing 0.1 M NaCl, pH 7.0, 25 ± 1 C. Protein solutions of 1 mg/ml were stored at 0 C for not longer than three days. Protein and buffer solutions were prepared with doubly distilled water. The second distillation was carried out over alkaline permanganate in an all glass system (Corning, Model AG-3, New York).

Apparatus

A sand blasted platinum blade and a radioactive (^{226}Ra) air electrode were used for the measurements of surface tension and surface potential respectively (1). A circular trough, however, was preferred to the rectangular one for the reasons given elsewhere (4,10).

The trough consisted of a crystallizing dish made of Pyrex glass and divided into two equal sections by a glass barrier annealed across the top of the dish (4); the film area was 37 cm^2. The barrier and inside of the trough were coated with paraffin (Fisher Scientific, mp 48 C). The subphase, 300 ml, was mixed with a magnetic stirrer.

Formation of Lipid Film and Injection of Protein

The lipid was applied in 1 μl fractions of 0.5 μg/μl solution in chloroform-methanol 85:15; the droplet, formed at the tip of a Hamilton microsyringe, was made to touch the accurately cleaned water surface. The lipid was so spread on one section area of the trough until the desired initial pressure (Π_i) was reached. The protein was injected, at time zero (through the service area), with the tip of a microsyringe or a pipette touching the bottom of the trough (10). The rise in film pressure ($\Delta\Pi$) was then recorded as a function of time.

Determination of Protein

At a given time, for a defined value of $\Delta\Pi$, the quantity of protein present in the subphase was measured with an accuracy of ± 0.1 μg, or better, by a modified micro-Lowry method (10). The trough consisted of a crystallizing dish in pyrex glass, 15.2 cm in diameter and 10 cm high. The area of the film was 180 cm^2, and the volume of the subphase was 500 ml. The trough partition was not necessary: the lipid film was made at 2 dynes/cm, and the protein, 200 μg, was injected from above the film by touching the lipid-water surface with the droplet of aqueous protein solution hanging from the tip of a Hamilton microsyringe (7,10). The hypophase consisted of 0.001 M NaCl instead of the phosphate buffer. In the presence of phosphate, a white precipitate formed upon the addition of the Folin reagent in the Lowry assay. Although the precipitate could be removed by centrifugation, accuracy and precision of the protein determination were not as good as in the absence of phosphate.

Since the dependence of $\Delta\Pi$ on lipid struc-

TABLE I

Quantity of Protein in Lipid-Protein Film[a]

Substance	Lipid μg	ΔΠ dynes/cm	Globulin, μg		
			Monolayer model	Found	Gibbs
Lecithin	21.6	6.6	3.06	67.0	248.0
Cholesterol	29.9	14.0	0.64	70.0	12.9

[a]The lipid monolayer was prepared at Π_i = 2 dynes/cm on a total area of 180 cm^2. Protein, rabbit γ-globulin, 200 μg, was injected from 200 μl of 0.01 M NaCl just below the lipid by the technique of Eley and Hedge (10). Hypophase 0.001 M NaCl. The Gibbs quantities were calculated for the same values of ΔΠ but from experiments in which the protein was injected deep into the hypophase: The valid assumption was made that the equilibrium concentration is the same as the initial protein concentration, since the quantity of protein in the film is negligible as compared to the quantity of protein in the hypophase. The model of Eley and Hedge suggesting that the total quantity of protein applied remains in the film could not be adopted here because of the large quantities of protein that are lost into the subphase when the protein is injected by the Eley and Hedge technique.

ture is similar in both the technique of Doty and Schulman and that of Eley and Hedge (4), the latter was preferred solely because in this case protein analysis is much simpler. At the desired time, 50 min, the film was removed, and aliquots of subphase containing between 1.0 and 2.0 μg of protein were processed for protein determination (10).

Action of Phospholipase A

The lecithin film was formed at 2 dynes/cm. The nonspecific protein was injected into the subphase at a final concentration of 10 μg/ml, and penetration was studied in the usual way. At 40 min, when the lipid protein film was nearly saturated, venom was injected into the subphase at a final concentration of 1 μg/ml. The changes in surface pressure and surface potential were recorded as a function of time (1). When the β-ester bond of lecithin is attacked by the enzyme, a decrease of surface potential takes place which is proportional to the number of lysolecithin molecules formed or lecithin molecules hydrolyzed per unit area (20). The extent of hydrolysis was verified by TLC (1). The film was collected on a platinum gauze and deposited in chloroform-methanol 2:1. The solvent was removed by evaporation under vacuum, and the residue was applied to a plate layered with silicic acid (Silica Gel H, Merck, Darmstadt, Germany). After resolution of the lipid mixture in chloroform-methanol-water-concentrated NH_4OH 70:30:4:1, staining with I_2 vapors revealed the relative quantities of lecithin and lysolecithin.

Action of Proteolytic Enzymes

The experimental setup was the same as that used in the study of phospholipase action. A lecithin monolayer was made at 2 dynes/cm pressure; the protein was injected in the subphase, and film penetration was studied in the usual way. At 40 min, the proteolytic enzyme was injected at the concentration indicated for each experiment, and the changes in surface potential and surface pressure were recorded as a function of time. According to the studies of Schulman and Rideal (21), it was to be expected that hydrolysis of peptide bonds in film proteins would cause a lowering of surface potential. Although at present it is difficult to provide a quantitative assay of proteolysis at the interface, the contention of Schulman and Rideal is correct because it is reconcilable with the theory of surface potentials (unpublished observations).

Furthermore, hydrolysis of film protein was shown by ninhydrin staining as follows. The lipid-protein film was collected on a platinum blade in the customary way. The blade, 5 cm long, was dipped across the interface 5 to 6 times consecutively; the protein, adsorbed onto the blade, was washed into a test tube with a few drops of water. The mixture was applied to strips of filter paper; these were sprayed with 0.2% ninhydrin in ethanol and heated in oven at 80 C. The difference between hydrolysis and non-hydrolysis was striking, it was not possible however to measure the extent or the kinetics of hydrolysis by this technique.

RESULTS

Influence of Chemical Structure of Lipid on Protein Penetration

With several proteins, rate and extent of surface pressure increase were clearly related to structural differences among lipids. With rabbit γ-globulin (Fig. 2), the ΔΠ values were in the order: cholesterol > phosphatidyl choline =

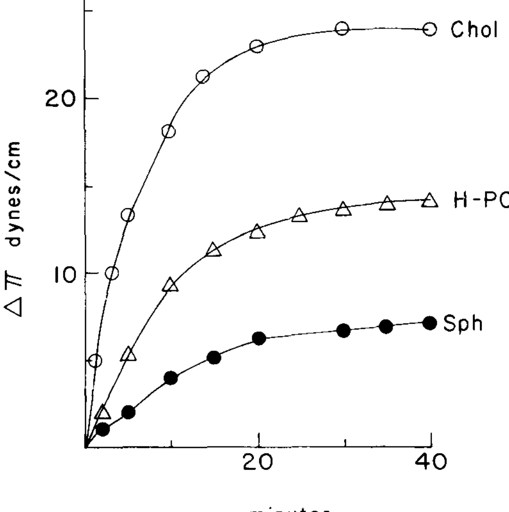


FIG. 2. Influence of lipid structure on the penetration of lipid monolayers by rabbit γ-globulin. Lipid: o cholesterol; Δ hydrogenated egg lecithin; ● sphingomyelin. Initial film pressure, 2 dynes/cm. Protein 10 μg/ml. Hypophase: 0.04 M phosphate buffer 0.1 M NaCl, pH 7.0, 25 C, Π_i = 2 dynes/cm. Film area 37 cm^2.

hydrogenated phosphatidyl choline = ganglioside > sphingomyelin. The same pattern, cholesterol > PC > sphingomyelin, was revealed by rabbit serum albumin and by other proteins (4,11). Albumin, however, (Fig. 3), at pH 6 to 10, showed a remarkably small penetration of ganglioside film, which in contrast was penetrated as readily as PC by γ-globulin, lysozyme and ribonuclease.

Similar results were obtained when the technique of Eley and Hedge was used for the injection of protein under the lipid film (Fig. 4). The smaller ΔΠ values of albumin (Fig. 3) as compared to those of γ-globulin (Fig. 2) and the smaller ΔΠ values in the technique of Eley and Hedge (Fig. 4) as compared to those in the technique of Doty and Schulman (Fig. 2) are consistent with previous observations (4).

Quantity of Protein in the Film

The quantity of protein in the film was calculated (Fig. 4) from the difference between quantity of protein applied to the film at time zero and quantity of protein in the hypophase at 50 min. The results of two experiments are presented in Table I, one with egg lecithin and one with cholesterol. The protein was rabbit γ-globulin, and the trough's area was 180 cm^2. The lipid monolayer at 2 dynes/cm pressure contained either 21.6 μg lecithin or 29.9 μg cholesterol. At 50 min, when most of the interaction had taken place, the quantities of protein found in the lecithin and cholesterol films were 67.0 μg and 70.0 μg, respectively. These quantities are much larger than the ones calculated from a geometrical model in which an average area of about 15 A^2 is attributed to each amino acid residue of the protein adsorbed at the interface in the form of a monolayer (22). According to that model, the area made available by the lipid in the compression from Π_i to Π_f is the one occupied by the extended protein, the nonpolar chains of which are intercalated with the hydrophobic chains of the lipid (7,8,23). The quantities of protein calculated were 3.06 μg in lecithin and 0.64 μg in cholesterol. The quantities of globulin found in the films were thus 21.9 and 110.0 times those calculated for lecithin and cholesterol films, respectively. They were also different from the quantities calculated by the Gibbs adsorption equation for protein monolayers (23). For reasons which are not fully explained, the Gibbs quantities were influenced by the molecular weight of the protein (24).

Action of Phospholipase A on Film Lipid

The data with two proteins, albumin and ribonuclease, are presented in Figure 5. Phosphatidyl choline in the lecithin-protein film was hydrolyzed readily, for a rapid decrease in surface potential, -Δ (ΔV), took place which was comparable with that observed in the case of a pure lecithin film. The magnitude of the change in the presence of albumin or ribonuclease, about 100 mv, or two thirds of the value observed with pure lecithin could be accounted for if one assumed that, after compression to the attained ΔΠ value at 40 min, the area occupied by lecithin is two thirds of the total area of the film, one third of which is occupied by the protein. TLC of the material extracted from the lipid-protein film (1) showed that more than 90% lecithin had been transformed to lysolecithin. For all practical purposes, the results obtained with γ-globulin and lysozyme were identical with those in Figure 5. Similar results were obtained when the venom had received the heat treatment.

The slight increase in film pressure which is seen during the action of the venom cannot be attributed solely to penetration of venom protein into the lipid, for increase in molecular volume and film pressure are expected when the hydrated products of lecithin hydrolysis are formed. Unlike *C. atrox*, the pressure increase caused by *N. naja* venom was very large, therefore, masking all other effects. Data with *C. atrox* venom are presented as this was the least surface active of the available venoms. This con-

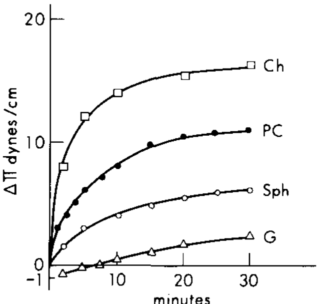


FIG. 3. Penetration of a rabbit serum albumin into monolayers of cholesterol (Ch), egg phosphatidyl choline (PC), sphingomyelin (Sph) and ganglioside (G). Protein 10 μg/ml. Subphase as in Figure 2.

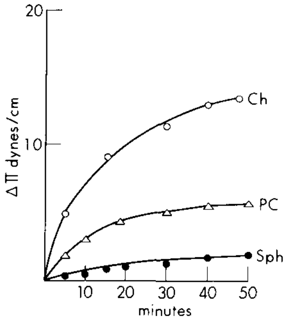


FIG. 4. Penetration of rabbit γ-globulin after injection of protein by the technique of Eley and Hedge. Hypophase 0.001 M NaCl. Π_i = 2 dynes/cm. Film area, 180 cm^2. The trend of the ΔΠ values, cholesterol > PC > sphingomyelin, was consistent irrespective of film area, volume of hypophase and technique of protein injection.

sideration, which is dictated by the disruptive effects of *N. naja* venom, ought to apply to the cases in which this and other surface active enzymes are used in the study of membrane and lipoprotein structures. In the absence of interpretable kinetic data (25), hydrolytic action of *N. naja* venom on lipid membranes may well reflect secondary effects and not necessarily the action of phospholipase A on the lipid of intact membranes, no matter how the venom-treated membrane structures appear in the electron microscope.

Action of Pronase and Trypsin on Film Protein

A decrease in surface potential, -Δ (ΔV), indicating proteolysis, was observed after action of pronase on lecithin-ribonuclease films (Fig. 6). The effect was less than trebled when the pronase concentration in the subphase was increased 5x, from 1 μg/ml to 5 μg/ml. Similar results were obtained with both proteolytic enzymes on lecithin-ribonuclease as well as on the lipid-protein films made with either albumin, globulin or lysozyme. A rise in film pressure was observed in every case (Fig. 6, lower panel). Experiments aimed at measuring the quantity of protein hydrolyzed, by means of chromatography and ninhydrin staining of the collected film, were not conclusive; a quantitative interpretation of the data must therefore await further experimentation.

DISCUSSION

A mechanism of film penetration can be approached from three standpoints: lipid structure, protein structure and lipid-protein arrangements.

Influence of Lipid Structure on ΔΠ

The marked dependence of film penetration on the chemical structure of the lipid, which has been pointed out consistently in the past (4,6,7,8,11), is summarized in Figures 2, 3 and 4.

In all the available studies and with a variety of proteins, the ΔΠ values for cholesterol were greater than those for other lipids. It has been argued that, since the intermolecular area of cholesterol films is very small, little protein would be needed to fill the intermolecular spaces and presumably produce the large and rapid increase in film pressure which is typical of this lipid (26,27). Although convincing, this explanation is not satisfactory. It has no experimental evidence and fails to take molecular mechanisms into account because it ignores the effects of possible lipid-protein interactions (4,28). The old contention cannot withstand criticism in the light of two new observations: (a) the very large quantity of protein which is incorporated in the cholesterol film (Table I), and (b) the contraction of cholesterol films in the early stages of the interaction. Both observations are illustrated later in this paper.

Egg lecithin and fully hydrogenated egg lecithin showed identical penetration curves. Although these lipids have different cross sections (area-molecule), their compression areas are nearly the same, 110 to 70 A^2 molecule and 80 to 44 A^2/molecule for PC and HPC, respectively. However, the information of equal compression areas does not constitute evidence for the claim that compressibility of the lipid is a valid criterion of film penetration. The conclusions must await further knowledge of the film topography for other parameters must thus be looked at. At present, the observation that the quantity of protein found in a very compressible lecithin film was smaller than the one in the least compressible cholesterol film

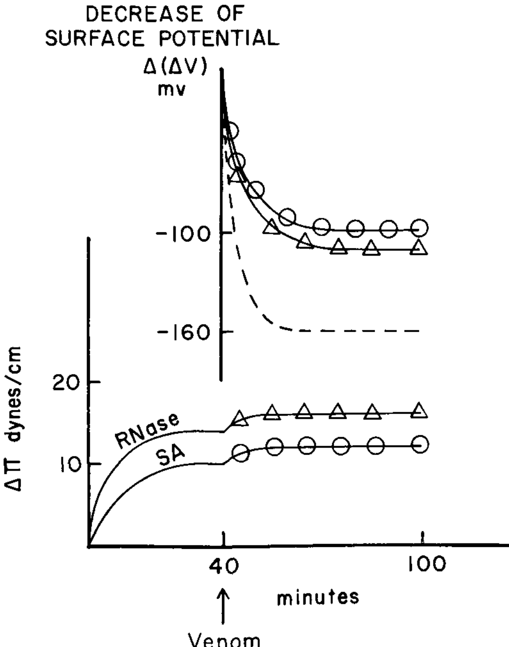


FIG. 5. Action of phospholipase A on egg lecithin-protein films: Δ Ribonuclease (RNase), 10 μg/ml; 0 rabbit serum albumin (SA), 10 μg/ml; — decrease of surface potential of lecithin film at 12 dynes/cm pressure under the action of *C. atrox*, 1.0 μg/ml, in the absence of protein. Subphase: 0.04 M potassium phosphate buffer, 0.1 M NaCl, pH 7.0, 25 C; *C. atrox* venom, 1.0 μg/ml, was injected at 40 min when the film pressure was about 9 dynes/cm for the albumin-lecithin film and 14 dynes/cm for the ribonuclease-lecithin film.

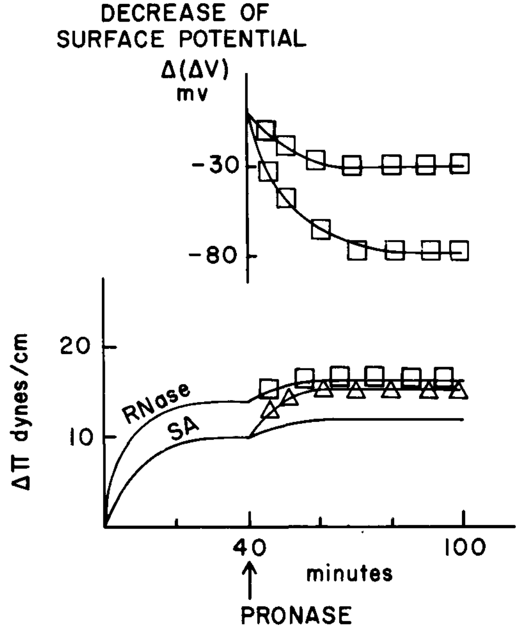


FIG. 6. Action of proteolytic enzymes on egg lecithin-protein films: ——— rabbit serum albumin (SA), 10 μg/ml, pronase 1 μg/ml. Δ rabbit serum albumin, 10 μg/ml, pronase 5 μg/ml. □ Ribonuclease (RNase), 10 μg/ml; lower panel, pronase 1 μg/ml; upper panel, upper curve, pronase 1 μg/ml; upper panel, lower curve, pronase 5 μg/ml. Subphase 0.04 M potassium phosphate buffer, 0.1 M NaCl, pH 7.0, 25 C.

(Table I) argues strongly against the criterion of the compressibility.

Sphingomyelin exhibited consistently small ΔΠ values with several proteins (4,11). In Figures 2, 3 and 4, the ΔΠ values for sphingomyelin are one half those for egg lecithin or hydrogenated egg lecithin. Yet, the latter and sphingomyelin have identical area-molecule and compression area (29,30). Unpublished data on impure sphingomyelin are in agreement with the data of Shah and Schulman (31). Binding of the free hydroxyl group of sphingomyelin with the protein in the subphase could inhibit film penetration. The higher surface viscosity (4) of sphingomyelin as opposed to that of lecithin, resulting probably from a particular orientation of sphingomyelin -OH groups at the air-water interface (32), may not be the sole cause of limited film penetration. Indeed, in the interaction with albumin, gangliosides, which have much lower surface viscosity than sphingomyelin, exhibited smaller ΔΠ values than sphingomyelin did (Fig. 3). In the interaction with rabbit γ-globulin, ribonuclease and lysozyme, ganglioside displayed the greater ΔΠ values of egg lecithin. The indication therefore is that ganglioside films and albumin at pH 7 and 25 C may enjoy some specific binding which inhibits albumin penetration.

Quantity of Protein Incorporated Into the Lipid Film

The protein found in the lipid-protein film was far more than the quantity expected if the protein were adsorbed as a monolayer in the intermolecular (compression) areas of the lipid. Although little protein would be required to produce a given value of ΔΠ (Table I), the quantity of protein associated with the lipid is about 22x and 110x the quantities calculated for lecithin and cholesterol monolayers, respectively. This means that excess protein either is packed in the hydrophobic regions of the lipid film and/or extends in the form of a matrix into the aqueous subphase.

The observation of thick protein films is in line with the suggestion of multilayer protein of Khaiat and Miller (9). I do not agree however

with these authors' contention that penetration studies carried out with an initial film pressure of 2 dynes/cm are meaningless, on the ground that the surface concentration of lipid at this pressure is nearly zero. First, with an area of 110 A^2/molecule, the surface concentration of egg lecithin at 2 dynes/cm is 9.1 x 10^{13} molecules/cm^2 and nowhere near zero; and lipid films at 2 dynes/cm are very stable. Secondly, an initial film pressure of 2 dynes/cm was chosen since the magnitude of the effects at this low pressure makes the observations more convenient and since it is really not known what film pressure may be more relevant to biological systems and membrane biogenesis (4). Penetration of protein at different film pressure has been studied (4,11 and unpublished results), and assessment of the more meaningful film pressures must await further investigations.

The foregoing data make two revelations. First, obeyance of Langmuir's adsorption equation by the $\Delta\Pi$ values in the form of a linear relationship of $1/\Delta\Pi$ to 1/ protein concentration (11), which had been verified with several proteins (unpublished data), is such that the $\Delta\Pi$ values bear no relation to the quantity of protein in the film. Secondly, use of the Gibbs equation as suggested by Eley and Hedge (24) yields great aberrations (Table I). For example, in the interaction of the apoprotein of rat plasma high density lipoprotein with lecithin monolayer at Π_i = 2 dynes/cm, the equilibrium quantity of film protein calculated from the Gibbs equation was 1.1 x 10^{-10} moles/cm^2 (11). This quantity, 2.5 $\mu g/cm^2$, corresponds to an area of 0.04 m^2/mg, or 20-fold the surface concentration of monolayer protein, 0.125 $\mu g/cm^2$ or 0.80 m^2/mg, and nearly 30x the effective quantity of apo-HDL found in lecithin monolayers at a $\Delta\Pi$ value of 20 dynes/cm.

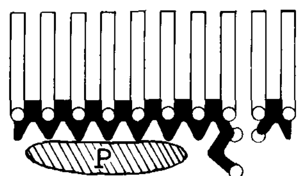
The data in Table I reveal that only one third of the protein applied was in the film. Appreciable loss of protein into the subphase was verified when different quantities of protein were applied, irrespective of the area of the film and of the volume of the subphase. This finding is contrary to a crucial assumption of Eley and Hedge (23) that all the protein was adsorbed to the lipid monolayer, and it invalidates the methods used for determining molecular weight of proteins (22,25,33,34) unless one ascertains that all the protein applied is in the film in a monolayer conformation. I found appreciable quantities of protein in the subphase when the apoproteins of the most surface active high density serum lipoproteins were spread as films. The facts that the quantity of protein in the film occasionally is that of a monolayer (10,11), and the relationships $\Delta\Pi$ vs. 1/c, $\Delta\Pi$ vs. lnc (11,23), and Π m^2/mg vs. Π (22,25) are linear, cannot be construed to mean that protein films are monolayers or that Π relates to the quantity of protein in the film (24). Although the protein monolayer has been previously suggested (22,33,34), much evidence is against it and its use for its molecular weight determination is incorrect. The results could be only a misleading coincidence, unless a satisfactory theory is worked out. Therefore, the increase in film pressure observed is not a measure of the quantity of protein penetrated; this must be determined independently. It was protein analysis which made it possible to distinguish between proteins that form thin films, e.g., the apoprotein of high density serum lipoprotein (10) and proteins which form thick films, e.g., the mitochondrial structural protein (10,35).

The protein films relevant to this discussion are the thick ones which derive from penetration of globulin into the lipid monolayer. The other three proteins, albumin, globulin and lysozyme, also formed thick films. Although the $\Delta\Pi$ values measured in film penetration are essentially those of the surface free energy of water, and as such they are proportional to the surface valence or number of protein groupings anchored at the interface, they do not necessarily reflect the quantity of protein present in the film nor do they express fully the energetics of anchoring. Beside the surface free energy of water ($\Delta\Pi$) which we determine by the surface tension measurements, two major contributions to the free energy of film formation derive from the hydration and solvent electrostriction of the anchoring groups and from dipole-dipole and other polar bindings. Aggregation of protein in a thick film expresses a number of complicated energetics which derive from dipole-dipole, dipole-charge and charge-charge interactions of polypeptide chains as these assume new asymmetric conformations in the proximity of the hydrophobic interface (10).

Organization of Protein Relative to Lipid

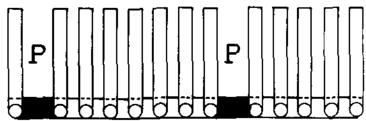
The only available model was proposed independently by Schulman (36) and Eley and Hedge (23). In that model, the extended polypeptide chains of denatured protein penetrate the lipid monolayer by intercalating the nonpolar side chains of the amino acids between the hydrophobic chains of the lipid. If the protein were to cover entirely the polar groups of the lipid as postulated by the model of Eley

MODELS



SCHULMAN

ELEY & HEDGE



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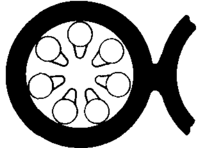


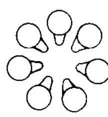
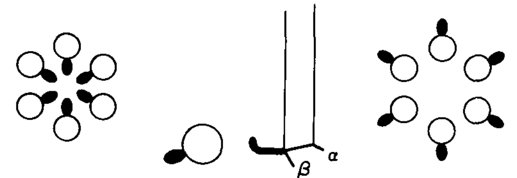


FIG. 7. Schematic representation of models of molecular organization of lipid-protein films. The letter P designates the protein filling some intermicellar spaces. No attempt is made to draw the protein's structure, for neither its conformation nor its orientation is known.



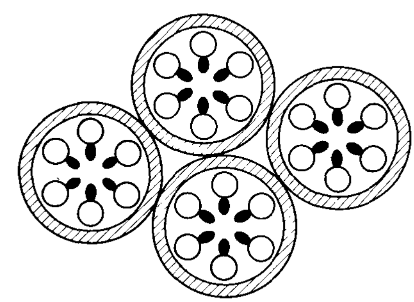


FIG. 8. Pictorial representation of the mechanism of free penetration. The lipid is phosphatidyl choline (PC). The phosphoryl choline group, heavy black, is oriented parallel to the interface: the β-ester group points into the aqueous. The lipid molecules are organized in surface micelles of one or another orientation, in which the cross section of the PC molecule is seen as an asymmetric dumbbell. Notice the protein's points of anchorage at interface under the structured form Z. In this arrangement, as in Figure 7, lecithin is attacked by phospholipase A readily, and the film protein is attacked by pronase and trypsin. The Ω protein is not shown.

and Hedge, the lipid (phosphatidyl choline) would not be accessible to lipases. The results obtained with phospholipase A (Fig. 5) indicate that all the β-ester bonds of egg lecithin were readily available to the enzyme after the lecithin film had been penetrated by either globulin, lysozyme, serum albumin or ribonuclease.

A model which is consistent with this observation is a mosaic structure in which packages of lipid alternate with packages of protein. Thereby lipid sites (lecithin) and protein sites are exposed to water, whereas protein must also be found inside the lipid milieu. In many respects this artificial model, which is supported by the thermodynamics of the water interface (10), is in accordance with the model that Lenard and Singer proposed for the blood red cell membrane (15,37).

Hydrolysis of film protein by pronase and trypsin was shown by a decrease of surface potential (Fig. 6, upper panel) and was confirmed by the ninhydrin staining of the lipid-protein film. Although one could expect proteolysis to bring about a loss of soluble peptides and a decrease in surface pressure (4), it is now clear that a small increase in surface pressure (Fig. 6, lower panel) or the absence of a change in film pressure (4) cannot be construed to mean absence of protein hydrolysis. The observed increase in film pressure could mean hydration of the products of hydrolysis, which remain in the film, as well as further penetration of peptides or protein fragments that are more surface active than the protein itself. Penetration of enzyme protein cannot be excluded. These new features complicate the quantitative analysis of the system but do not impair the validity of the proposed model. The data say that all the lipid was attacked by phospholipase A, the protein was attacked by pronase and by trypsin; the extent of proteolysis is irrelevant.

The schematic representation of the model in Figure 7 does not offer either geometry or dimensions of lipid and protein particles. Although it appears as if the protein raps itself

around the lipid, one cannot exclude the possibility that protein particles are surrounded by lipid. Although there is no evidence at present for either case, it is fair to say that each case will be determined by two conditions, the relative quantities of lipid and protein in the film, and the specificity of the protein for either the lipid or the water (10). Some proteins tend to extend and some tend to aggregate at the interface, e.g., the apoprotein of high density lipoprotein and the mitochondrial structural protein, respectively (10,35). In membrane systems containing large quantities of protein relative to the lipid, provisions must be made for protein-protein interactions. These can lead to aggregation of protein beyond monolayer thickness in the form of a matrix extending out of the monomolecular contour. This concept supports the conclusions of Zahler et al. (38) on mitochondrial structural protein and of Khaiat and Miller (9) on lecithin-ribonuclease films.

Models of Penetration of Protein Into Lipid Monolayers

It has become increasingly apparent that a transport mechanism of protein and peptides into the air-water interface can be conceived independently of specific lipid-protein interactions and can rest on protein-water and protein-protein interactions, and primarily on specific peptide sequences and conformations which are responsible for the interfacial asymmetry and thus the affinity of protein for the interface (10,11,39). Because of the intricacies of the processes attending the formation of lipid-protein assemblies, it is convenient at present to treat two classes of phenomena separately: molecular organization of protein at the interface and interaction of protein with the lipid monolayer. The mechanism underlying the transport of protein from the hypophase to the interface (10) will not be discussed here. Rather, the foregoing experiments point out a marked dependence of the $\Delta\Pi$ values on the type of lipid and thus bear out the importance of the lipid structure and of some lipid-protein interactions.

In the light of these concepts it is now possible to describe three mechanisms for the incorporation of protein into lipid films: free penetration, binding-mediated penetration and binding-inhibited penetration (32,40). Although these mechanisms draw from observations made with nonspecific proteins, they encompass lipid-protein interactions which may be typical of all lipid-protein assemblies.

Free Penetration

Free penetration is essential with phosphatidyl choline (Fig. 8). In this Figure, the lecithin molecules are organized in surface micelles. Discontinuity in the lipid film is suggested by the concept that the relationships between amphipathic molecules and water are expressed in packages (10) and is supported by a recent conclusion on the micellar structure of lecithin films (41). In a sequence of events X $\rightarrow$ Y $\rightarrow$ Z, globular protein X first extends or expands into Y in contact with the lipid, and this expanded form restructures itself into Z, in the intermicellar lipid spaces. The net result is a mosaic in which packages of protein alternate with or surround clusters of lipid. The surface micelles could be of any shape; for simplicity they can be pictured round as in Figure 8 or rectangular. These surface units resemble open cylinders or trenches, the bottom of which consists of the phosphoryl choline groups. These are oriented parallel to the interface as in Finean's walking stick (31,42).

In this molecular model of lecithin, the β-ester bond, which anchors in the water next to the phosphate, is readily accessible to phospholipase A, whereas the α-ester is located higher up in the hydrophobic region. If the phosphoryl choline group were oriented perpendicular to the interface as in the model proposed by Shah and Schulman (29,43), it would not be possible for the lipase, with an area of about a thousand A^2/molecule to gain access into the upper region of the α and β ester bonds without enormous pressure increases, which are not seen with *C. atrox*. The large pressure increase observed with *N. naja* could be due to the direct lytic factor which is present in the venom (44) and which would operate as a highly surface active cationic peptide (39). The lecithin film is attacked readily by phosphalipase A at 12 to 20 dynes/cm and at a rate which could not be accounted for by penetration by the lipase protein. Another important, probably the soundest, consideration is that the salt linkage or ion pair of the neutralized phosphoryl choline group, being less hydrated and thus more surface minded than the individual ions, is expected to make an interface in the hydrophobic regions of the film rather than to extend into the water. If the choline group were pointing down, the molecular area of hydrogenated phosphatidyl choline would be 36 A^2 instead of the 44 A^2 which we observe at high pressure, and 50 A^2 instead of 80 A^2 at 2 dynes/cm. The evidence can be found in the work of Quarles and Dawson (45) who observed a marked decrease in film pressure meaning a decrease in molecular area after that choline was cleaved off lecithin by phospholipase D, even though the phosphatidic acid

formed did not leave the film. Since the topography of phosphatidyl choline monolayers and of any monolayer is not known, quantitative arguments of molecular orientation based on molecular dipole moments or on molecular area assignments (29,31,43) are not valid. We measure only average molecular area, average surface tension and average surface potential. The meaning of any of these measurements is not available in terms of definite molecular correlates.

An important consequence of the molecular orientation of lecithin is the organization of the protein. Under certain conditions, lecithin films containing up to 50 mole % cholesterol behaved as pure lecithin films towards the penetration of γ-globulin (5). Although there is not direct evidence, it is possible that cholesterol is anchored on the lecithin's phosphate as in Zull's and Finean's models (31,42,46) with consequent filling of the lecithin cylinders. It follows that, when cholesterol is not present, the trenches could be filled with protein (Z form).

A feature of the free penetration mechanism is a possible differentiation into ΔΠ-mechanism and non-ΔΠ-mechanism. The ΔΠ-mechanism pertains to those proteins or protein fragments that form very thin films with protein quantities even smaller than the monolayer quantities. The protein, form W (10), has a high surface valence, namely many points of anchorage that cause the observed increase in film pressure. The non-ΔΠ-mechanism brings large quantities of protein into thick films without or beyond the benefit of surface free energy. Penetration by the protein takes place either through and into hydrophobic channels, whose low dielectric constant favors a spontaneous restructuring of the expanded protein from Y to Z, or in the new form Ω of matrices extending into the aqueous phase and determining abundant water compartments (10,17,47). A balance between W, Z and Ω forms could be a feature of membrane proteins: W for surface anchoring, Z for filling and Ω for capping.

Thus the X → Y → W or ΔΠ-mechanism operates by depositing a thin layer of anchored asymmetric protein structures at the periphery of the surface lipid micelles. In contrast, the X → Y → Z and X → Ω non-ΔΠ-mechanisms bring protein into a thick film. Part of this is in a hydrophobic matrix, such as the upper region of the intermicellar spaces and the lecithin cylinders or trenches above the phosphoryl choline groups, and part forms the maze of a hydrofilic matrix which extends into the aqueous subphase. The non-ΔΠ-mechanism accounts for the film protein in excess of the monolayer quantities, whereas the whole free penetration mechanism rests on the ability of the protein to establish itself at the interface independently of the lipid. This (lecithin) undoubtedly presents to the protein a medium of low dielectric constant in which the X → Y → Z, X → Y → W and X → Y → Ω processes are favored. Some sophisticated and not so obvious polar interactions between lecithin and protein cannot be excluded and must be looked for.

Two predictions are now possible. First, regardless of whether the one or the other form prevails, namely the compact form Z, the spongy form Ω in a thick film, or the extended high valence form W in a thin film, the protein by itself can acquire an asymmetric, amphipatic, conformation that enables it to produce specialized membranous structures in the absence of lipid. Second, the insertion of form Z into intermicellar and intramicellar hydrophobic spaces can hinder the motions of the hydrophobic chains of the lipid. This effect can be related to broadening of the NMR line of the protons in the CH_2 groups of lipid as it was observed with some lipid-protein and with the cholesterol-lecithin systems (48). In contrast, the thin form W inserted in the lipid-water interface with or without the Ω caps, would neither reach nor affect the hydrophobic chains of the lipid. This could explain the observation that in certain serum lipoproteins, the protein does not cause broadening of the NMR line of the methylene protons of the lipid (48-50).

Another important effect of the free penetration mechanism is that the polar groups of lecithin are in contact with water and are not coated with protein. Since lecithin is so abundantly represented in biological membranes, this lipid could be nature's device to bring protein into the membrane and still preserve a lipid-water interface.

Binding-mediated Penetration

Binding-mediated penetration is a mechanism conspicuous with monolayers of cholesterol, cerebrosides and ceramide lactosides. It is characterized by large quantities of protein in the film (Table I) and large ΔΠ values and rates of pressure rise (Fig. 2 and Ref. 4). A relevant feature of this mechanism is a rapid loss of film pressure in the earliest stages of the interaction. When rabbit γ-globulin in a concentration of 5 μg/ml was injected under a cholesterol film at 2 dynes/cm, the pressure fell to zero in the first 10 seconds and rose rapidly thereafter.

An explanation is provided in Figure 9. Before introduction of protein (upper panel),

the cholesterol or long chain alcohol-type molecules are distributed at the interface. Soon after injection, protein is adsorbed under the lipid film by electrostatic interaction, which includes hydrogen bonding. A film contraction ensues with the typical loss of film pressure (central panel). The lipid is now organized in clusters probably bound to the protein. The action of the free alcohol of several lipids may be one in which the OH groups of the lipid stimulate drastic conformation changes in the protein. This then becomes extremely surface active and penetrates into the lipid film in large quantities. The molecular organization of lipid and protein in a cholesterol-protein film is not known and cannot be surmised at present. Two effects are extremely important in this mechanism: film contraction with loss of pressure (Fig. 9, central panel) and massive protein transport (Fig. 9, low panel). The former, I wish to call the Bangham-Papahadjopoulos effect, since it has a significant analogy with the film contraction which Bangham and Papahadjopoulos described in the interaction of Ca^{++} with monolayers of acidic lipids (51). In both cases, the opening of gaps in the film (Fig. 9 central panel) bears a relation to a possible nonspecific diffusion transport of ions and proteins. Although observed since my early acquaintance with these systems, the -$\Delta\Pi$ effect was not reported earlier (4) for two reasons: there was no explanation and its omission in the $\Delta\Pi$-time curves (as in Fig. 2) facilitates the presentation of data. A detailed description of this phenomenon does not belong to this communication. However, it may suffice to know that the effect is clear with low protein concentration, 1 to 5 μg/ml, whereas at higher protein concentrations the -$\Delta\Pi$ effect may be masked by a +$\Delta\Pi$ effect associated with rapid and massive protein penetration.

In the transport of protein into the cholesterol film, with a compression area of cholesterol less than 1/20 of that of lecithin, the cholesterol film picks up more protein than phosphatidyl choline does. In the case of cholesterol a complete reorganization of the film must take place. Cholesterol remains in the film, for in experiments with ^{14}C-cholesterol, after removal of the film, the entire solution was taken to dryness, and no radioactivity was found in the subphase, whereas large quantities of protein were in the film. An implication of this mechanism is that when protein is adsorbed to cover the entire water surface of a lipid film, the lipid-water interface is abolished and a new interface must be formed at the expense of the total reorganization of the lipid-protein film. The net result could be a

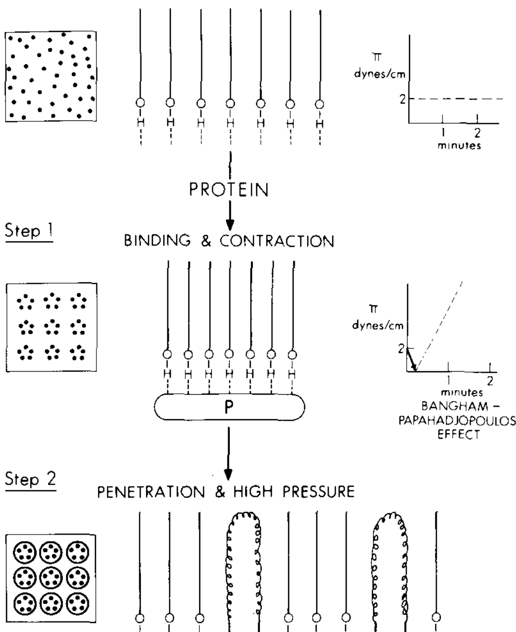


FIG. 9. Pictorial representation of the binding-mediated mechanism, including the Bangham-Papahadjopoulos effect.

lipid-protein assembly of the Benson model type (14) or any mosaic, as in the lower panel of Figure 7. This mechanism, which has some thermodynamic basis (17,47), is in contrast to any Danielli-Davson or Eley and Hedge model of a lipid-protein assembly in which the protein is adsorbed by massive electrostatic interactions that mask the entire lipid-water interface. Should this happen, the consequences of the binding-mediated penetration must set in. However, protein can be adsorbed on the lipid surface provided that the interaction occurs with polar groups which extend into the aqueous phase and are distributed sparingly on the surface of the lipid monolayer. This case is an aspect of the binding-inhibited penetration in which the main lipid (lecithin) surface is not disturbed and abundant water compartments are instituted. The latter separate the lipid surface from the omega protein caps (17,47 and Fig. 9 in Ref. 10).

Binding-inhibited Penetration

Binding-inhibited penetration is a mechanism characterized by a small $\Delta\Pi$ value. It has been verified with ganglioside-albumin at pH 6 to 9 (Fig. 3), with a specific lactoside-antibody system (10), and it probably also applies to sphingomyelin. In a typical model (10), the protein binds with the lipid's more hydrophilic

groups which extend into the subphase; thereby, the Π interface is little affected as it is contributed by the less hydrophilic polar groups of the lipid. The bound protein, extrinsic Ω form (17,47), blocks the routes of access to the interface, thus little protein may enter the Π interface of the film. The net result is a small if any penetration or little increase in film pressure. The ΔΠ-mechanism is so limited or entirely blocked. Noticeable with the ganglioside-albumin system (Fig. 3) is a -ΔΠ effect, indicative of a film contraction in the early stage of the interaction. Relevant to this mechanism and to the formation of thick protein films is the electrostatic adsorption of basic cytochrome c under monolayers of acidic lipids (52) at the high film pressures at which penetration and ΔΠ-mechanism are excluded.

CONCLUSIONS

Rate and extent of penetration of protein into the air-water interface of lipid monolayers, the quantities of protein found in the lipid film, and the relative organization of lipid and protein are influenced markedly by the chemical structure of the lipid. With regard to the mode of incorporation of protein in the lipid monolayers, three mechanisms are visualized: free penetration in phosphatidyl choline, binding-mediated penetration in cholesterol and binding-inhibited penetration of albumin in ganglioside. The qualitative and quantitative information which monolayer studies provide about the orientation of lipids and proteins at water interfaces is unique and must be brought to bear very strongly on the discussion of both membrane biogenesis and molecular organization of membrane components in vivo.

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The Synthesis of Phosphatidylethanolamine and Phosphatidylserine Containing Acetylenic or Cyclopropane Fatty Acids and the Activity of These Phosphatides in Blood Coagulation[1]

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ABSTRACT

Six different phosphatides were made by combination of phosphorus oxychloride and either *t*-butyloxycarbonylaminoethanol or the phthalimidomethyl ester of anisyloxycarbonyl-L-serine with three different diglycerides. The diglycerides were *rac*-1,2-distearoloylglycerol, *rac*-1-stearoloyl-2-stearoylglycerol, and *rac*-1,2-di(9,10-methyleneoctadecanoyl) glycerol. The phosphatides were freed of protective groups by methods that did not cause rupture of the cyclopropane rings. The resulting phosphatides were purified by chromatography and evaluated for their effects on blood coagulation in vitro after solubilization with sodium desoxycholate. The phosphatidyl(distearoloyl)serine and the phosphatidyl di(9,10-methyleneoctadecanoyl) serine were as active as beef brain phosphatidylserine in the antithromboplastin test and the Hicks-Pitney test. The phosphatidyl(stearoloyl, stearoyl)serine was slightly less active in the Hicks-Pitney test. The phosphatidylethanolamines accelerated coagulation in the Hicks-Pitney test. The cyclopropane phosphatidylethanolamine was also tested in a test of prothrombin conversion using all purified components and was found to be active. The high activity of the cyclopropane phosphatidylserine is specially important because of its resistance to autoxidation.

INTRODUCTION

Phosphatidylserine has been shown to be a potent anticoagulant in vivo (1,2) and in vitro (3,4). The anticoagulant activity of phosphatidylserine depends on its degree of microdispersion (solubilization) in aqueous media, water clear sols being most active (1,3,5). [The object of solubilization is to produce water clear solutions with particles of colloidal dimensions. The relation between solubilization and activity and the effect of agents like sodium desoxycholate is discussed in Reference 1.] Phosphatides with long chain saturated fatty acids like phosphatidyl(distearoyl)serine cannot be solubilized. [Phosphatidyl(dihexanoyl)serine can be solubilized (6) but it has little activity in blood clotting tests (unpublished work of this laboratory). Phosphatidyl(dihexanoyl)ethanolamine can also be solubilized (7) but its activity if any is unrecorded.] The active phosphatides that can be solubilized ordinarily contain double bonds. These can be solubilized with the aid of sodium desoxycholate or albumin (3).

Unfortunately, the unsaturated phosphatides are subject to easy oxidation and this is particularly true of the more potent preparations like phosphatidyl(dilinoleoyl)serine (4). Phosphatidylserine is an anticoagulant with potential therapeutic activity, but there is no record of any attempt to develop it for this purpose. This may be partly due to the difficulty in standardization and storage which autoxidation would cause.

Phosphatidylethanolamine is also of great interest because of its demonstrated procoagulant activity (3) and its capacity to shorten the secondary bleeding time in hemophilic dogs (unpublished work of this laboratory).

Our interest in the acetylenic fatty acids as possible components of synthetic phosphatides arose from the statement of Meade (8) that "oxygen attacks simple acetylenes far less readily than the corresponding olefins presumably because the methylenic attack which leads to the symmetrical low energy intermediate -CH-CH-CH- has no equally powerful driving force in acetylenes." This statement is supported by the experimental work of Kuhn

[1]Presented at the AOCS Meeting, Minneapolis, October 1969.

TABLE I

Analysis of Products

Substance	Carbon		Hydrogen		Nitrogen		Phosphorus	
	Theory	Found	Theory	Found	Theory	Found	Theory	Found
Rac-stearoyl,stearoloyl PE	66.10	65.99	10.69	10.28	1.87	1.94	4.15	4.22
Rac-distearoloyl PE	66.46	66.17	10.20	10.55	1.89	1.95	4.18	4.21
Rac-stearoyl,stearoloyl PS	64.01	63.97	9.97	10.02	1.77	1.82	3.93	3.95
Rac-distearoloyl PS	64.34	64.66	9.51	9.60	1.76	1.85	3.95	4.24
Rac-di(9,10-methylene-octadecanoyl) PE	66.89	66.56	10.71	10.47	1.82	1.88	4.01	4.03
Rac-di(9,10-methylene-octadecanoyl) PS	64.75	64.71	10.13	10.10	1.72	1.69	3.79	3.79

and Meyer (9) who measured the oxygen uptake of solutions of oleic acid and stearolic acid in the presence of hemin when shaken with aqueous buffer and oxygen at 37 C. They found no oxidation of stearolic acid (Table II in Ref. 9; also 10). Khan et al. (11), on the other hand, found that methyl stearolate absorbed more oxygen than methyl oleate. In these experiments, oxygen was bubbled through at 75 C. This apparent contradiction will have to be resolved by further experiments.

According to the literature on dihydrosterculic acid and lactobacillic acid (12) the cyclopropane ring resists the attack of powerful oxidizing agents like potassium permanganate, monoperphthalic acid (12) and chromic acid (13). We decided to synthesize phosphatidylserine and phosphatidylethanolamine containing acetylenic and cyclopropane fatty acids to find out if these phosphatides could be solubilized and tested in blood coagulation systems.

The cyclopropane phosphatides might also be of interest for the study of bacterial metabolism since it has been shown by Zalkin et al. (14,15) that phosphatidylethanolamine is a precursor in the formation of cyclopropane fatty acids.

EXPERIMENTAL PROCEDURES AND RESULTS

Materials and Methods

Stearolic Acid. This was purchased from Farchan Research Laboratories, Willoughby, Ohio. Two samples were used. The first sample assayed 99.4% C_{18} monoyne by gas liquid chromatography (GLC). This was used to prepare *rac*-phosphatidyl(stearoloyl, stearoyl) ethanolamine and *rac*-phosphatidyl(stearoloyl, stearoyl)serine. The distearoloyl phosphatides were made from a second sample of stearolic acid which contained 95.2% C_{18} monoyne, 3.6% of C_{18} monoene and 1.2% of C_{18} diene. A small quantity of *rac*-phosphatidyl(distearoloyl)serine was also made from the first (99.4%) sample of stearolic acid. This material did not differ from the other phosphatidylserine (from the 95% sample) in dispersibility or effect in blood coagulation.

Stearic acid (99.5% pure) was purchased from Stearinerie Dubois Fils, Scoury, Commune de Ciron (Indre), France.

Cis-dl-9,10-Methyleneoctadecanoic Acid. This was made by the Simmons-Smith reaction (16,17) as modified by Christie and Holman (18). Christie and Holman describe the synthesis on a very small scale. For 20 g of methyl oleate, we employed 50 g of zinc dust converted to the copper couple with 200 g of diiodomethane in 400 ml of anhydrous ether. The product was freed of diiodomethane and purified on a florisil column as described (18). The product contained 87.4% of cyclopropane fatty ester as determined by GLC. The ester was saponified with alkali and the free acid was converted to the acid chloride with oxalyl chloride. The free acid had the infrared spectrum indicated in the literature (19).

t-Butyloxycarbonylaminoethanol was made as described by Daemen et al. (20). The phthalimidomethyl ester of *p*-methoxybenzyloxycarbonyl-L-serine was made as described (4).

Silicic acid was either Bio-Sil from Bio-Rad Laboratories, Richmond, Calif., or silicic acid type CC-7 from Mallinckrodt Chemical Works, St. Louis, Mo. In each case it was washed thoroughly with solvent as described by Rouser et al. (21). Silica Gel H for thin layer chromatography (TLC) was a product of Merck of Darmstadt, furnished by Brinkmann Instruments, Inc., Westbury, N.Y. DEAE cellulose was Whatman No. DE-23 from Reeve Angel, Inc., Clifton, N.J.

All operations were conducted under nitrogen, using the glove box of I2R Inc., Cheltenham, Pa.

TABLE II

GLC Analysis of Fatty Acid Content

Substance	C_{18} monoyne	C_{18} satd.	C_{18} monoene (?)[a]	C_{18} diene (?)[a]	C_{19} cyclopropane
Rac-stearoyl, stearoloyl PE	49.3%	50.7%	---	---	
Rac-distearoloyl PE	95.2%		3.6%		
Rac-stearoyl stearoloyl PS	49.1%	50.9%	---	---	
Rac-distearoloyl PS					
Sample 1	92.8%		5.8%	1.1%	
Sample 2	99.8%				
Rac-cyclopropane PE					91.1%
Rac-cyclopropane PS					93.3%

[a]The question mark indicates that the retention times were not quite identical to those of standard esters.

Carbon, hydrogen, nitrogen and phosphorus analyses were by Galbraith Laboratories, Inc., Knoxville, Tenn.

GLC Analyses. The analyses were made with an Aerograph 1522 (Varian Aerograph, Walnut Creek, Calif.) chromatograph equipped with dual hydrogen flame ionization detectors. The column was an 8 ft x 1/8 in. o.d. (0.093 in i.d.) stainless steel coiled tube packed with 12% stabilized diethylene glycol succinate (DEGS) on 60-80 mesh acid washed Chromosorb W and maintained at 175 C. The areas under the peaks of the individual components were determined by an electronic integrator (Infotronics, Model CRS-11HSB, Houston, Texas) coupled to a digital printer. The methyl esters of the fatty acids and phospholipids were prepared by the method of Luddy et al. (22). Identifications were made from comparison with reference samples when available and from published data on retention volumes.

Rac-1,2-Distearoloylglycerol. Rac-1-(2′-tetrahydropyranyl)-glycerol was made according to Barry and Craig (23). Stearoloyl chloride was made from stearolic acid and oxalyl chloride. Acylation of the glycerol derivative with the stearoloyl chloride was performed by the method used by Baer and Buchnea for the acylation of other glycerol derivatives (24). The tetrahydropyranyl protecting group was removed from the product by the method of Paltauf and Spener (25) to give the *rac*-1,2-distearoloylglycerol. This product was purified on a column of silicic acid by elution with *n*-hexane followed by 5% ether in hexane and 30% ether in hexane. The fractions were examined by TLC using a boric acid impregnated Silica Gel H (26) and the solvent *n*-hexane-ether-acetic acid 7:20:7 by comparison with a standard mixture of triglyceride, 1,2,- and 1,3-diglycerides and monoglyceride furnished by Applied Sciences, Inc., State College, Pa. The fraction eluted from the column with 30% ether in hexane was found to be the 1,2-diglyceride and showed only a single spot on GLC. This fraction was used for the synthesis of phosphatides.

Rac-1-Stearoloyl-2-Stearoylglycerol. 2-Stearoylglycerol was made according to Bogoslovskii et al. (27) from 1,3-benzylideneglycerol which was made according to Johary and Owen (28). To a solution of 15.0 g of 2-stearoylglycerol in 6.9 ml of pyridine and 200 ml of benzene stirred at 30 C was added dropwise in the course of 40 min, a solution of 10 g of stearoloyl chloride in 100 ml of benzene. The mixture was stirred for two days at room temperature. It was then diluted with anhydrous ether and centrifuged to remove pyridine hydrochloride. The ether-benzene was washed four times with water and dried over sodium sulfate. Evaporation gave an oil which was dissolved in 185 ml of hexane and cooled in ice water to give some recovered 2-stearoylglycerol. The filtrate from this was evaporated to an oil which was taken up in 175 ml of 95% ethanol. After stirring for 10 min at 30 C the mixture was centrifuged giving a trace of undissolved oil which was discarded.

The ethanol solution was evaporated to give a crystalline residue, mp 34-36 C. TLC of this material on silica gel impregnated with boric acid, using the solvent *n*-hexane-ether-acetic acid 70:20:7 showed only one spot which ran opposite authentic 1,2-diglyceride.

Analysis Calculated for $C_{39}H_{72}O_5$ (620.97): C, 75.42; H, 11.69. Found: C, 75.67; H, 11.75.

Rac-1,2-di(cis-dl-9,10-Methyleneoctadecanoyl)Glycerol. This was made using the cyclopropane fatty acid chloride as described above for the distearoloylglycerol. The purifi-

TABLE III

Procoagulant Activity of Solubilized[a] Phosphatidyl(Distearoloyl)Ethanolamine and Phosphatidyl(Stearoyl,Stearoloyl)Ethanolamine in the Modified Hicks-Pitney Test (3)

Substance tested	Micrograms in incubation mixture	Incubation time in minutes			
		2	4	6	8
		Substrate clotting time in seconds			
Distearoloyl PE	100	>50	17.0	11.2	8.8
	5	45.5	13.8	9.0	8.0
Stearoyl,stearoloyl PE	100	>80	36.5	30.0	15.8
	5	59.8	31.2	20.5	15.0
Controls					
Crude phosphatides (40)	6	85.0	12.0	7.8	7.8
Buffered saline (3)	---	>90	46.7	30.5	22.8
Sodium desoxycholate (3)	100	>90	68.0	33.0	23.0

[a]Each substance tested was solubilized in a solution of sodium desoxycholate in buffered saline (3).

cation was also similar. The product showed only one spot on TLC running with the same R_f as a standard 1,2-diglyceride.

Synthesis of Protected Phosphatides. The combination of the diglycerides with phosphorus oxychloride and the phthalimidomethyl ester of anisyloxycarbonyl-L-serine or *t*-butyloxycarbonylamino ethanol was conducted as described by Baer and Buchnea (29) except that the product was worked up in chloroform instead of ether (30,31).

Removal of the Phthalimidomethyl Group. This was done with 95% hydrazine dissolved in ethanol by incubation at 37 C for two days as described (31).

Removal of BOC and Anisyloxycarbonyl Protecting Groups. The product from the phosphorus oxychloride synthesis (in the case of phosphatidylethanolamine) or from the removal of the phthalimidomethyl group (in the case of phosphatidylserine) was dissolved in 97% formic acid and the solution was kept at room temperature for 3 hr (32). The solution was then freeze-dried to remove formic acid.

The phosphatidylserines were purified on a column of DEAE cellulose acetate by the method of Rouser et al. (21,33,34), the desired fraction being eluted with glacial acetic acid. The acetic acid was removed by freeze-drying. The product was examined by TLC using Silica Gel H and the solvent system of Grisdale and Okany (35), staining with ninhydrin and the phosphorus stain of Long et al. (36). The phosphatidylserines ran with the same R_f as a standard preparation of phosphatidylserine from beef brain. However, they contained a trace of impurity at the origin. To remove this impurity, the phosphatidylserines were chromatographed on silicic acid as described previously (4,31). The analyses of the products are shown in Table I and II.

The phosphatidylethanolamines were eluted from a DEAE cellulose acetate column with 10% methanol in chloroform and this fraction

TABLE IV

Procoagulant Activity of Solubilized[a] Phosphatidylethanolamine Containing Cyclopropane Fatty Acids in the Modified Hicks-Pitney Test (3)

Substance tested	Micrograms in incubation mixture	Incubation time in minutes			
		2	4	6	8
		Substrate clotting time in seconds			
Cyclopropane PE	200	85.3	35.2	24.0	7.5
	100	>90	35.0	9.5	9.0
	50	>90	60.5	48.0	43.0
Controls					
Crude phosphatides (40)	6	27.2	8.5	7.8	7.5
Buffered saline (3)	---	>90	89.6	44.0	37.0
Sodium desoxycholate (3)	200	>90	>90	>90	57.6

[a]Each substance was solubilized in a solution of sodium desoxycholate in buffered saline (3).

TABLE V

Anticoagulant Activities of Solubilized[a] Phosphatidyl(Distearoloyl)Serine, Phosphatidyl (Stearoloyl,Stearoyl)Serine and Beef Brain Phosphatidylserine in the Modified Hicks-Pitney Test (3) Versus Coagulant Phosphatides

		Incubation time in minutes			
	Micrograms in incubation mixture	2	4	6	8
Substance tested		Substrate clotting time in seconds			
Distearoloyl PS	10.0	>90	>90	>90	>90
	5.0	>90	71.8	82.2	38.0
	1.0	59.0	14.0	8.0	7.8
Stearoyl,stearoloyl PS	12.5	>90	>90	>90	>90
	10.0	>90	88.2	44.2	42.2
	5.0	>90	41.5	38.0	13.0
	1.0	>90	18.5	10.8	9.2
Beef brain PS (3)	10.0	>90	>90	>90	>90
	5.0	>90	81.0	50.0	52.5
	1.0	>90	31.0	24.2	10.8
Controls					
Crude phosphatides (40)	6	>90	19.0	8.8	8.5
Crude phosphatides + Desoxycholate	6 10	50.0	13.5	7.8	7.8
Buffered saline	---	>90	43.5	24.0	20.0

[a]Each substance tested was solubilized in a solution of sodium desoxycholate in buffered saline. All substances were tested for their anticoagulant activity against the acceleratory activity of crude phosphatides.

was further purified on Mallinckrodt CC-7 silicic acid. The pure material was eluted with 10% methanol in chloroform. The analyses of the phosphatidylethanolamines are shown in Tables I and II. The yield of pure phosphatide in these syntheses was about 500 mg from 5 g of diglyceride.

All of the six synthesized phosphatides were pure when compared to standard preparations by chromatography on Whatman SG-81 silica impregnated paper by the method of Marinetti (37). A load of 250 µg was employed.

Hydrolysis of Mixed-Acid Phosphatide. This was done using Russell's viper venom as described by DeHaas and Van Deenen (38) on a sample of *rac*-phosphatidyl(stearoloyl, stearoyl) ethanolamine using an equimolar amount of palmitic acid as a standard. TLC showed the production of equal amount of lysophosphatide and unchanged phosphatide as expected from the hydrolysis of a racemic phosphatide. GLC of the fatty acid methyl esters showed equal amounts of palmitic and stearic acids.

Procoagulant Activity of the Synthetic Phosphatidylethanolamines

The *rac*-phosphatidyl(distearoloyl)ethanolamine was shown to be a potent accelerator of thromboplastin generation. As little as 5 µg produced substrate clotting times of 9 sec in the test system shown in Table III. The *rac*-phosphatidyl(stearoloyl, stearoyl)ethanolamine was considerably less active. The solubilized phosphatidylethanolamine containing cyclopropane fatty acids was active in tests of thromboplastin generation at 100 µg in the incubation mixture (Table IV). At lower concentrations of the phosphatidylethanolamine the procoagulant activity was not evident. The phospholipid was also effective in the conversion of prothrombin to thrombin in a test system described elsewhere (39) which employs all purified clotting factors. The cyclopropane phosphatidylethanolamine (100 µg) was able to produce 5.1 units of thrombin after 1 min incubation, whereas crude phosphatides (40) produced 4.5 units. At lower concentrations of phosphatidylethanolamine the prothrombin converting activity fell off sharply.

Anticoagulant Activity of the Phosphatidylserines

All the synthetic phosphatidylserines were tested for their ability to interfere with thromboplastin generation and for their ability to overcome the strong procoagulant effect of brain thromboplastin. In addition, the cyclopropane phosphatide was tested for its ability to inhibit prothrombin conversion in a test system employing all purified components (39). In all cases there was a potent anticoagulant effect. In the prothrombin conversion test there was marked inhibition of rate and amount of

TABLE VI

Anticoagulant Activity of Solubilized Cyclopropane Phosphatidylserine Compared to That of Beef Brain Phosphatidylserine in the Modified Hicks-Pitney Test (3) Versus Coagulant Phosphatides (40)

Substance tested	Micrograms in incubation mixture	Incubation time in minutes: 2	4	6	8
		Substrate clotting time in seconds			
Cyclopropane PS	10	>90	>90	>90	71.2
	7.5	>90	>90	>90	62.9
Beef brain PS	20	>90	>90	>90	98.0
	10	>90	69.5	46.5	12.8
Controls					
Crude phosphatides (40)	6	32.5	7.5	7.5	7.5
Buffered saline (3)	---	90	67.8	43.5	40.0
Sodium desoxycholate (3)	20	90	90	60.2	41.4

[a]Each substance was solubilized in a solution of sodium desoxycholate in buffered saline (3).

prothrombin conversion. With 10 μg of the cyclopropane phosphatide in the test system, 0.88 units of thrombin were obtained after 1 min of incubation of the prothrombin converter (plus inhibitor) and prothrombin, while the control (without cyclopropane PS) produced 2.0 units of thrombin. After 10 min of incubation, the control produced 6.24 units of thrombin while 10 μg of added cyclopropane PS reduced this to 3.68 units of thrombin. The anticoagulant effect against thromboplastin generation and against brain thromboplastin are shown in Tables V and VI.

In the Hicks-Pitney test the action of phosphatidylserine against the strong acceleratory activity of a crude thromboplastin fraction from brain tissue was examined. The strong anticoagulant activity of 10 μg of *rac*-phosphatidyl(distearoloyl)serine (Table V) or of *rac*-phosphatidyl di(9,10-methylene-octadecanoyl) serine (Table VI) is clear at 10 μg where the activity is similar to that of phosphatidylserine from beef brain. At lower concentrations the activity diminished, and with only 1 μg of phosphatidylserine in the test the substrate clotting times were similar to that of the control containing only the acceleratory crude phosphatides (Table V).

Table VII shows that the cyclopropane phosphatidylserine is equivalent to beef brain phos-

TABLE VII

Anticoagulant Activity of Solubilized[a] Cyclopropane Phosphatidylserine Compared to That of Beef Brain Phosphatidylserine in the Antithromboplastin and Recalcification (3) Tests

Substance tested	Microgram in clotting test	Antithromboplastin test; clotting time, sec	Recalcification test; clotting time, sec
Cyclopropane PS	200	176	2,750
	100	66	985
	50	48	485
	10	25	300
	1	14	210
Beef brain PS	200	173	3,440
	100	84	1,405
	50	32	335
	10	21	280
	1	14	215
Controls			
Desoxycholate	100	14	165
Buffered saline	---	14	215

[a]Each substance was solubilized in a solution of sodium desoxycholate in buffered saline (3).

phatidylserine in the antithromboplastin and recalcification tests. Similar results were obtained with both the phosphatidyl(distearoloyl)serine and phosphatidyl(stearoloyl, stearoyl)serine (not shown). However, the mixed acid phosphatidylserine was not as active in the Hicks-Pitney test (Table V) as the phosphatide containing two stearoloyl residues.

All the tests employed phosphatidylserines solubilized with the aid of sodium desoxycholate. Similar results were obtained when the synthetic phosphatidylserines were solubilized with albumin solution (3).

Small amounts of dilute alkali were employed to aid in the solubilization with albumin. Phosphatidylserine with protonated carboxyl group is not readily solubilized with albumin solution, whereas sodium or potassium salts of phosphatidylserine are (3).

DISCUSSION

The purpose of this work was to synthesize phosphatides having potent biological activity and fatty acids that would not be autoxidizable. In this paper, we report synthetic phosphatides containing triple bonds or cyclopropane rings rather than double bonds.

The synthesis followed the general scheme devised originally by Baer and Buchena (29) which has been used in our earlier work (30). In this scheme, phosphorus oxychloride is combined with a diglyceride and a protected base, either *t*-butyloxycarbonylaminoethanol or the phthalimidomethyl ester of anisyloxycarbonyl-L-serine. For the removal of protective groups, hydrazine is employed to remove the phthalimidomethyl group as described originally by Nefkens et al. (41). The method of Halpern and Nitecki (32) for removing the *t*-butyloxycarbonyl protecting group was found to be equally applicable to the anisyloxycarbonyl group. This gave an improvement in yield when compared to our previous method employing hydrogen chloride.

The removal of the *t*-butyloxycarbonyl and anisyloxycarbonyl protecting groups by formic acid is essential for the success of the synthesis of cyclopropane phosphatides. Prior to the appearance of the paper of Halpern and Nitecki (32), we had prepared pure phosphatides containing cyclopropane fatty acids by removal of the protecting groups using hydrogen chloride in chloroform. These phosphatides were homogeneous by TLC and paper chromatography. Unfortunately, GLC showed the phosphatidylethanolamine made in this way to contain only 23% of cyclopropane fatty acids with at least 10 other unidentified components. Our products also contained some halogen. It is well known that hydrogen chloride rearranges the cyclopropane ring to methyl olefines (13). Using formic acid to remove protecting groups, it was possible to get phosphatides containing 93% of cyclopropane fatty acids. This is probably as good as can be expected, since the Simmons-Smith reaction is not quantitative (42).

The mixed acid phosphatide, *rac*-phosphatidyl(stearoloyl, stearoyl)serine was hydrolyzed with phospholipase A to determine whether any rearrangement occurred in the synthesis. Since the product of the hydrolysis was stearic acid, no rearrangement in fact occurred. It is well established that this enzyme acts exclusively in the 2-position of 3-phosphoglycerides (43).

One of the major problems in studying the coagulant or anticoagulant activity of phospholipids or considering their use as therapeutic agents is their tendency to autoxidation. The most active of the naturally occurring or synthetic phosphatides with coagulant (3) or anticoagulant (3,4) activity have all contained unsaturated fatty acids. As autoxidation proceeds, their color gradually changes from white to yellow. This is accomplished by a loss of clot accelerating activity of the phosphatidylethanolamines and the appearance of lysophosphatide and other hydrolysis products in the TLC of phosphatidylserine (44). It is hoped that substitution of cyclopropane rings for double bonds will eliminate the problem of autoxidation.

The synthetic phosphatidylserines and phosphatidylethanolamines with triple bonds had the same, strong biological activity as compounds with two double bonds (4,33) or as phosphatidylserine from beef brain in in vitro tests of blood coagulation. The phosphatides containing triple bonds in both fatty acids had more activity (procoagulant for phosphatidylethanolamine and anticoagulant for phosphatidylserine) than those having a triple bond in only one of the fatty acids.

The cyclopropane phosphatidylserine also had as much anticoagulant activity in vitro as beef brain phosphatidylserine. The latter has been shown to be a potent anticoagulant in vivo (1,2). The cyclopropane phosphatidyl ethanolamine had less activity than the PE with triple bonds.

Activity of phosphatides in blood coagulation tests is related to the colloidal state of the phospholipid particles in the aqueous dispersion and particularly to the size and shape of the dispersed particles and their surface charge (3,45-48). It would appear that these character-

istics are similar for the phosphatides containing two triple bonds or two cyclopropane rings to those prevailing with the corresponding naturally occurring phosphatides having strong biological activity. The triple bond and the cyclopropane ring probably resemble the double bond in producing optimal separation of the fatty acid chains in the micelles. Another factor related to solubilization and biological activity may be the lowering of the transition temperature from crystalline to liquid crystalline produced by double bonds in the phospholipid molecule (49,50).

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SHORT COMMUNICATIONS

Inhibition of Renal Cortical Rb^+ Transport by Phospholipase C and its Relationship to (Na^+ + K^+)-Adenosine Triphosphatase

ABSTRACT

Treatment of isolated renal cortical tubules with a low concentration of phospholipase C (one which does not lead to a chemically detectable loss of tubular phospholipid) results in a markedly inhibited Rb^+ transport function which is referable to the loss of a phospholipid component of the tubules. This treatment also results in an inhibition of the (Na^+ + K^+)-adenosine triphosphatase enzyme of the tubules. The data are consistent with the concept that it is the loss in (Na^+ + K^+)-adenosine triphosphatase activity which is responsible for phospholipase C-induced inhibition of Rb^+ transport.

It was recently reported from our laboratory (1) that treatment of isolated rabbit renal cortical tubules with a protease free preparation of phospholipase C from *C. welchii* causes marked inhibition of the ability of the tubules to transport Rb^+ from the incubation medium into the cell. This inhibitory effect occurred even when phospholipase C was reduced to levels that caused no chemically detectable destruction of tubule phospholipid. Nevertheless, this effect was shown to be related to activity of the enzyme since agents that inhibit the phospholipase C activity of the *C. welchii* enzyme preparation cause an equivalent loss in the preparation's ability to inhibit Rb^+ uptake. It was also shown that phospholipase C inhibition of Rb^+ uptake was not due to a generalized destruction of renal tubular structure and function nor to an inhibition of the plasma membrane transport processes in general. The conclusion was therefore drawn that a phospholipid structure or structures on the outer surface of the plasma membrane of renal cortical tubule cells is intimately involved in the uptake of Rb^+ by these cells.

From a theoretical point of view, there are several ways that such a phospholipid structure could function in Rb^+ transport, e.g., by serving as a carrier molecule or affecting the conformation of a carrier molecule or influencing the structure of the membrane or affecting the activity of an enzyme involved in some aspect of the transport or a combination of these. That it does so by playing a role in the functioning of (Na^+ + K^+)-adenosine triphosphatase in the plasma membrane of the renal cortical cells seemed likely for the following reasons: (a) (Na^+ + K^+)-adenosine triphosphatase has been found to depend on the presence of lipids to be active (2); (b) Rb^+ can replace K^+ in the activation of the enzyme (3); (c) this adenosine triphosphatase is involved in K^+ transport across cell membranes (4); and (d) Rb^+ is believed to be transported by the same system that transports K^+ (5). The findings presented below indeed show that, when renal cortical tubules are treated with levels of phospholipase C which cause no chemically detectable loss of phospholipids but a markedly depressed Rb^+ uptake, inhibition of the (Na^+ + K^+)-adenosine triphosphatase activity of the tubules occurs. Moreover, the extent of the depression of the adenosine triphosphatase activity correlates rather well with the amount of inhibition of Rb^+ uptake.

Rabbit renal cortical tubules were prepared as described in an earlier paper (1). Commercial phospholipase C was purified by the method of Ispolatovskya et al. (6). Treatment of the tubules with phospholipase C, measurement of Rb^+ uptake and measurement of the phospholipid content of the renal tubules were all carried out as described previously (1). Cell free homogenates were prepared from isolated tubules in the following manner: The tubules were suspended in 0.001 M $NaHCO_3$ (approximately 100 mg of tubule wet weight per 1 ml of solution) in a glass homogenization tube and homogenized by four strokes of a motor driven teflon pestle. This fraction is called the whole homogenate. An aliquot of the whole homogenate was further fractionated into a crude membrane fraction by a modification of the differential centrifugation method of Manitius et al. (7).

TABLE I

Effect of Phospholipase C (Plase C) on Renal Tubules Rb^+ Uptake and $(Na^+ + K^+)$-Adenosine Triphosphatase Activities[a]

						$(Na^+ + K^+)$-*Adenosine triphosphatase* (μmoles P_i/mg tubular protein)[d]							
						Whole homogenate fraction				Crude membrane fraction			
		Phospholipid content		Rb^{+}[c] sequestered[c]		No DOC treatment		DOC treatment[e]		No DOC treatment		DOC treatment[e]	
Experiment	Plase C treatment[b] (U/ml)	Lipid P (μEq/mg protein)	% loss due to Plase C	μEq/g dry wt tubules	% loss due to Plase C	Plase C treatment	% loss due to Plase C	Plase C treatment	% loss due to Plase C	Plase C treatment	% loss due to Plase C	Plase C treatment	% loss due to Plase C
1	0 0.17	0.37 0.37	0	50 31	38	115 101	12	260 173	34	253 163	36	341 152	54
2	0 0.14	0.25 0.25	0	44 31	31	84 82	3	224 164	27	193 139	28	310 160	48
3	0 0.17	0.32 0.29	9	47 26	45	— —	—	114 66	43	251 130	48	582 267	54

[a]All values are the average from duplicate determinations.

[b]Tubule suspensions containing 10 mg of protein per ml were treated with purified phospholipase C as described (1).

[c]After incubation with ^{86}Rb for 5 min at 25 C as described (1).

[d]$(Na^+ + K^+)$-*Adenosine triphosphatase* activity was defined as the difference between total adenosine triphosphatase and Mg^{++} adenosine triphosphatase. It was expressed as the amount of P_i liberated during 20 min incubation at 37 C. The incubation medium for measuring *total adenosine triphosphatase* activity contained 100 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 3 mM ATP, 10 mM NaCN, 0.1 mM EDTA, 21 mM tris buffer at pH 7.5; for Mg^{++} adenosine triphosphatase contained 15 mM NaCl, 3 mM $MgCl_2$, 3 mM ATP, 10 mM NaCN, 0.1 mM EDTA, 1 mM ouabain and 125 mM tris buffer at pH 7.5. Both systems were adjusted to 2 ml.

[e]The whole homogenate and crude membrane preparations were incubated at 0 C with 2.4 mM sodium deoxycholate (DOC), 3 mM EDTA and 50 mM imidazole buffer (pH 7.5 at 20 C) for 30 min (11), then processed for $(Na^+ + K^+)$-adenosine triphosphatase assay.

The (Na^+ + K^+)-adenosine triphosphatase activity of the whole homogenate and crude membrane fractions was measured by a modification of the methods of Bonting et al. (8), and Skou (9). Prior to assay, half of the fraction was treated with deoxycholate by the method of Jorgensen (11) because it has been shown that much of the (Na^+ + K^+)-adenosine triphosphatase of kidney is not measured without prior treatment with detergent. The concentration of deoxycholate used was that which yielded maximum (Na^+ + K^+)-adenosine triphosphatase activity. Adenosine triphosphatase assay was made on both deoxycholate-treated and untreated preparation for each of the tissue fractions studied. The reaction was started by adding the enzyme in an amount sufficient to hydrolyze 10% to 20% of the total ATP present in 20 min at 37 C. At the end of the incubation period, 0.2 ml of 50% trichloroacetic acid were added, and the P_i liberated was determined by the method of Fiske and Subbarow (10). With the exception of an initial rapid burst of activity similar to that reported for the adenosine triphosphatase of myosin B (12), the (Na^+ + K^+)-adenosine triphosphatase activity was linear for 60 min. For the routine assays reported in Table I, the incubation time was 20 min. The protein content of the various cellular fractions was determined by the method of Lowry et al. (13).

The same population of tubules, either untreated or treated with a level of phospholipase C that caused almost no detectable loss in phospholipid, were used to study Rb^+ uptake and (Na^+ + K^+)-adenosine triphosphatase activity. The results are reported in Table I. Phospholipase C treatment markedly depresses Rb^+ uptake by the tubules. Although only three experiments are reported in Table I, the results are similar to 23 such experiments on Rb^+ uptake reported previously; moreover the previous work clearly shows that this effect is due to phospholipase C activity and not to some contaminant in the enzyme preparation (1). This phospholipase C treatment of the tubules also lowers the (Na^+ + K^+)-adenosine triphosphatase activity of homogenates prepared from these tubules (Table I). With whole homogenates treated with deoxycholate, there is a good correlation between the extent of inhibition of (Na^+ + K^+)-adenosine triphosphatase activity and Rb^+ uptake. However when whole homogenates are not treated with deoxycholate prior to adenosine triphosphatase assay, the correlation is poor (both on the basis of the data in Table I and data from several other experiments in which the only measurement made was the adenosine triphosphatase activity of whole homogenates without prior deoxycholate treatment). The crude membrane fraction has a (Na^+ + K^+)-adenosine triphosphatase specific activity 1 1/2 to 2 times that of whole homogenates and this activity is inhibited by the treatment of the tubules with phospholipase C. The level of adenosine triphosphatase inhibition in crude membrane fractions treated with deoxycholate and those not so treated correlates well with the extent of loss in Rb^+ uptake.

Martonosi et al. (14) reported that phospholipase C inhibited (Na^+ + K^+)-adenosine triphosphatase of a kidney microsome preparation only if the microsomes had been incubated with a high concentration of phospholipase C (10 mg of microsomal protein per mg of phospholipase C) for a long period of time. This finding contrasts sharply with the exquisite sensitivity of the (Na^+ + K^+)-adenosine triphosphatase to phospholipase C action in intact cells. Our previous work (1) establishes that a phospholipid structure on the outer surface of the plasma membrane of tubule cells is involved in Rb^+ transport; presumably this structure is also involved in the (Na^+ + K^+)-adenosine triphosphatase.

In summary, it was previously shown that phospholipase C induces a decrease in Rb^+ uptake by kidney tubules and that this inhibition of Rb^+ uptake results from a small destruction of phospholipid and not from a contaminant of the purified phospholipase C preparation (1). Similar treatment of the tubules with phospholipase C causes a loss in (Na^+ + K^+)-adenosine triphosphatase activity when this enzyme is assayed in whole homogenate or crude membrane fractions prepared from the tubules. The inhibition of this adenosine triphosphatase activity correlates well with the inhibition of Rb^+ uptake. It is postulated that it is the change in the (Na^+ + K^+)-adenosine triphosphatase activity which is responsible for the phospholipase C-induced inhibition of Rb^+ transport.

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Liberation of Aldehydes From Alk-1-enyl Glyceryl Ethers by Acid Hydrolysis

ABSTRACT

Labeled alk-1-enyl glyceryl ethers were used *in conjunction* with *thin* layer chromatography to study the liberation of aldehydes from alk-1-enyl glyceryl ethers by two acid hydrolysis procedures. Both methods gave similar results, but neither liberated the aldehydes quantitatively. Only 75-85% of the alk-1-enyl glyceryl ether radioactivity was liberated as free aldehydes. Several nonaldehyde products were detected and one appeared to be a cyclic acetal.

Synthetic alk-1-enyl ethers (vinyl ethers) are hydrolyzed by acid to aldehydes and alcohols (1). Karnovsky et al. (2) reported the use of methanolic HCl to release aldehydes from the unsaponifiable lipid fractions of biological origin. Since then, the acid lability of the alk-1-enyl glyceryl ethers has been used to an advantage for the analyses of neutral glycerides and phosphoglycerides containing these ethers. Schmid and Mangold (3) have reported a technique for the selective acid hydrolysis of neutral plasmalogens (alk-1-enyl glyceryl ether diesters) and separation of the products (aldehydes and diglycerides) on a thin layer chromatoplate. The technique has since been applied to the phosphatides containing alk-1-enyl glyceryl ethers (plasmalogens) (4-6), alk-1-enyl acyl glycerides (7) and free alk-1-enyl glyceryl ethers (8,9).

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[2] Under contract with the U.S. Atomic Energy Commission.

The liberation of aldehydes from the intact plasmalogens is reported to be quantitative (4,6). However, when free alk-1-enyl glyceryl ethers were exposed to HCl fumes, the developed chromatoplate showed other compounds in addition to free aldehydes (10). Unlike the intact neutral plasmalogens and plasmalogens, the free alk-1-enyl glyceryl ethers contain free hydroxyl groups that can lead to the formation of cyclic acetals (11,12). An investigation into the quantitative aspects of the acid hydrolysis of alk-1-enyl glyceryl ethers on adsorbent layers was attempted with a labeled aldehyde, a product of the hydrolysis reaction (10). However, until now, the lack of radioactive labeled alk-1-enyl glyceryl ethers has not permitted an accurate evaluation of the reaction.

Labeled alk-1-enyl glyceryl ethers were obtained from the experiments with Ehrlich ascites cells in which we reported the route of plasmalogen biosynthesis (13,14). The phosphatidyl ethanolamine class was reduced with lithium aluminum hydride (12) and the labeled alk-1-enyl glyceryl ethers were separated from the hydrogenolysis products by thin layer chromatography (TLC) (13,14). The only detectable contaminant of the alk-1-enyl glyceryl ethers was 6.2% alkyl glyceryl ether, for which the data have been corrected. The disappearance of all the alk-1-enyl glyceryl ether radioactivity (Fig. 1C) after acid treatment indicates the absence of other labeled impurities. The tritium and carbon-14 label was located in the hydrocarbon chain as established earlier (13,14). The distribution of radioactivity along the developed TLC plates was determined by counting the radioactivity in successive 2 mm sections of adsorbent layer (13).

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Labeled alk-1-enyl glyceryl ethers were used *in conjunction with thin* layer chromatography to study the liberation of aldehydes from alk-1-enyl glyceryl ethers by two acid hydrolysis procedures. Both methods gave similar results, but neither liberated the aldehydes quantitatively. Only 75-85% of the alk-1-enyl glyceryl ether radioactivity was liberated as free aldehydes. Several nonaldehyde products were detected and one appeared to be a cyclic acetal.

Synthetic alk-1-enyl ethers (vinyl ethers) are hydrolyzed by acid to aldehydes and alcohols (1). Karnovsky et al. (2) reported the use of methanolic HCl to release aldehydes from the unsaponifiable lipid fractions of biological origin. Since then, the acid lability of the alk-1-enyl glyceryl ethers has been used to an advantage for the analyses of neutral glycerides and phosphoglycerides containing these ethers. Schmid and Mangold (3) have reported a technique for the selective acid hydrolysis of neutral plasmalogens (alk-1-enyl glyceryl ether diesters) and separation of the products (aldehydes and diglycerides) on a thin layer chromatoplate. The technique has since been applied to the phosphatides containing alk-1-enyl glyceryl ethers (plasmalogens) (4-6), alk-1-enyl acyl glycerides (7) and free alk-1-enyl glyceryl ethers (8,9).

The liberation of aldehydes from the intact plasmalogens is reported to be quantitative (4,6). However, when free alk-1-enyl glyceryl ethers were exposed to HCl fumes, the developed chromatoplate showed other compounds in addition to free aldehydes (10). Unlike the intact neutral plasmalogens and plasmalogens, the free alk-1-enyl glyceryl ethers contain free hydroxyl groups that can lead to the formation of cyclic acetals (11,12). An investigation into the quantitative aspects of the acid hydrolysis of alk-1-enyl glyceryl ethers on adsorbent layers was attempted with a labeled aldehyde, a product of the hydrolysis reaction (10). However, until now, the lack of radioactive labeled alk-1-enyl glyceryl ethers has not permitted an accurate evaluation of the reaction.

Labeled alk-1-enyl glyceryl ethers were obtained from the experiments with Ehrlich ascites cells in which we reported the route of plasmalogen biosynthesis (13,14). The phosphatidyl ethanolamine class was reduced with lithium aluminum hydride (12) and the labeled alk-1-enyl glyceryl ethers were separated from the hydrogenolysis products by thin layer chromatography (TLC) (13,14). The only detectable contaminant of the alk-1-enyl glyceryl ethers was 6.2% alkyl glyceryl ether, for which the data have been corrected. The disappearance of all the alk-1-enyl glyceryl ether radioactivity (Fig. 1C) after acid treatment indicates the absence of other labeled impurities. The tritium and carbon-14 label was located in the hydrocarbon chain as established earlier (13,14). The distribution of radioactivity along the developed TLC plates was determined by counting the radioactivity in successive 2 mm sections of adsorbent layer (13).

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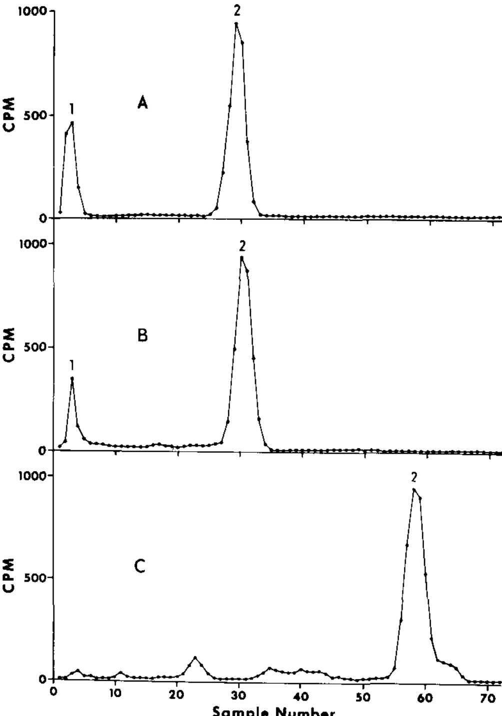

FIG. 1. Liberation of free aldehydes from radioactive alk-1-enyl glyceryl ethers by acid hydrolysis according to two different procedures: A, hydrolysis extraction procedure of Anderson et al. (10); B, hydrolysis on the chromatoplate as described by Schmid and Mangold (3); and C, hydrolysis by the Schmid and Mangold procedure (3) and development of the chromatoplate in a more polar solvent system (diethyl ether-water, 100:0.5 v/v). Chromatoplates A and B were developed in a hexane-diethyl ether (90:10 v/v) solvent system and all separations were carried out on Silica Gel G adsorbent layers. Peak 1 is discussed in the text. Peak 2 represents the free long chain aldehydes.

When labeled alk-1-enyl glyceryl ethers were spotted on a chromatoplate and exposed to HCl fumes, as described by Schmid and Mangold (3), and developed, 83.6 ± 2.9% of the radioactivity was found in the free aldehyde region of the chromatoplate. Eighty ± 5.3% of the radioactivity occurred in the aldehyde peak when the hydrolysis extraction procedure by Anderson et al. (10) was used. A comparison of the distribution of radioactivity (Fig. 1A and 1B) indicates that most of the nonaldehyde radioactivity remained near the origin in the 90:10 solvent system. The distribution of radioactivity on a chromatoplate exposed to HCl fumes and developed in a more polar solvent system (Fig. 1C) showed (a) only a small amount of radioactivity at the origin attributable to activity in the glycerol; (b) the contaminating alkyl glyceryl ether appearing between samples number 20 and 25; (c) the absence of alk-1-enyl glyceryl ethers opposite the standard appearing between samples number 25 and 31; (d) 10% of the activity appearing in the region of cyclic acetals and long chain alcohols between samples number 33 and 45; and (e) a peak of radioactivity adjacent to the aldehyde peak. The distribution of radioactivity from the hydrolysis extraction procedure (10) was similar.

The results indicate that alk-1-enyl glyceryl ethers are completely converted to other compounds when treated either with acid on the TLC plate (3) or by hydrolysis extraction (10). Aldehydes are the primary products of each hydrolysis procedure; however, competing reactions give rise to other compounds. One of the major nonaldehyde compounds appears to be a cyclic acetal. Cyclic acetals that can exist in isomeric forms (15) have been prepared from alk-1-enyl glyceryl ethers (12) and have R_f values corresponding to the radioactivity appearing between samples number 31 and 45 of Figure 1C. Contrary to the conclusions reached by Anderson et al. (10), aldehydes are not quantitatively liberated from alk-1-enyl glyceryl ethers by either method. The discrepancy between our results and theirs probably lies in the fact that their conclusions were drawn from studies with a labeled aldehyde, a product of the acid hydrolysis, which precluded their encountering reactions of the alk-1-enyl glyceryl ethers that would not yield aldehydes. More recently, Bandi (16) has reported the quantitative hydrolysis of alk-1-enyl glyceryl ethers using a procedure almost identical with the procedure described by Anderson et al. (10), except for the longer hydrolysis time. Although we have not tried this slightly modified procedure, it too may not be quantitative. The alk-1-enyl glyceryl ether sample on the thin layer chromatoplate, apparently used to assess the quantitative aspects of the hydrolysis (16), showed a small spot at the origin where we observed radioactivity (Fig. 1A and 1B).

Our results indicate that the quantitative estimation of the percentage of alk-1-enyl glyceryl ethers from the aldehydes liberated from alk-1-enyl glyceryl ethers by either of these methods will be low.

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The Effect of Hydrogen Peroxide on Serum Lecithin-Cholesterol Acytransferase Activity[1]

ABSTRACT

Glomset has reported an acyltransferase in plasma which effects the transfer of a fatty acid from the beta position of lecithin to cholesterol, forming cholesteryl esters. Peroxide is shown to have an inhibitory effect on the enzyme activity in a concentration of 0.1 M. There is a variable effect with a concentration of 0.01 M, with a stimulation of activity in some sera, while in others, an inhibition. The addition of a synthetic saturated lecithin does not cause a reversal of the inhibition at 0.1 M H_2O_2.

Exposure of human serum to 0.1 M hydrogen peroxide has been shown by Clark et al. to result in a disruption of the α-lipoprotein pattern on ultracentrifugation (1). Glomset has shown that the plasma lecithin-cholesterol acyltransferase reaction is associated with the same high density lipoproteins (2), a finding which is corroborated by the alterations in the lipoprotein pattern in the congenital deficiency of the enzyme (3-5).

The effect of hydrogen peroxide on the acyltransferase reaction was studied in vitro. Two milliliter samples of pooled human serum were incubated with labelled cholesterol, and varying amounts of hydrogen peroxide and synthetic dipalmitoyl L-α-lecithin at 37 C for a period of 24 hr.

4-^{14}C-Cholesterol (New England Nuclear Corporation, NEC-018, specific activity: 58 mc/mM) was added to the incubation vial in benzene in the amount of 0.1 μc (0.17 x 10^{-3} μM or 0.668 μg cholesterol) per 2 ml serum, and the benzene was evaporated to dryness under a stream of nitrogen, prior to adding the serum. Synthetic dipalmitoyl L-α-lecithin (No. 10070) was obtained from General Biochemicals. Hydrogen peroxide, 30% solution, analytical reagent grade, was obtained from the Mallinckrodt Chemical Company.

TABLE I

Variation in Response to Peroxide[a]

Experiment	No H_2O_2, %	0.01 M H_2O_2, %	0.1 M H_2O_2, %
A	30.7	4.9	5.2
B	22.9	27.2	8.8
C	18.6	20.0	4.4
D	33.0	29.3	6.6
E	21.5	24.6	8.6
F	29.0	14.7	---
G	29.9	19.3	---
H	26.5	21.8	---
I	15.6	---	1.8
J	12.8	---	3.0

[a]Each experiment represents an individual or pooled sera. The results are expressed as per cent conversion of 4-^{14}C-cholesterol to ester after 24 hr incubation at 37 C.

[1]Presented at the Aerospace Medical Association Annual Meeting, San Francisco, May 1969.

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[a]Each experiment represents an individual or pooled sera. The results are expressed as per cent conversion of 4-^{14}C-cholesterol to ester after 24 hr incubation at 37 C.

[1]Presented at the Aerospace Medical Association Annual Meeting, San Francisco, May 1969.

TABLE II

Reversibility of Peroxide Inhibition With Dipalmitoyl Lecithin[a]

Peroxide	Experiment	No lecithin, %	Lecithin (500 mg%) %
None	A	12.8 ± 0.3	26.9 ± 0.7
	B	22.2 ± 1.9	40.8 ± 3.2
0.1 M H_2O_2	A	3.0 ± 0.2	8.4 ± 0.5
	B	4.2 ± 0.7	5.0 ± 0.5

[a]Human sera from three donors were pooled and incubated with 4-^{14}C-cholesterol for 24 hr at 37 C in the presence of added dipalmitoyl L-α-lecithin. Each experiment was performed in triplicate, and the results are expressed with the standard error of the mean.

The lipids were extracted with chloroform-methanol (2:1 v/v) and fractionated by the Leeder and Clark modification (6) of the Hirsch and Ahrens method (7). Radioactivity was determined in a scintillation system, using 5 g/L PPO and 0.1 g/L dimethyl POPOP in toluene. The percentage of conversion of free cholesterol to cholesteryl ester was expressed as the percentage of radioactivity in the cholesteryl ester fraction of the total radioactivity in all the fractions.

In each of the individual or pooled sera assayed, there was a significant drop in the conversion of free 4-^{14}C-cholesterol to the ester in the presence of 0.1 M H_2O_2. The effect of 0.01 M H_2O_2 was variable; in some sera, a suppression of enzymatic activity was observed, and in others there appeared to be a slight augmentation of the esterification of cholesterol. An indication of the variability of response in human sera at 0.01 M and the consistent inhibition at 0.1 M H_2O_2 is shown in Table I.

One mechanism by which peroxide may inhibit the enzyme activity may be by the production of the hydroperoxide of the unsaturated fatty acid, making the lecithin unacceptable to the enzyme. We studied the effect of added dipalmitoyl L-α-lecithin on the enzyme activity, to determine if the enzyme was suppressed in the presence of a saturated lecithin. The presence of a saturated lecithin provided little protection (Table II).

We determined that the observed decrease in the esterification of cholesterol in the presence of peroxide was not due to changes in the chromatographic separation of the lipids induced by peroxidation by treating serum with peroxide after incubation. Peroxide-treated samples had 32.3 ± 5.6% in the cholesteryl ester fraction, whereas the control samples had 25.4% ± 2.1%.

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Inhibitors of Cholesterol Synthesis and Myelin Formation[1]

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ABSTRACT

Inhibitors of cholesterol biosynthesis AY 9944 and 20,25 diazacholesterol were administered by stomach tube to suckling rats in varying doses during the time of rapid myelination (15-22 days of age). Purified myelin was prepared from the brains and spinal cords, and the sterol content analyzed. Up to 50% of the myelin sterol consisted of desmosterol in rats treated with 20,25 diazacholesterol, while 7-dehydrocholesterol comprised at least one third of the myelin sterol in rats administered AY 9944. Myelin from rats treated with both compounds contained desmosterol, 7-dehydrocholesterol, $\Delta^{5,7,24}$ cholestatriene-3-β-ol and an unknown sterol, the four comprising about 45% of the total sterol. The proportion of phospholipid: galactolipid-total sterol in myelin from the drug-treated rats was not significantly different from the normal, although much less myelin was recovered. Brain and spinal cord slices from 22 to 25-day rats pretreated with inhibitors showed decreased uptake of U-^{14}C-glucose into all myelin components. The decreased uptake was approximately proportional in all lipids and the protein was also affected. It is proposed that myelin composition is fixed, and that a deficiency of one of the lipid components will limit the assembly of the whole lipid portion of the membrane.

INTRODUCTION

In recent years a number of reports of the lipid compositions of purified central nervous system myelin has appeared from various laboratories. Although several different procedures were used for myelin isolation and for lipid separations and assay, the published lipid composition of adult myelin is surprisingly consistent from species to species (1). Myelin with an abnormal composition has been found only in severe pathological conditions (2-4).

[1]Presented at the AOCS Meeting, San Francisco, April 1969.

In 1953 Finean proposed that the lipid interior of myelin is largely composed of cholesterol-phospholipid complexes (5). It was later shown with the use of molecular models that close packing is possible with the phospholipid- or sphingolipid-cholesterol complexes and it was suggested that this may explain the metabolic stability of the myelin membrane (6). Eng and Smith have suggested that the slower metabolizing lipids of myelin may exist in the membrane complexed with cholesterol, while those with shorter half-lives might be uncomplexed (7).

It was of interest, therefore, to determine the degree of interaction between lipids entering the myelin membrane of animals in which the synthesis of cholesterol had been inhibited. AY 9944 (Ayerst) which inhibits the Δ^7 reductase of 7-dehydrocholesterol and 20,25 diazacholesterol (G. D. Searle), shown to inhibit the reduction of the Δ^{24} double bond were used as hypocholesteremic agents. The chemical composition and metabolism of myelin of animals fed these drugs was investigated. A detailed analysis of the sterols found in brain and spinal cord subcellular fractions has been reported elsewhere (8).

EXPERIMENTAL PROCEDURES

Animal and Drug Treatment

Groups of 4-6 suckling Wistar rats from the same litter and matched carefully by weight (22 g average body weight at 15 days) were administered 1 or 2 mg AY 9944 [(*trans*-1,4-bis-(2-chlorobenzylaminomethyl)cyclohexane dihydrochloride] or 20,25 diazacholesterol in 0.25 ml water 1,2,3 or 4 times by stomach tube between 15 and 21 days of age, the period of the maximum rate of myelin formation. In one experiment, 1 mg each of both drugs was given on day 15, 18 and 22. Two days after the final drug treatment (22nd or 23rd-day of life) the rats were killed by decapitation. Their brains and spinal cords were quickly removed and pooled and myelin was prepared by methods described previously (9). In all cases body weight, brain weight and spinal cord weight of the drug-treated animals was comparable to that of the untreated rat.

TABLE I

Desmosterol Content in Myelin

Experiment	Treatment	Control % Desmosterol in total sterol	20,25 Diazacholesterol-treated, % Desmosterol in total sterol
Brain myelin			
1	1 mg/rat day 15, 20	4.78	46.58
2	2 mg/rat day 15, 20	1.47	41.12
3	2 mg/rat day 15, 18, 21	ND[a]	37.5[b]
4	2 mg/rat day 15, 18, 21	ND	46[b]
5	2 mg/rat day 15, 18, 21	ND	49[b]
Spinal cord myelin			
1		2.84	48.67
2		1.54	45.94
3		ND	ND
4		ND	50[b]
5		ND	50[b]

[a]ND, not determined.

[b]Desmosterol determined by spectrophotometric method. All other values determined by GLC (8).

Chemical Determinations

Methods for lipid extraction, thin layer chromatographic (TLC) separations of lipids and methods for measurement of radioactivity are described elsewhere (10). 7-Dehydrocholesterol was determined either by gas liquid chromatography (GLC) (8) or by spectrophotometric measurement of the lipid extract at 282 mμ. Desmosterol was measured by GLC or by the following spectrophotometric method devised in this laboratory. The eluted sterol mixture from the thin layer plate was taken to dryness in the dark in a 30 ml beaker and 5 ml of chloroform and 2 ml of cold acetic anhydride-H_2SO_4 (9:1) was added. After reaction for 25 min in the dark, the color was read in a Beckman DU spectrophotometer at 420 mμ and 620 mμ. The ratio of optical densities at 420 and 620 was matched against a line graph of ratios obtained from 3 or 4 known mixtures of desmosterol and cholesterol. Intermediate points were determined by computer. This method which is less accurate than GLC is useful only in the absence of 7-dehydrocholesterol. The composition of the sterol mixture from myelin of rats fed both drugs was determined by GLC (8). The identification of sterols is based on their fragmentation patterns, obtained using an LKB model 9000 gas chromatograph-mass spectrometer, (LKB, Stockholm), compared with those of pure reference compounds (11). Of the sterol reported here as partially identified, it can be said, on the basis of its fragmentation pattern, that it is a cholestadien-

TABLE II

Desmosterol and 7-Dehydrocholesterol Content in Myelin

Experiment	Treatment	Control, % Desmosterol in total sterol	AY 9944-treated, % 7-Dehydrocholesterol in total sterol
Brain myelin			
1	1 mg/rat day 15, 20	1.16	32.94
2	2 mg/day 15, 20	1.47	34.20
3	2 mg/rat day 15, 17, 19, 21	2.5	31.6
4	2 mg/rat day 15, 18, 21	ND[a]	37.5[b]
5	2 mg/rat day 15, 18, 21	ND	38.0[b]
6	2 mg/rat day 15, 18, 21	ND	38.6[b]
Spinal cord myelin			
1		0.98	37.75
2		1.54	38.37
3		1.4	32.7
4		ND	ND
5		ND	38[b]
6		ND	37[b]

[a]ND, not determined.

[b]Determined by spectrophotometric measurement at 282 mμ. Other values obtained by GLC (8).

3-β-ol with one double bond in the steroid nucleus and the other in the side chain. Methods for measuring myelin metabolism in vitro have been described previously (10).

RESULTS

Myelin Sterol Composition

In agreement with previous results (12) myelin in normal young rats contained a small amount of desmosterol (Table I). We have shown earlier that this sterol rapidly decreases with age, until at 90 days of age only a trace remains. 20,25 Diazacholesterol given in various doses at the time of rapid myelination dramatically increased the desmosterol content to as high as 50% of the total myelin sterol. No desmosterol was found in myelin from rats fed AY 9944, but a third or more of the sterol incorporated into the myelin sheath was present as 7-dehydrocholesterol (Table II). The steroid composition of myelin from rats fed both drugs was more complex. Five different sterols including a partially identified cholestadienol appeared in both the homogenate and myelin of drug treated rats (Table III). Four were identified as cholesterol, desmosterol, 7-dehydrocholesterol and $\Delta^{5,7,24}$ cholestatriene-3-β-ol, the latter comprising 31% and 35% of the brain and spinal cord myelin sterol respectively. The presence of small amounts of desmosterol and 7-dehydrocholesterol suggested that the drug inhibition of the two reductive pathways was not complete. In all, the noncholesterol sterols accounted for 44.7% and 46.8% of the total sterols of brain and spinal cord myelin respectively from drug-treated rats.

Class Composition of Myelin

In all cases where drugs were administered to suckling rats, the final myelin recovery was considerably lower than in the control, varying from 50-75% of the control values in brain and 70-90% in the spinal cord.

That myelin which was recovered from the drug-treated animals contained exactly the same proportions of phospholipid-galactolipid-sterol-protein as that found in the normal preparations (Table IV). Although much of the sterol was present in the form of the precursors, the total was essentially that of the normal. More detailed analyses of several preparations showed also that the distribution of the individual phospholipids (lecithin, ethanolamine phosphatide etc.) was unchanged as was the cerebroside-sulfatide ratio. Also normal proportions of galactolipid and phospholipid were obtained from one preparation of myelin

TABLE III

Sterol Composition of Brain and Spinal Cord Homogenates and Myelin From Rats Administered AY 9944 and 20,25 Diazacholesterols

Sample	Cholesterol, %	Desmosterol, %	7-Dehydrocholesterol, %	Unknown sterol, %	5,7,24-Cholestatriene-3-β-ol, %	Total sterol, mg/100 mg dry wt.
Brain homogenate						
control	97.3	2.7	ND[a]	ND	ND	7.12
drug-treated	56.3	6.6	3.8	3.8	29.5	6.49
Spinal cord homogenate						
control	97.4	2.6	ND	ND	ND	8.97
drug-treated	61.7	2.5	3.7	4.3	27.8	7.64
Brain myelin						
control	97.6	2.5	ND	ND	ND	13.43
drug-treated	55.3	5.5	3.5	4.1	31.6	12.32
Spinal cord myelin						
control	96.6	3.4	ND	ND	ND	13.39
drug-treated	53.2	2.1	4.4	5.0	35.3	13.11

[a]ND, nondetectable, $<$0.2% of total sterol.

TABLE IV

Class Composition of Myelin Preparations[a]

Preparations	Phospholipid, % of total lipid ± SD	Galactolipid, % of total lipid ± SD	Total sterol, % of total lipid ± SD	Protein, % dry wt[b]
Brain Myelin				
Control	53.1 ± 1.02	25.3 ± 0.45	21.2 ± 0.77	20.2
Azasterol	52.9 ± 0.66	25.5 ± 1.02	21.5 ± 1.00	20.45
AY 9944	54.9 ± 1.01	25.0 ± 1.37	20.1 ± 2.53	19.5
Spinal cord myelin				
Control	54.3 ± 1.52	23.4 ± 0.30	22.2 ± 1.62	17.5
Azasterol	54.5 ± 2.70	24.5 ± 3.97	21.0 ± 1.10	17.5
AY 9944	55.7 ± 1.95	24.4 ± 0.83	19.1 ± 2.13	16.4

[a]Figures represent average compositions of three preparations.
[b]One determination.

from rats treated with both AY 9944 and azasterol, and, as shown in Table III, the total sterol expressed as per cent dry weight was reduced only slightly. It appeared, therefore, that myelin composition is relatively fixed.

In Vitro Studies

In view of the finding that the drug feeding resulted in lowered myelin recoveries, it was of interest to investigate in more detail the mechanism for the decreased myelin production. Rats pretreated with AY 9944 or 20,25 diazacholesterol, 2 mg/dose, on the 19th and 22nd day of age were killed on the 23 day and brain and spinal cord slices were prepared and incubated with U-^{14}C-glucose. Determination of the specific activities of the total lipids and proteins of myelin isolated and purified from the incubated slices showed a decreased uptake of U-^{14}C-glucose into the myelin components of the drug-treated rats (Table V). Uptake into the myelin lipids in the different experiments ranged from 62-90% of normal and from 69-98% of the normal values of the proteins. The respective components of the mixed non-myelin membranes of drug-treated rats showed in some cases a decreased uptake, but more often this was nearly the same as the control

TABLE V

Uptake of U-^{14}C-Glucose Into Lipids and Proteins of Myelin and Non-myelin Mixed Membranes[a]

Membranes	Expt. No.	Control	AY 9944 Treated		20,25 Diazacholesterol	
		dpm	dpm	% of Control	dpm	% of Control
Lipids						
Myelin brain	1	1530	1220	77	1362	89
	2	1630	1162	71	1120	69
Spinal cord	1	1630	1015	62	1372	84
	2	1445	1298	90	1305	90
Non-myelin membranes						
Brain	1	2195	2380	108	2180	100
	2	2445	2200	90	2190	90
Spinal cord	1	4800	3555	74	4520	94
	2	4300	4830	112	4480	104
Proteins						
Myelin brain	1	485	383	79	367	74
	2	510	351	69	375	73.5
Spinal cord	1	463	493	98	481	82
	2	447	397	89	402	90
Non-myelin membranes						
Brain	1	686	675	98	729	106
	2	930	701	75	682	73
Spinal cord	1	1112	1210	109	1145	103
	2	1260	1090	86	1145	91

[a]Each figure represents specific activity of myelin components from pooled slices of two rat brains or spinal cords.

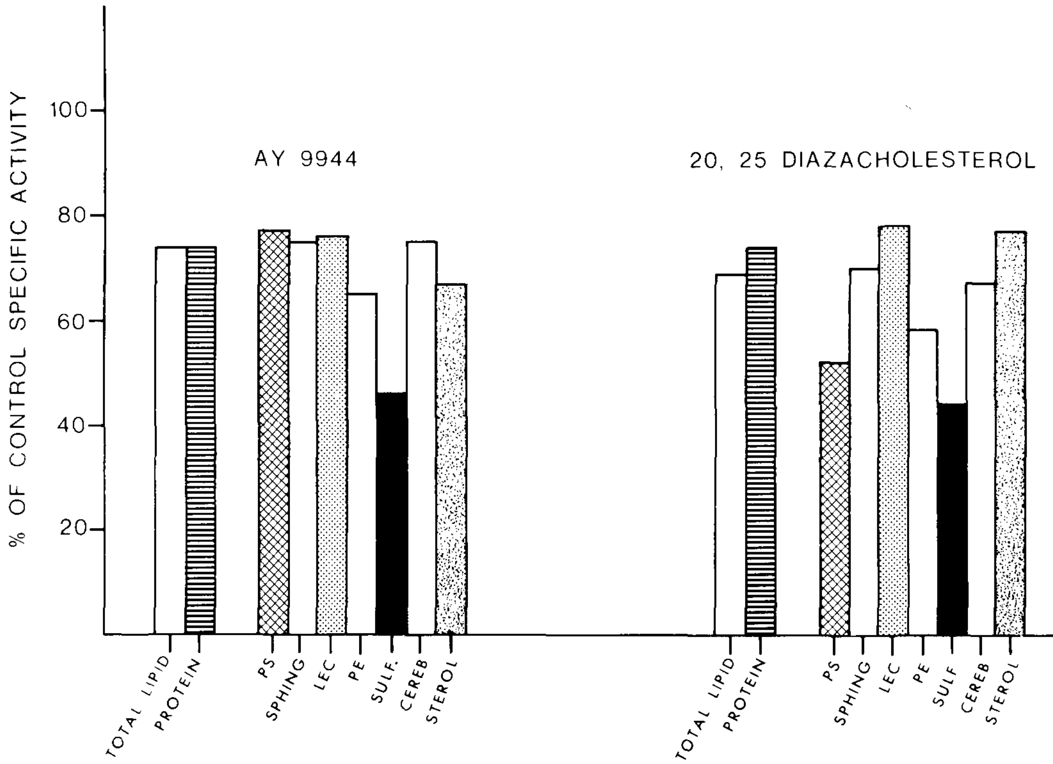

FIG. 1. Uptake of U-^{14}C-glucose into brain myelin components. Each bar represents the average of two experiments. PS, phosphatidyl serine; Sphing, sphingomyelin; Lec, lecithin; PE, ethanolamine phosphatide; Sulf, cerebroside sulfate; Cereb, cerebroside.

values, and on the whole, appeared to show much less effect from the hypocholesteremic agents.

When the lipid extracts of brain myelin were separated into their components by TLC, each lipid species of the myelin from drug-treated animals showed a specific activity which was less than normal (Fig. 1). In the case of the AY 9944-treated animals, total lipid mixture, proteins, phosphatidyl serine, sphingomyelin, lecithin, ethanolamine phosphatide and cerebroside showed specific activities almost exactly proportionally decreased, while that of the sulfatide was much lower. The lipids from the 20,25 diazacholesterol treated myelin showed a more irregular pattern, again with the sulfatide most affected.

DISCUSSION

The drugs appeared to inhibit cholesterol synthesis according to their previously proposed mechanisms. The appearance of desmosterol in myelin from 20,25 diazacholesterol-treated rats agreed with previous findings that this drug inhibits the Δ^{24} reductase (13,14). AY 9944 has been found to act on an enzyme of an alternative reductive pathway, Δ^7 reductase (15,16), thereby causing 7-dehydrocholesterol to accumulate. Administration of both 20,25 diazacholesterol and AY 9944 partially blocked both pathways and another sterol appeared, $\Delta^{5,7,24}$ cholestatriene-3-β-ol which may be the common precursor of desmosterol and 7-dehydrocholesterol (17,18). This compound has recently been identified in pig tissue from animals treated with both AY 9944 and 20,25 diazacholesterol (19).

The precursors appeared to be incorporated easily into the myelin membrane, desmosterol comprising up to 50% of the total myelin sterol. On the other hand, only about 38% of the cholesterol was replaceable by 7-dehydrocholesterol. The sum of the $\Delta^{5,7}$ and the $\Delta^{5,7,24}$ compounds in myelin of rats receiving the combined drug treatment was 35.1% in the brain and 39.7% in spinal cord, very close to the amount of maximum 7-dehydrocholesterol replacement. These myelin preparations also incorporated additional precursor in the form of desmosterol as well as the partially identified cholestadienol. These data suggest that the

shape of the desmosterol molecule with its additional double bond only in the side chain may pack better into the membrane than does 7-dehydrocholesterol. Previous results have shown that desmosterol is also retained longer in the myelin membrane than is 7-dehydrocholesterol (8).

Although no differences in body weight or wet weight of brain and spinal cords could be found at the time of autopsy, much less myelin was recovered from the brains and spinal cords of drug-fed animals, in some cases as little as 60% of the normal amount. In the case of the rats on the combined drug treatment, only 53% of the normal amount of myelin was obtained from the brains, although 89% was recoverable from the spinal cords. This myelin was normal in appearance when viewed by the electron microscope.

The myelin that was obtained from the drug-treated animals, however, was shown to have almost exactly the same chemical composition as the littermate controls. Although slightly lower amounts of total sterols were obtained in spinal cord myelin from AY 9944-treated rats, this was variable in the three batches and the statistical treatment showed no significant difference.

The drug-induced cholesterol deficiency decreased the uptake of U-^{14}C-glucose into myelin components by amounts ranging from 44-90% of normal. The total lipid was decreased almost proportionately to the myelin protein, while the sulfatide incorporation was reduced by a larger amount.

The possibility exists that the drugs exerted a direct toxic effect on the nervous tissue to inhibit myelin synthesis. In one experiment where the rats were not pretreated, but the drugs put directly into the incubation medium, there was no effect on the incorporation of U-^{14}C-glucose into the myelin. Pretreatment of the animals for a few days was found to be necessary for the in vitro effects. The in vitro effects were best seen in young animals not more than 25 days of age. The drug effect was much more variable and sometimes ineffective when 30 to 35-day old rats were used.

We propose that the inhibitory mechanism is mediated directly by a sterol deficiency. The fact that the smaller amount of myelin that was formed was completely normal in respect to composition except for the substitution of sterol precursors for a portion of the cholesterol indicated that the lipid interactions inherent in the myelin membrane are fairly rigid. It is well-known that cholesterol can complex with phospholipids and sphingolipids (6,20), and the role of cholesterol in membranes has been explored (21). There is not sufficient cholesterol in myelin to complex with all the phospholipid and sphingolipid on a 1:1 basis, and it has not been possible from these experiments to determine whether certain lipids might be present in an uncomplexed form. The data, on the other hand, indicate a proportionate decrease in the lipid assembly on to the membrane approximately equal to the decrease of sterol incorporation. It is proposed, therefore, that all lipids must be available in a fixed ratio before myelin synthesis can occur and when one component is limited, synthesis of the whole complex is also limited by this amount. The protein apparently participates in these interactions also.

The uptake of U-^{14}C-glucose into non-myelin membranes of brain and spinal cord showed occasional decreases in the drug treated animals, but on the whole, were much less affected. There are several explanations for this. These membranes, though still increasing in amount, are not being synthesized as rapidly as myelin at the particular age used. Furthermore these membranes contain about 14% sterol, less than the 20% found in the myelin, therefore, they may be less dependent on a supply of sterol. It is also possible that because of a looser type of molecular structure, the interdependencies of the various lipids and proteins in membranes other than myelin are less strict. The higher metabolic activity of this membranous fraction supports this idea. Further study of this fraction is necessary before conclusions can be made regarding the biosynthesis of its components.

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Cyanolipids of *Koelreuteria paniculata* Laxm. Seed Oil[1]

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ABSTRACT

Koelreuteria paniculata Laxm. (Sapindaceae) seed oil is a mixture of cyanolipids (42%) and ordinary triglycerides. The cyanolipid portion contains two classes of components. One of these (25% of the oil) is a mixture of diesters composed of two fatty acid moieties (predominantly C_{18} and C_{20} monoenoic) esterified with an unsaturated five-carbon dihydroxynitrile (1-cyano-2-hydroxymethylprop-1-ene-3-ol). The other class (17% of the oil) consists of cyanolipids having one fatty acid moiety (predominantly C_{20} monoenoic) esterified with 1-cyano-2-methylprop-1-ene-3-ol. Monoesters based on this same hydroxynitrile were previously isolated from *Stocksia brahuica* seed oil and characterized. Hydrogenation of the diesters was accompanied by varying degrees of hydrogenolysis of the ester groups. The hydrogenated diester was reduced with lithium borohydride and the dihydroxynitrile portion was isolated. Acetolysis of the hydrogenated diester in glacial acetic acid with sulfuric acid catalyst yielded an acetylated γ-lactone. The double bond of the dihydroxynitrile moiety in the diester does not react with bromine in carbon tetrachloride.

INTRODUCTION

Cyanolipids were first reported to occur in *Schleichera trijuga* (Sapindaceae) seed oil (1-4), but the structure of these particular lipids has not been elucidated. A cyanogenetic lipid was recently isolated from *Cordia verbenacea* (Boraginaceae) seed oil and has been characterized (5,6) as a diester containing two fatty acid moieties esterified with 1-cyano-2-hydroxymethylprop-2-ene-1-ol. Another cyanolipid, which contains one fatty acid esterified with 1-cyano-2-methylprop-1-ene-3-ol, has been isolated from *Stocksia brahuica* (Sapindaceae) seed oil (7). Significantly, these cyanolipids are major constituents of the seed oils (35%) and the hydroxynitrile portion of both types of esters is isoprenoid in nature.

We now wish to report the isolation and identification of two cyanolipid fractions from *Koelreuteria paniculata* Laxm. (Sapindaceae) seed oil. One of these fractions is a mixture of diesters containing two fatty acid moieties esterified with an isoprenoid dihydroxynitrile; they have the structure designated CLF-1 (cyanolipid fraction-1) in Figure 1. The other fraction (CLF-2) is comprised of monoesters whose hydroxynitrile moiety is identical with that of the cyanolipid isolated from *Stocksia* oil. Although both CLF-1 and CLF-2 are mixtures, the individual components differ only in fatty acid composition; therefore each fraction is treated as if it were homogeneous.

EXPERIMENTAL PROCEDURES

Spectrometry

Infrared (IR) spectra were determined with a Perkin-Elmer Model 137 spectrophotometer on 1% solutions in $CHCl_3$. Nuclear magnetic resonance (NMR) spectra were obtained with a Varian HA-100 spectrometer; $CDCl_3$ or a mixture of $CDCl_3$ and C_6D_6 (10:1) were the solvents used. Chemical shifts were measured from internal tetramethylsilane (τ 10.0). Mass spectral analyses were done on a Nuclide 1290-G instrument. A Beckman DK-2A spectrophotometer was used to determine the ultraviolet (UV) spectra.

Oil Recovery and Methyl Ester Formation

Oil was recovered from finely ground seeds by a 12-hr extraction with petroleum ether (bp 30-60 C) in a Soxhlet apparatus. Methyl esters were prepared from the oil and from CLF-1 and CLF-2 by refluxing them for 3 hr with 3% H_2SO_4 in methanol. The esters were recovered by ether extraction.

Gas Liquid and Thin Layer Chromatography

Analyses of methyl ester samples by gas liquid chromatography (GLC) were performed essentially as described by Miwa et al. (8). Direct GLC analysis of triglycerides and CLF was achieved with an F&M Model 5750 chromatograph equipped with hydrogen flame detectors. The column (0.3 cm O.D. x 19.7 cm, stainless steel) was packed with 3% OV-1 on

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[2]No. Utiliz. Res. Dev. Div., ARS, USDA.

Gas-Chrom Q (Applied Science Laboratories, Inc.) and the temperature was programmed from 150 to 400 C at 4 deg/min. Helium was the carrier gas.

Analytical thin layer chromatography (TLC) was done on 0.25-mm layers of Silica Gel G. The developing solvents varied and will be described below. Spots were detected by treating developed plates first with iodine vapor and then by spraying the plates with a saturated solution of CrO_3 in 50% aqueous H_2SO_4 and charring them for 45 min at 120-130 C. Preparative TLC involved 1-mm Silica Gel G layers developed with an appropriate solvent system. Sample bands were visualized by viewing developed plates over an incandescent light in a darkened room. This method is suitable for major components on heavily loaded plates, and eliminates possible contamination of the sample with spray reagents. Desired components were recovered from the silica by standard procedures.

Formation and Detection of HCN

Lipid material to be tested (75-100 mg) for formation of HCN was placed in a test tube with 1 ml of dilute NaOH or H_2SO_4. A strip of filter paper dipped in an alkaline sodium picrate solution (0.5%) was partially dried and was then suspended over the mixture in the stoppered test tube. The test tube and contents were warmed at 35-50 C for 0.5-1 hr. A positive test is indicated by a color change of the filter paper from yellow to brick red (9).

Bromination Reaction

A 0.040-g sample of CLF-1 (Fig. 1) dissolved in 2 ml of CCl_4 was treated dropwise with Br_2 in CCl_4 until color persisted in the solution. After the solution stood for 1 hr, the solvent and excess Br_2 were blown off with a stream of nitrogen. The residue was placed in an evacuated desiccator for 4 hr; yield, 0.061 g. This entire process was repeated; yield, 0.061 g.

Hydrogenation of CLF-1

To obtain hydrogenated CLF-1 (I, Figure 1) containing less than 10% of the partial hydrogenolysis product II, it was necessary to hydrogenate small samples (ca. 40 mg) in hexane (10 ml) with 5 mg of palladium-on-carbon (10%) catalyst. The reaction was essentially complete in 15-20 min at room temperature and atmospheric pressure. Hydrogenations were done repeatedly until the desired amount of hydrogenated product was obtained.

$LiBH_4$ Reduction of Hydrogenated CLF-1 (I)

Crude hydrogenated material (I, 0.370 g, containing some partial hydrogenolysis

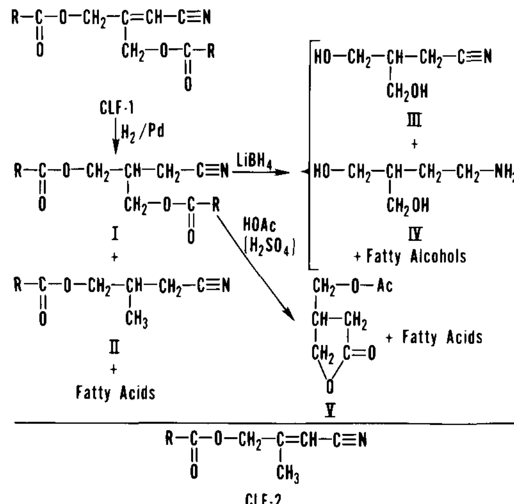

FIG. 1. Diagram of structures and reactions. R = fatty acyl groups–dienoic, monoenoic or saturated; CLF = cyanolipid fraction.

product) in 10 ml of dry ether was added slowly and with stirring to 100 ml of dry ether saturated with $LiBH_4$. The resulting mixture was stirred for 16 hr under a reflux condenser fitted with a drying tube and then was refluxed in a warm water bath for 5.5 hr. Excess $LiBH_4$ was destroyed by cautious dropwise addition of 2N HCl to the stirred mixture. Then the acidified solution was extracted with ether and this extract was washed six times with water. The fatty alcohols remained in the ether phase. Reextraction of the combined aqueous washes with ether afforded the short chain reduction product III; yield, 0.006 g. Because this yield was poor, the original acidic aqueous phase from the reduction was concentrated to about 15 ml, basified with NaOH, and extracted thoroughly with ether and chloroform. Another 0.008 g of product (IV) was secured.

Acetolysis of Hydrogenated CLF-1 (I)

A sample of hydrogenated CLF-1 (I, 0.236 g) which contained only 10-15% of partially hydrogenolyzed material (II) was treated for 5 hr at slow reflux with 15 ml of glacial acetic acid containing 0.2 ml of concentrated H_2SO_4. The cooled solution was diluted with water, and all the products were extracted into ether. The ether extract was washed repeatedly with saturated $NaHCO_3$ solution; since the γ-lactone acetate (V) is quite water soluble, it was washed out of the ether extract. These washes were added to the original aqueous phase. Solid $NaHCO_3$ was added to the combined aqueous solutions to neutralize residual acetic acid. Extraction of the resulting

basic solution with ether yielded 0.021 g of product containing the γ-lactone acetate (V). Essentially all the fatty acids remained in the first ether extract.

RESULTS AND DISCUSSION

Analysis and Fractionation of K. paniculata Seed Oil

IR analysis of this seed oil revealed a nitrile absorption band (2230 cm^{-1}) of moderate intensity and weak unassigned bands at 965 and 1030 cm^{-1} in addition to bands normally found in spectra of triglycerides. Treatment of the oil with dilute base or acid failed to generate HCN as shown by negative picrate tests (9). The oil, when analyzed by TLC with an ether-hexane (1:3) solvent system, gave a spot associated with ordinary triglycerides at R_f 0.79 and two other major spots at R_f 0.54 and 0.45. These two spots were due to the cyanolipids designated CLF-1 (R_f 0.54) and CLF-2 (R_f 0.45).

Temperature-programmed GLC analysis of *Koelreuteria* oil revealed three groups of homologous constituents. The first set of peaks to emerge from the column appeared at 208, 223 and 234 C; the 223 C peak accounted for more than 95% of the total of these three. A second set of three unidentified peaks had retention characteristics (308, 316 and 324 C) similar to those of the nitrile diesters from *Cordia* oil (5). The third set of peaks on the chromatogram emerged between 350 and 382 C, and these are the normal triglyceride components of the seed oil.

GLC analysis of methyl esters derived by methanolysis of *Koelreuteria* oil gave the following composition: $C_{16:0}$, 5%; $C_{16:1}$, Trace; $C_{18:0}$, 1%; $C_{18:1}$, 34%; $C_{18:2}$, 10%; $C_{18:3}$, 3%; $C_{20:0}$, 2%; $C_{20:1}$, 44%; $C_{20:2}$, Trace; and $C_{22:1}$, 1%.

A number of attempts to separate the triglycerides from the unusual components by column chromatography resulted in a concentrate which, at best, contained about 80% cyanolipids (CLF-1 plus CLF-2) and 20% triglycerides. However, we found that preparative TLC plates could be loaded with up to 200 mg of oil and that the resolution was sufficient with ether-hexane (1:2) to give fractions of about 95% purity. The more mobile (R_f 0.54) of the unusual components (CLF-1) was recovered in 25% yield from the oil; the other component (CLF-2) was recovered in 17% yield.

Analysis of CLF-1 and Hydrogenated CLF-1 (I)

The IR spectrum of CLF-1 showed a medium intensity nitrile band (2230 cm^{-1}), a strong ester carbonyl band (1740 cm^{-1}), and a weak unassigned band at 1010 cm^{-1}. CLF-1 appeared to be optically inactive since it gave no rotation (in hexane) at the sodium D-line with a visual polarimeter. Mass spectral data indicated that the most abundant molecular ions were m/e 641, 669 and 697, and also that the m/e 669 peak was by far the largest of the three. This molecular weight range implies that two fatty acid moieties are associated with each molecule of CLF-1. Methanolysis of CLF-1 gave a methyl ester mixture of the following composition (by GLC): $C_{16:0}$, 6%; $C_{18:0}$, 1%; $C_{18:1}$, 24%; $C_{18:2}$, 6%; $C_{18:3}$, 1%; $C_{20:0}$, 3%; $C_{20:1}$, 55%; $C_{20:2}$, 1%; $C_{22:0}$, Trace; and $C_{22:1}$, 3%. The conclusion that CLF-1 is a mixture of diesters is supported by its GLC characteristics since it gives peaks that correspond closely to those observed for the *Cordia* diesters (5,6). Evidence that CLF-1 contained one nitrogen atom per molecule (assuming a mean mol wt of 669) was supplied by the Kjeldahl determination, which indicated 2.1% of nitrogen.

The UV spectrum of CLF-1 in cyclohexane had an inflection point at 208 mμ, ϵ = 14,340 (mol wt = 669). This absorption compares with that observed for the *Stocksia* cyanolipid (7) at 208 mμ, ϵ = 13,070. The presence of a double bond conjugated with the nitrile grouping is thereby established (10). A conjugated nitrile structure is also indicated by a large decrease in the intensity of the IR nitrile band and a shift in its position (11) from 2230 cm^{-1} to 2240 cm^{-1} upon hydrogenation of CLF-1. The hydrogenated material (I) shows no UV absorption maximum.

Hydrogenation uptake was erratic and depended on the amount of hydrogenolysis that occurred. The hydrogenolysis product (II) could not be separated by preparative TLC from hydrogenated CLF-1 (I), but the amount of II could be kept to a minimum by hydrogenating less than 0.040 g of sample at a time. When samples larger than 0.040 g were hydrogenated, the amount of hydrogenolysis product increased, but the yield was not reproducible. Analytical TLC and IR were used to estimate the degree of hydrogenolysis.

NMR Spectra of CLF-1 and Hydrogenated CLF-1 (I)

The NMR spectrum of CLF-1 in $CDCl_3$ is shown in Figure 2. Signals due to protons of the fatty acid R groups are observed at τ 9.13 (terminal methyl), τ 8.74 (shielded methylenes), τ 8.38 (β to carbonyl), τ 8.05 (α to double bonds), τ 7.64 (α to carbonyl), and τ 4.68 (vinyl). Methylene protons H_b and H_c, which are adjacent to the oxygen atoms of the dihydroxynitrile moiety, give the two signals at τ 5.32 and 5.13. This difference in shielding is

caused by the stereochemistry of the methylene groups; one of them is *cis* to the nitrile grouping and the other is *trans* (Fig. 2). As a result of this stereochemical difference between the two methylene groups, the protons of one group couple more strongly with the vinyl proton (τ 4.45) than do protons of the other methylene group. This unequal coupling explains the appearance of one signal as a doublet and the other as an apparent singlet. Irradiation of the τ 5.32 signal (inset A) collapses the vinyl proton signal to a singlet. Inset B shows reduction of the τ 5.32 signal to a singlet by irradiation of the vinyl proton signal. Integration indicates one vinyl proton, four methylene protons adjacent to oxygen and four methylene protons adjacent to carbonyl groups. Long range coupling, which is not apparent from Figure 2, also occurs between protons H_b and H_c. This extremely small coupling (less than 0.5 Hz) can be detected when the τ 5.32 and 5.13 signals are amplified and displayed on a 250 Hz sweep spectrum.

These NMR data, coupled with the mass spectral data and the GLC composition of methyl esters derived from CLF-1, indicate that CLF-1 has the structure shown in Figure 1. From this combination of results, it can now be seen that the molecular ions observed in the mass spectrum at m/e 641, 669 and 697 are due to a dihydroxynitrile moiety esterified with two C_{18} monoenes (641), a C_{18} and a C_{20} monoene (669), and two C_{20} monoenes (697), respectively.

Hydrogenated CLF-1 (I, FIG. 1) in $CDCl_3$ was also analyzed by NMR. The distinguishing features of this spectrum were a pair of overlapping doublets (J = 2 Hz) with chemical shifts of τ 5.87 and 5.92 and a rough doublet (J = 1.5 Hz) at τ 7.57. The signals at τ 5.87 and 5.92 are due to the protons of the two methylene groups adjacent to oxygen atoms in I. This difference in shielding of the two methylene groups can be explained by the possible predominance of a conformational rotamer of I in which the nitrile grouping is considerably closer to one methylene than to the other. The τ 7.57 band is assigned to protons of the methylene group bearing the cyano function. All three signals (τ 5.87, 5.92 and 7.57) show evidence of fine structure, which indicates that long range coupling (J is very small) occurs between these two types of methylene protons.

The τ 7.57 peak is sharpened somewhat by irradiation of the τ 5.9 region. Irradiation near τ 7.57 collapses the downfield pair of doublets to singlets at τ 5.88 and 5.91. Since their signals overlap, the protons of the methylene adjacent to the cyano group, as well as the methine proton, are irradiated by this technique. Presumably a complex methine proton signal is obscured by the τ 7.57 signal and the resonance at τ 7.72 due to protons of the methylene groups adjacent to the ester carbonyl groups. These observations are consistent with structure I. A small amount of II was also present because a very weak methyl proton doublet was detected at τ 8.90.

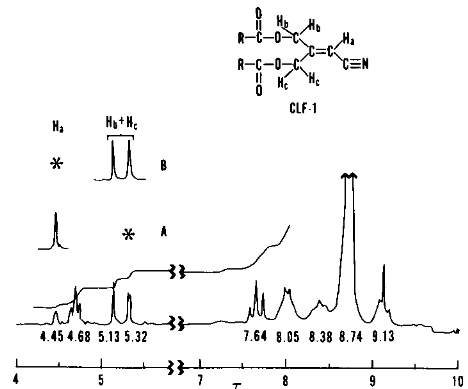

FIG. 2. 100 MHz NMR spectrum of *Koelreuteria paniculata* cyanolipid CLF-1 in $CDCl_3$. Insets A and B: Portions of decoupled spectra; asterisks mark points of irradiation.

Acetolysis of Hydrogenated CLF-1 (I)

Strong γ-lactone absorption (1775 cm^{-1}), ester carbonyl absorption (1740 cm^{-1}), and acetate absorption (1370 and 1020 cm^{-1}) were observed in the IR spectrum of the product derived by acetolysis of hydrogenated CLF-1. TLC of the product with methanol-chloroform (2:98) indicated that it contained one major component, R_f 0.38, and two minor components, R_f 0.17 and 0.88. Preparative TLC gave three fractions, and IR analysis of the R_f 0.38 band revealed that it was the γ-lactone acetate, V. The remaining two bands, while not isolated in quantities large enough to analyze, are probably the corresponding nonacetylated γ-lactone (R_f 0.17) and a γ-lactone with a methyl group in place of the $-CH_2OH$ group (R_f 0.88). The latter compound is the result of contamination of I with II.

The NMR spectrum of V (Fig. 1) from preparative TLC showed a sharp singlet (3H) at τ 7.96 due to acetate methyl protons. Two sets of partially overlapped signals occur in the τ 7.0-7.8 region. These can be separated into an eight line pattern centered at τ 7.52 and attributable to nonequivalent protons of the methylene group adjacent to the carbonyl, and a complex series of bands due to the methine proton centered near τ 7.2. The eight line

pattern is the AB portion (J_{AB} = 17 Hz) of an ABX-type system. Protons of the methylene adjacent to the ring oxygen similarly give an eight line (J_{AB} = 10 Hz) pattern centered at τ 5.76. A doublet (J = 4.5 Hz) at τ 5.92, which is superimposed on this eight line pattern, is assigned to protons of the methylene group attached to the acetate oxygen atom. Integration reveals that the eight line pattern at τ 5.76, plus the doublet at τ 5.92, is equivalent to four protons and that the signals in the τ 7.0-7.8 region are equivalent to three protons. When the methine proton signal is irradiated, the downfield signals are reduced to an AB quartet (J = 10 Hz) at τ 5.73 and a singlet at τ 5.89.

The acetolysis of CLF-1 was not attempted because double bond migration would probably result in an inseparable mixture of products (7).

$LiBH_4$ Reduction of Hydrogenated CLF-1 (I)

IR analysis of the fatty alcohol portion of the reduction products indicated that about 30% of the original ester groupings had not been reduced; this observation partially accounts for the poor yield of short chain products. The hydroxynitrile portion isolated (III, Fig. 1) showed strong hydroxyl (3700 cm^{-1}) and medium intensity nitrile (2260 cm^{-1}) absorption in the IR (in $CHCl_3$). The NMR spectrum of III demonstrated that it was a mixture because a methyl proton doublet was observed at τ 8.95. This signal was due to the monohydroxynitrile formed by reduction of some partially hydrogenolyzed material (II) present as an impurity in I. Other NMR signals were observed at τ 7.96 (methine), τ 7.60 (methylene adjacent to cyano group), τ 6.72 (probably hydroxyls), and τ 6.46 (methylenes adjacent to oxygen). This spectrum appears to be consistent with a mixture of III and the corresponding nitrile with one $-CH_2OH$ group replaced by a methyl group. TLC on silica gel with methanol-chloroform (1:3) also indicated that two major components were present, R_f 0.36 and 0.62.

The second short chain product isolated from the borohydride reduction had a strong aminelike odor presumably due to IV. Its IR spectrum showed hydroxyl (3700 cm^{-1}) and weak nitrile (2260 cm^{-1}) absorption, as well as bands at 1605, 1580 and 1055 cm^{-1} which may represent the amino group (12). Broad nondescript maxima appeared in the 3000-3500 cm^{-1} region.

TLC analysis [methanol-chloroform (1:3)] of this amine fraction reveals two major components, which are probably IV and the corresponding amine derived from II. Two minor components, which have the same R_f as the two nitriles described above, are also present.

Reportedly, $LiBH_4$ reduces ester linkages (13) while leaving nitrile groups intact (14). The cyanolipid from *Stocksia* (7) was apparently reduced in accordance with these generalizations. Comparable treatment of *Koelreuteria* CLF-1, however, resulted in some reduction of nitrile to amino groups.

Complete Hydrogenolysis of CLF-1

Isoamyl amine was isolated in reasonable yield by hydrogenolysis of the *Stocksia* cyanolipid with Adams catalyst (7). When applied to *Koelreuteria* CLF-1 however, this reaction yielded hydrogenated material (I), some partial hydrogenolysis product (II) by loss of one acyloxy group, but no detectable amount of isoamyl amine. Apparently neither the hydrogenated material nor the partial hydrogenolysis product undergoes further hydrogenolysis.

Bromination of CLF-1

CLF-1 consumed 2.2 mole equivalents of Br_2 (assuming mean mol wt = 669). The NMR spectrum of the product, however, indicated that the double bond of the dihydroxynitrile portion of CLF-1 was unaltered. The chemical shifts and multiplicities of signals were precisely as they had been in the starting material. Only the signal due to fatty acid vinyl protons changed; it became a pair of triplets at τ 5.76 and 5.88 (each 2H) instead of one 4H triplet at τ 4.68 (Fig. 2).

The double bond of the *Cordia* dihydroxynitrile moiety, which was terminal and nonconjugated, took up bromine readily (6). Evidently the failure of the *Koelreuteria* double bond to react must be at least partially due to the electron-withdrawing effect of the nitrile grouping in conjugation with the double bond. Compounds having a double bond conjugated with an electron-withdrawing substituent show diminished reactivity toward Br_2 in CCl_4 (15,16) and sometimes even toward Br_2 in acetic acid (16,17).

Analysis and Identification of CLF-2

The IR spectrum of CLF-2 revealed strong nitrile absorption (2230 cm^{-1}, $CHCl_3$) and was superimposable on the IR spectrum of *Stocksia* nitrile (7). UV analysis in cyclohexane gave λ_{max} = 208 mμ, ϵ = 12,600 (assuming mean mol wt = 389) as compared to ϵ = 13,070 for the *Stocksia* cyanolipid. Temperature-programmed GLC analysis of CLF-2 revealed that this fraction was responsible for the three peaks at 208, 223 and 234 C seen on the whole oil chromatogram. The major molecular ion was

observed in the mass spectrum of CLF-2 at m/e 389; this corresponds to a hydroxynitrile esterified with a C_{20} monoenoic acid. Additional molecular ions were observed at m/e 391, 417 and 419 for esters containing $C_{20:0}$, $C_{22:1}$ and $C_{22:0}$ acids, respectively. Methanolysis of CLF-2 gave a methyl ester mixture, which had the following composition by GLC: $C_{16:0}$, 1%; $C_{18:0}$, Trace; $C_{18:1}$, 3%; $C_{18:2}$, 1%; $C_{20:0}$, 6%; $C_{20:1}$, 84%; $C_{20:2}$, 2%; $C_{22:0}$, 1%; and $C_{22:1}$, 2%.

The NMR spectrum of CLF-2 (in $CDCl_3$) is the most definitive structural evidence. Fatty acid protons gave signals at τ 9.14 (terminal methyl), τ 8.74 (shielded methylenes), τ 8.01 (α to double bonds), τ 7.63 (α to carboxyl), and τ 4.68 (vinyl). Signals due to protons of the hydroxynitrile moiety are observed at τ 8.09 (3H, doublet, vinyl methyl), τ 5.20 (2H, singlet, methylene attached to oxygen), and τ 4.73 (1H, multiplet, vinyl). Irradiation of this vinyl proton signal reduces the methyl signal to a singlet. By analysis of CLF-2 in $CDCl_3$-C_6D_6 (9:1) a spectrum is obtained which is superimposable on that reported for the *Stocksia* cyanolipid (7). Thus CLF-2 has the structure depicted in the lower segment of Figure 1.

Additional Considerations

The nitrile portions of the cyanolipids isolated thus far (5-7) are related since all are isoprenoid. Differences occur in the positioning of the double bond and in the number and points of attachment of acyloxy groups which incorporate fatty acid moieties. *Koelreuteria* oil is the only one shown to contain more than one of these novel cyanolipids. As we suggested previously, the dimethyl acetal isolated from *Cardiospermum halicacabum* mixed methyl esters by Hopkins et. al (18) may have been derived from a cyanolipid related to the ones we have isolated (6,7). Similarly, the unusual lipids of *S. trijuga* seed oil (1-4) may also be related to these cyanolipids.

Because of the isoprenoid nature of the nitrile moiety, and because the biogenetic possibilities for these cyanolipids are many, it would be worthwhile to determine how and why some plants produce such large amounts of these compounds. Hopkins and Swingle (19) report that a number of seed oils of the *Sapindaceae* (including *Koelreuteria*) are rich in C_{20} monoenoic fatty acids.

The cyanolipid-containing sapindaceous seed oils which we have investigated (*Koelreuteria* and *Stocksia*) also have a high C_{20} monoene content, and it seems rather curious that these C_{20} acids are concentrated in the cyanolipid ester fractions. Whereas the whole oil from *Koelreuteria* seed contains 44% of the C_{20} monoenoic acid, the triglyceride fraction has only 27% of this acid. In *Stocksia* oil (which contains 41% of C_{20} monoene), the percentage of C_{20} monoenoic acid in the triglyceride fraction is only 15%. The inference from these observations is that sapindaceous seed oils having little or no C_{20} acids (19) would not contain cyanolipids to any great extent. This point has not yet been shown experimentally.

Further work is being done on the structure of sapindaceous cyanolipids and will be reported later.

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Lipoxygenase From *Zea mays:* 9-D-Hydroperoxy-*trans*-10, *cis*-12-Octadecadienoic Acid From Linoleic Acid

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ABSTRACT

Lipoxygenase from the germ of corn, *Zea mays*, oxidized linoleic acid to primarily 9-D-hydroperoxy-*trans*-10,*cis*-12-octadecadienoic acid.

INTRODUCTION

Lipoxygenase (E.C. 1.13.1.13) in corn has received limited attention compared to soybean lipoxygenase, which has been thoroughly investigated. Franke and Frehse (1), Fritz and Beevers (2) and Wagenknecht (3) studied lipoxygenase activity in corn, but none of them characterized the oxidation product.

Previous work by Gardner (4) has indicated that corn lipoxygenase may oxidize linoleic acid to one product, 9-hydroperoxyoctadecadienoic acid. He found a sequential enzyme system in corn germ extracts, which initiated with the oxidation of linoleic acid by lipoxygenase and terminated with the formation of 9-hydroxy-10-oxo-*cis*-12-octadecenoic acid and 9-(*cis*-9,*cis*-12-octadecadienoyl)-10-oxo-*cis*-12-octadecenoic acid. The substitution of hydroxyl at the 9-carbon and the *cis*-12 unsaturation indicated a 9-hydroperoxidation as the intermediate step. We report here a more conclusive proof that the predominant product of lipoxygenase activity in corn is 9-hydroperoxyoctadecadienoic acid. We also demonstrate that the product is *trans*-10,*cis*-12, and that the asymmetric 9-carbon has a D-optical configuration.

METHODS

Oxidation of Linoleic Acid

A lipoxygenase activity was partially purified from corn germ. The germ was hand-dissected from a commercial hybrid dent corn, which was air-dried at room temperature after harvest and subsequently stored at 1 C. After the germ was hexane-defatted by grinding in cold hexane, the dried residue was homogenized with 0.1 M phosphate buffer (pH 6.9) in the proportion of 7 g/100 ml. The homogenate was centrifuged at 8,000 x *g* for 15 min and the resulting supernatant fractionated with $(NH_4)_2SO_4$ at 0 C according to the nomogram constructed by diJeso (5). The fraction collected between 42% and 53% $(NH_4)_2SO_4$ saturation contained most of the lipoxygenase activity. The precipitate was dissolved in a volume of phosphate buffer equivalent to the original.

Linoleic acid (99+% pure from The Hormel Institute, Austin, Minn.) was oxidized with the purified extract according to a procedure described previously (4). Absorption of the reaction solution at 234 nm was monitored periodically for conjugated diene. When the absorption became relatively constant with time (usually 30 to 40 min), extraction was done with chloroform-methanol (2:1). More thorough extraction of the fatty acids was achieved by acidifying the solution with 1 N HCl to pH 4-5, and was done except where noted under Results.

Preparation of Derivatives

Linoleic acid hydroperoxide was reduced by an equivalent weight of $NaBH_4$ in 50% methanol buffered with 0.05 M potassium borate (pH 9). After 1 hr the solution was acidified to pH 4-5 and extracted with chloroform; then the extract was washed with water.

Hydroxyoctadecadienoic acid was methyl esterified with diazomethane (6).

Methyl hydroxyoctadecadienoate was hydrogenated in hexane with H_2 and 10% palladium catalyst on charcoal.

Chromatography

Silicic acid columns were prepared as described previously (4). The product mixture from lipoxygenase oxidation of linoleic acid was applied to a column in admixture with small amounts of hexane (ca. 10 ml) and silicic acid (1.5 g). The column was eluted by a combination stepwise-gradient method as outlined by Gardner (4).

Methyl hydroxyoctadecadienoate was eluted from the column by a less polar solvent system than above. The mixing chamber was filled with 70 ml hexane. The reservoir was filled consecutively with 200 ml hexane, 300 ml 10% ether, 300 ml 15% ether and 500 ml 20% ether in hexane.

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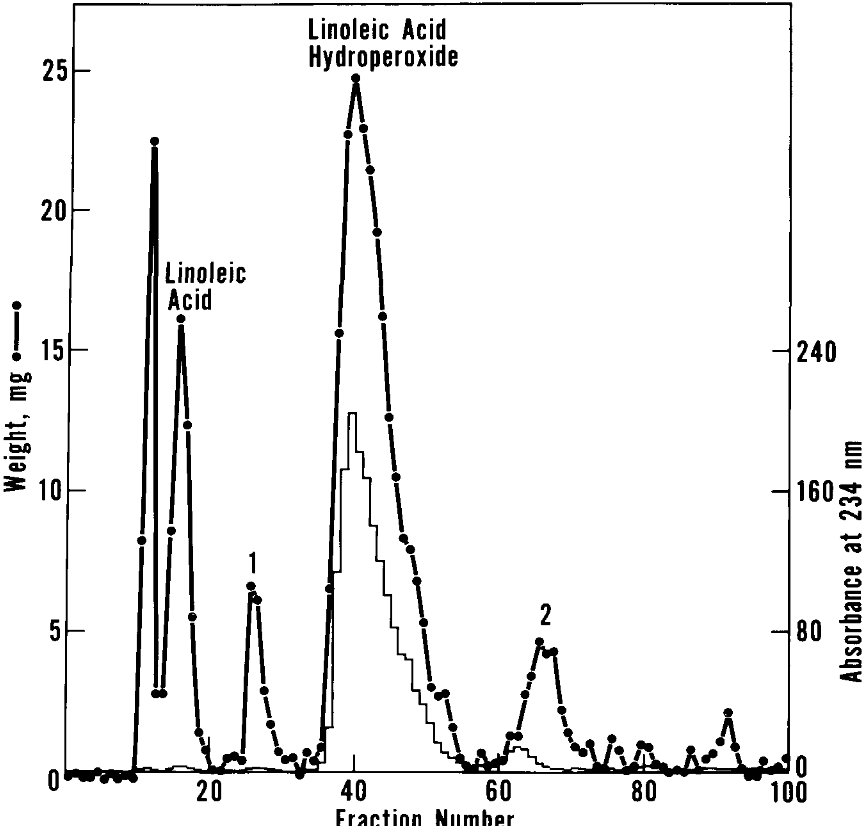

FIG. 1. Silicic acid column chromatography of products obtained after oxidation of linoleic acid by corn lipoxygenase; 390 mg total weight was applied to the column. Ten-milliliter fractions were collected. 9-(*cis*-9,*cis*-12-Octadecadienoyl)-10-oxo-*cis*-12-octadecenoic acid (1) and 9-hydroxy-10-oxo-*cis*-12-octadecenoic acid (2).

Methyl hydroxystearate was eluted from the column with the same system used to separate methyl hydroxyoctadecadienoate, except that elution with 200 ml hexane was omitted.

Products were analyzed by thin layer chromatography (TLC) on analytical plates prepared as described by Mangold and Malins (7). Fatty acids were separated by development with isooctane-ether-acetic acid (50:50:1), and esters with isooctane-ether (60:40). Separations of 9- and 13-hydroxy-esters were improved by double development. Hydroperoxides were detected by a spray composed of KI and starch (8). All compounds on the plates were observed after charring with a 50% H_2SO_4 spray. The 9- and 13-hydroxy-esters that were separated by TLC were in turn quantitated by densitometry according to Downing's method (9).

Spectral Methods

Infrared (IR) spectra were recorded with Perkin-Elmer Model 621 and 700 spectrometers. Spectra of isolated compounds were determined as a 1% solution in both CCl_4 and CS_2 in a 1-mm thick NaCl cell.

Proton magnetic resonance (PMR) spectra ($CDCl_3$) were recorded on a Varian HA-100 spectrometer.

The mass spectrum of methyl 9-hydroxystearate was obtained as described by Dolev et al. (10).

Optical rotations were measured in a 0.2-dm cell at 546 nm with a Bendix Model 1100 polarimeter.

RESULTS AND DISCUSSION

Isolation of Hydroperoxyoctadecadienoic Acid

After oxidation of linoleic acid by corn lipoxygenase, the product—hydroperoxyoctadecadienoic acid—was isolated by column chromatography (Fig. 1). The hydroperoxide was located by the absorption of diene conjugation at 234 nm. The molar absorptivity was ca.

TABLE I

Column Chromatographic Separation of Products Derived From Hydroperoxyoctadecadienoic Acid by Reduction and Esterification

Compound	Weight, %		Fraction number[a]
Methyl hydroxyoctadecadienoate	91.8		75-107
13-hydroxy-*cis, trans*[b]	1.5	100	75- 83
9-hydroxy-*cis, trans*[b,c]	93.6		75-107
9-hydroxy-*trans, trans*[b]	4.9		85-107
Other products	8.2		---

[a]When compounds were not completely separated by column chromatography, the extent of overlapping fractions was estimated by thin layer chromatography (TLC).

[b]Percentage determined by TLC densitometry after double development of the plates.

[c]The 9-hydroxy-*cis, trans* isomer may be mixed with a small amount of 13-hydroxy-*trans, trans* isomer.

25,000, which is the value expected based on the published absorptivity of linoleic acid hydroperoxide (11). TLC analyses of individual fractions from the peak (fractions 37-61) resulted in the migration of one spot (R_f = 0.40), which was highly reactive to the peroxide-sensitive spray composed of KI and starch.

Other compounds eluting from the column were small amounts of 9-hydroxy-10-oxo-*cis*-12-octadecenoic acid and 9-(*cis*-9,*cis*-12-octadecadienoyl)-10-oxo-*cis*-12-octadecenoic acid (4).

Isolation of Methyl Hydroxyoctadecadienoates

Methyl hydroxyoctadecadienoate was synthesized from the hydroperoxyoctadecadienoic acid. Fractions 37 to 61 (Fig. 1) were reduced to the corresponding hydroxy-acid with $NaBH_4$. The reduction product was methyl esterified with diazomethane.

The methyl hydroxyoctadecadienoate was isolated by column chromatography. Other components eluting from the column amounted to 8.2% of the total. An IR spectrum of the isolated hydroxyoctadecadienoate showed absorption peaks at 3620, 950 and 985 cm^{-1} characteristic of a *cis,trans* conjugated dienol (12). Also indicative of a conjugated dienol was strong ultraviolet (UV) absorption at λ_{max} 233-4 nm.

The hydroxyoctadecadienoate that eluted from the column was actually three components, which were barely separable by TLC. The percentage of the total and probable identity of the three components are given in Table I. We confirmed the results of Morris et al. (13), who separated the 9- and 13-hydroxyoctadecadienoates and slightly resolved the *cis, trans* from the *trans,trans* isomers by TLC and surmised their identities. However, we apparently obtained greater separation of the *cis,trans* from the *trans,trans* isomer. The upper TLC spot (R_f = 0.345) was postulated to be methyl 13-hydroxy-*cis,trans*-octadecadienoate since it migrated with the hydroxyoctadecadienoate prepared from the product of soybean lipoxygenase oxidation. The middle spot (R_f = 0.310) was the major component. As visualized by TLC, this component eluted free of most others in a pooled sample of fractions 80 to 89. The pooled sample was primarily methyl 9-hydroxy-*trans*-10,*cis*-12-octadecadienoate; however, this sample may have been mixed with a quantity of the 13-hydroxy-*trans,trans* isomer, which was unresolved by TLC. As explained in the following sections, the presence of a maximum of 15% to 16% of the 13-hydroxy-*trans,trans*-octadecadienoate was possible as surmised from the products obtained after hydrogenation. Little evidence was found of *trans,trans* absorption by PMR or IR spectroscopy. The lower spot (R_f = 0.278) appeared to be methyl 9-hydroxy-*trans,trans*-octadecadienoate. IR spectra taken of fractions containing a higher percentage of the lower TLC spot had an increased ratio in absorption of 985 to 950 cm^{-1} indicative of increased admixture of *trans,trans* with *cis,trans*.

Demonstration of α,β-trans-γ,δ-cis Hydroxy-Diene

The PMR spectrum of methyl 9-hydroxyoctadecadienoate (column fractions 80 to 89) established that one *cis* and one *trans* double bond are present and that the *trans* double bond is adjacent to the hydroxyl-bearing carbon atom. Irradiation of carbinol proton H_9 at δ 4.15 decoupled it from H_{10} at δ 5.63 and caused the H_{10} doublet-split-doublet to collapse to a simple doublet with a splitting of 15.4 Hz, which result is typical of a *trans* proton coupling accross a double bond. Thus the double bond adjacent to the hydroxyl carbon is

trans. Irradiation of the allylic methylene protons H_{14} at δ 2.18 decoupled H_{13} and caused the triplet-split-doublet at δ 5.42 to collapse to a 10.8 Hz doublet, a value typical of a *cis* double bond coupling. The spectrum is similar

$J_{12,13}=10.8$ $J_{10,11}=15.4$

$$-H_2C-\overset{H}{C}=\overset{H}{C}-\overset{H}{C}=\underset{H}{C}-\overset{OH}{CH}-$$

$J_{13,14}=7.5$ $J_{11,12}=10.8$ $J_{9,10}=6.6$

	14	13	12	11	10	9
δ	2.18	5.42	5.95	6.48	5.63	4.15

to that found for the methyl ester of coriolic acid (14), which has a methyl 13-hydroxy-*cis*-9,*trans*-11-octadecadienoate structure. PMR data for the corn lipoxygenase product demonstrated clearly that the *trans* double bond was adjacent to the oxygenated carbon. Previously the *trans* configuration had been assumed to be adjacent to the hydroperoxide group, but other investigations with lipoxygenase products did not prove this assumption.

Isolation of Methyl Hydroxystearate

The entire sample of methyl hydroxyoctadecadienoate was pooled for hydrogenation. The products, methyl hydroxystearates, were isolated by column chromatography between fractions 57 and 81. Other products (Table II) were due to hydrogenolysis commonly observed in the reduction of activated hydroxyls (15).

Fractions 57 to 81 were composed of 9- and 13-hydroxystearates (Table II). 9-Hydroxystearate was clearly the major portion making up 83% of the total. As can be surmised from Table II, 9- and 13-hydroxystearates were obtained pure in selected fractions.

The IR spectra of the two hydroxystearates were identical, except in the region of carbon chain C-H rock absorptions where the 13-hydroxystearate absorbed more strongly at 785 cm^{-1}.

Proof of the 9-Hydroxyl

The pure hydroxystearate, isolated from fractions 69 to 81, was subjected to mass spectral analysis. The fragmentation analysis identified the sample as 100% methyl 9-hydroxystearate. The mass spectrum was identical to the one reported by Dolev et al. (10) for authentic methyl 9-hydroxystearate. Intense mass peaks appeared at 155, 158 and 187 due to cleavage at the 9-hydroxyl. No mass peaks attributable to 13-hydroxyl cleavage were found.

Optical Rotation and Absolute Configuration

The optical rotation of methyl 9-hydroxy-*trans*-10,*cis*-12-octadecadienoate was determined. $NaBH_4$ reduction of the hydroperoxide to the hydroxyoctadecadienoate probably would not disturb the asymmetric carbon, as the mechanism of $NaBH_4$ reduction of hydroperoxides is undoubtedly a hydride attack of the hydroperoxide oxygen. The hydroxyoctadecadienoate was as pure as could be acquired from the column separation (fractions 80 to 89), and was determined to be dextrorotatory, $[\alpha]^{25}_{546}$ = +5.3° (c , 3.25% hexane). The rotation was comparable in direction and magnitude to methyl 9-hydroxy-*cis*,*trans*-octadecadienoate from *Calendula* oil, $[\alpha]^{20}_{D}$ = +3.6° (c, 19.4% chloroform) (12) and to methyl dimorphecolate, $[\alpha]^{25}_{D}$ = +5° (c, 5.0% chloroform) (16). Inspection of optical rotatory dispersion measurements of methyl dimorphecolate in hexane (17) showed that this rotation at 546 nm was also similar to the product derived from corn lipoxygenase.

The absolute optical configuration of the corn lipoxygenase product, 9-hydroperoxyoctadecadienoic acid, was established by determining the rotation of its derivative, methyl 9-hydroxystearate. Baker and Gunstone (18) synthesized a stereo-specific 9-D-hydroxystearate, which was determined to be levorotatory by Schroepfer and Bloch (19). The sample of methyl 9-hydroxystearate derived from the lipoxygenase product was levorotatory, $[\alpha]^{25}_{546}$ = -0.22° (c, 4.62% methanol). The rotation of methyl 9-hydroxystearate obtained from the corn lipoxygenase product was similar to those obtained from methanolic solutions of 9-D-hydroxystearate derived from naturally occurring oils; *Calendula* oil ($[\alpha]^{25}_{546}$ = -0.29 to -0.32°) (12), *Strophanthus* oil ($[\alpha]^{23}_{546}$ = -0.18°) (19) and *Dimorphothecae* oil (20). We concluded the product of corn lipoxygenase is 9-D-hydroperoxyoctadecadienoic acid.

The two products of linoleic acid oxidation by soybean lipoxygenase, 13-hydroperoxyoctadecadienoic and 9-hydroperoxyoctadecadienoic acids, reportedly have opposite optical configurations. Hamberg and Samuelsson (21) showed that one of the products was 13-L-hydroperoxyoctadecadienoic acid. Veldink et al. (22) reported that the minor product of soybean lipoxygenase oxidation was 9-D-hydroperoxyoctadecadienoic acid since the 9-hydroxystearate derived from the hydroperoxide was

TABLE II

Column Chromatographic Separation of Products Derived From Methyl Hydroxyoctadecadienoate by Hydrogenation

Compound	Weight, %		Fraction number[a]
Methyl hydroxystearate	77		57-81
13-hydroxy-[b]	17	100	57-67
9-hydroxy-[b]	83		63-81
Methyl oxostearate	19		23-34
Methyl stearate	4		12-17

[a]When compounds were not completely separated by column chromatography, the extent of overlapping fractions was estimated by TLC.

[b]Percentage determined by TLC densitometry after double development of the plates.

levorotatory. Optical activity confirmed the enzymic origin of the 9-hydroperoxide in soybean systems. The 9-hydroperoxide from the soybean oxidation appeared to be identical to the major product from the corn enzyme.

Most naturally occurring 9-hydroxyoctadecadienoates investigated to this date have D-configuration, such as dimorphecolate (20), 9-hydroxy-*cis,trans*-octadecadienoate from *Calendula* (12). The finding of a 9-D-specific lipoxygenase activity strengthens the arguments of Morris et al. (13) and Powell et al. (23), who have suggested that the naturally occurring 9- and 13-hydroxyoctadecadienoates may be biosynthesized from linoleic acid through the action of lipoxygenase.

Both the D- and L-configurations of 13-hydroxyoctadecadienoic acid have been found in seed oils. D-Coriolic acid occurs in *Coriaria* oil (14) and L-coriolic acid, in *Monnina* oil (24). Although 13-L-specific lipoxygenase activity is known in soybeans, whether there is a 13-D-specific lipoxygenase activity remains to be demonstrated.

Further Studies on Positional Specificity

Although we propose that corn lipoxygenase oxygenates the 9-carbon of linoleic acid, Table II shows that the 13-carbon is oxygenated about 17%. In another study (4) of a series of enzyme reactions beginning with corn lipoxygenase, no evidence was found in the products that a 13-hydroperoxide intermediate existed. Transformation of the 9-hydroxyoctadecadienoate to the 13-hydroxyoctadecadienoate is known to occur in acidic media, as well as geometric isomerization of the *cis,trans* to *trans, trans* (23). Factors in the work-up of the products, including chemical reactions, time and silicic acid column separations, could be involved in some rearrangement that causes interconversion of the 9- to the 13-isomer. Autoxidation resulting in 13-carbon substitution also could occur to a small extent during the time of lipoxygenase oxidation, especially since the linoleic acid would be exposed to high oxygen tension in the presence of hydroperoxide. Zimmerman and Vick (25), reporting on the products of flaxseed lipoxygenase, discussed the possibility that minor products from lipoxygenase oxidations may be due to autoxidation.

In subsequent experiments an attempt was made to eliminate suspect factors that might lead to rearrangement and racemizations. However, artifacts arising from autoxidation could not be controlled. After oxidation of linoleic acid with corn lipoxygenase, the products were extracted without acidification of the reaction mixture (pH 6.9). The product mixture was immediately reduced with $NaBH_4$ followed by hydrogenation. The reduced mixture was then methyl esterified. All reactions were completed before any separations were made. Other components in the mixture were sufficiently separated by TLC from the hydroxystearates so that the percentage of 13- and 9-hydroxystearates could be determined by densitometry (13-hydroxystearate R_f = 0.288, 9-hydroxystearate R_f = 0.241). Separations were improved further by double development, which aided densitometry quantitation. This TLC method could prove to be a simple and rapid way to survey various lipoxygenase preparations for positional specificity. In duplicate corn-lipoxygenase oxidations 12.2% and 13.1% 13-hydroxystearates were obtained compared with 17% from the previous experiment (Table II).

Since the 13-carbon was also oxygenated, one must speculate whether the 13-hydroperoxide was formed either as an artifact, or by lipoxygenase activity. Nevertheless, an 83% to 88% yield of the 9-hydroperoxide is indicative of a high degree of specificity for 9-carbon oxidation. Since soybean lipoxygenase is known to produce up to 100% of the 13-hydroperoxide (10), there are probably at least two types of

lipoxygenase activity, which are responsible for preferential oxidation of either the 9- or 13-carbon.

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The Positional Distribution of Fatty Acids in the Phospholipids and Triglycerides of *Mycobacterium smegmatis* and *M. bovis* BCG

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ABSTRACT

Position 1 of the phospholipid and triglyceride fractions isolated from *Mycobacterium smegmatis* and *M. bovis* BCG was esterified principally with C_{18} related fatty acids (18:0, 18:1 and 19Br). Position 2 was occupied principally by C_{16} fatty acids. The third position of the triglycerides was esterified with a preponderance of C_{20}+ fatty acids. Seventy-six per cent of position 3 fatty acids in BCG and 43% in *M. smegmatis* triglycerides contained fatty acids of greater than 20 carbon atoms.

INTRODUCTION

Utilizing enzymatic or enzymatic and chemical means, the sterospecific distribution of fatty acids in phospholipids and triglycerides may be accurately determined. In the case of phospholipids, the sterospecific enzyme phospholipase A (E.C. 3.1.1.4) is used to hydrolyze fatty acids from the 2 position. Positional distribution of fatty acids on the 1, 2 and 3 positions of triglycerides may be determined by the enzymatic and chemical methods developed by Brockerhoff (1,2) as modified by Christie and Moore (3).

Our investigations have revealed that the triglycerides of *Mycobacterium smegmatis* and *M. bovis* BCG are extremely assymetric molecules having very long chain fatty acids (C_{20} and up) distributed essentially on the 3 position of L-glycerol. In addition, a close similarity was found to exist between the 1,2 positional distribution of fatty acids of both the phospholipids and triglycerides. This distribution will be discussed in relationship to possible lipid biosynthetic pathways operable in the mycobacteria.

MATERIALS AND METHODS

Thin layer chromatographic (TLC) standards were obtained from the Hormel Institute (Austin, Minn.). All solvents were distilled in glass before use. *Ophiophagus hanna* venom (Sigma Chemical Co., St. Louis, Mo.), steapsin (deactivated (4), and Porcine Lipase 448 (Nutritional Biochemicals Co., Cleveland, Ohio) were used in the lipase experiments.

Cultivation of Bacteria

M. smegmatis ATCC 19420 was grown in 2.8 liters Fernbach flasks containing 1 liter of modified Youmans medium (5) on a rotary shaker at 27 C for six to six and one half days. The cells were harvested by centrifugation and stored frozen at -20 C.

M. bovis BCG (Glaxo strain) was obtained from the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service. These organisms were grown as stationary surface cultures on Sautons medium (6) containing 5% glycerol for three weeks by Lederle Laboratories (Pearl River, N.Y.). Cells were kept frozen at -20 C until used.

Crude Lipid Preparation

Thawed bacterial cell paste was extracted with 4 to 5 vol of chloroform-methanol (2:1 v/v) on a shaker overnight at 27 C. Cell bodies were separated by filtration and the solvents were removed by rotary evaporation. The dried lipid extract was treated several times with boiling acetone in order to obtain hot acetone soluble and insoluble fractions.

Triglyceride Preparation

Hot acetone soluble lipid was chromatographed on Florisil columns (7) (100-200 mesh, deactivated with 7% water (w/w); Fisher Scientific Co., Medford, Mass.). The columns were eluted with hexane and hexane-diethyl ether (80:20 v/v). The hexane-ether fraction was further purified by TLC on 0.5 mm Adsorbosil 1 plates (Applied Science Labs., State College, Pa.), developing solvent, hexane-ether (80:15 v/v). The triglyceride preparations gave essentially single spots by TLC in petroleum ether-diethyl ether-glacial acetic acid (90:10:1 v/v/v).

Phospholipid Preparation

Hot acetone insoluble lipids were streaked on 0.25 mm Adsorbosil 3 plates (10% magnesium silicate binder) and developed with chloro-

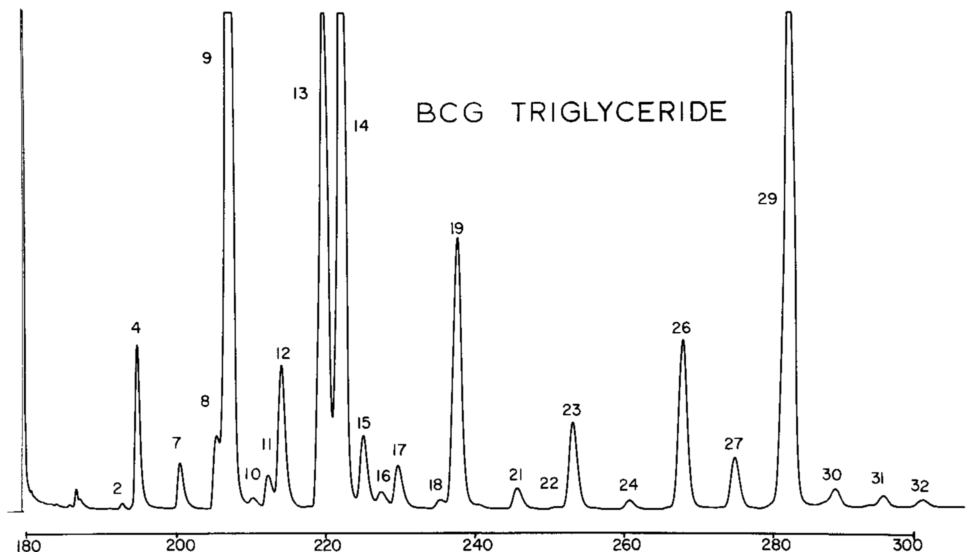

FIG. 1. GLC recording of fatty acid methyl esters from *M. bovis* BCG triglyceride. Conditions under Materials and Methods. Numbers correspond to fatty acids listed in Table I.

form-methanol-water (75:25:4 v/v/v). Phospholipids were located with Rhodamine 6G spray (0.01% w/v) under ultraviolet light. Phospholipid bands were scraped, eluted with chloroform-methanol (1:1 containing 5% water) and rechromatographed on 0.5 mm Adsorbosil 3 plates in chloroform-methanol-water (65:25:4 v/v/v). Cardiolipin was rechromatographed on 0.25 mm Adsorbosil 3 plates in chloroform. Cardiolipin, which remained at the origin, was scraped and eluted.

The cardiolipin and phosphatidylethanolamine were homogeneous by TLC in several solvent systems. The phosphatidylinositolmannosides gave two or three phosphorous containing spots by TLC (8,9).

Stereospecific Analysis of Triglycerides

The methods of Brockerhoff (1,2) as modified by Christie and Moore (3) were used, with the exception that the experiments were scaled up in order to use 60 mg or 100 mg of trigly-

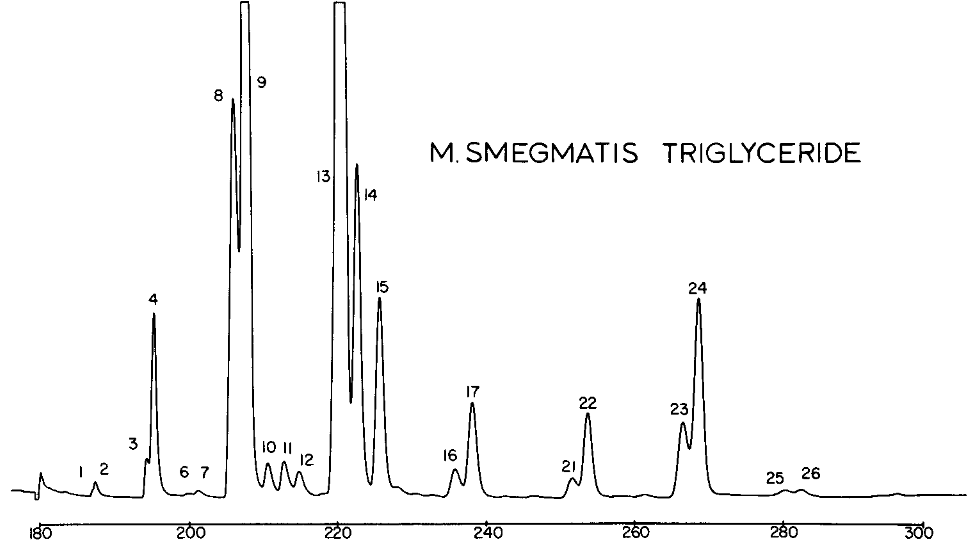

FIG. 2. GLC recording of fatty acid methyl esters from *M. smegmatis* triglyceride. Conditions under Materials and Methods. Numbers correspond to fatty acids listed in Table III.

TABLE I

Stereospecific Analysis of Triglycerides From *M. bovis* BCG[a]

	Fatty acid composition[b] (wt %)	Total[c]	Position 1[d]	Position 2[e]	Position 2[f]
1	12:0	Trace[g]	Trace	---	---
2	14Br	Trace	---	---	---
3	14:1	Trace	Trace	---	---
4	14:0	2.2	0.7	6.0	4.5
5	15Br	Trace	---	---	Trace
6	15:1	Trace	0.3	Trace	---
7	15:0	0.8	0.3	2.2	2.0
8	16:1	1.7	2.1	1.6	1.0
9	16:0	19.4	10.5	56.8	57.0
10	17Br	0.3	0.3	0.3	0.1
11	17:1	0.7	1.9	0.5	0.1
12	17:0	3.2	2.5	6.8	6.9
13	18:1	13.9	35.7	2.1	1.1
14	18:0	17.6	26.2	19.0	20.1
15	19Br	1.9	4.4	0.2	0.3
16	19:1	0.5	1.1	0.1	---
17	19:0	1.1	1.3	0.2	0.5
18	20:1	0.3	0.4	0.2	0.1
19	20:0	7.1	4.9	1.5	1.8
20	21Br	0.1	Trace	---	---
21	21:0	0.4	0.4	0.3	0.6
22	22:1	Trace	0.1	0.1	0.2
23	22:0	2.3	0.9	0.5	0.9
24	23:0	0.2	Trace	Trace	0.1
25	24:1	Trace	---	---	---
26	24:0	4.9	1.4	0.5	0.9
27	27Br	1.5	0.4	Trace	0.2
28	26:1	0.1	---	---	---
29	26:0	17.7	4.0	1.0	1.4
30	29Br	0.8	---	---	---
31	32Br	0.4	---	---	---
32	X	0.6	---	---	---
33	Y	Trace	---	---	---

[a]GLC conditions under Materials and Methods.

[b]Tentative identification based on retention time data.

[c]Average of five experiments.

[d]Average of three experiments; from lysophosphatidylphenol.

[e]Average of two experiments; fatty acids liberated by phospholipase A from phosphatidylphenol.

[f]Average of three experiments; 2-monoglyceride from pancreatic lipase hydrolysis.

[g]Less than 0.1%.

cerides in the initial reaction. As cautioned by Christie and Moore (3), fresh Grignard reagent (ethyl-magnesium-bromide and super-dry diethyl ether) (10) was used for each experiment and great care was taken to exclude moisture from glassware and lipid samples in the preparation of diglycerides by Grignard reaction.

Pancreatic Lipase Hydrolysis

To an 8 ml vial with Teflon lined screw cap was added 60 mg of triglyceride, 2.0 ml Tris buffer [0.5 M tris(hydroxymethyl) methylamine; pH 7.45], 0.4 ml 22% calcium chloride solution, 0.6 ml 0.2% bile salts (ox bile extract, Nutritional Biochemicals Co.) and 0.5 ml pancreatic lipase [either a 10% solution-suspension of deactivated steapsin (4) or a solution of Porcine Lipase 448 (5 mg/ml in 0.005M $CaCl_2$)]. The material was ultrasonicated for 10-15 sec and an additional 0.5 ml of enzyme was added. The vials were sealed and placed in a Mickle shaker (Brinkmann Instruments Inc., Westbury, N.Y.) set at maximum oscillations for 2 hr (37 C).

The monoglycerides were isolated from 0.5 mm Adsorbosil 1 plates which had been developed in unlined TLC tanks with diethyl ether-benzene-ethanol-glacial acetic acid (11) (40:50:2:0.2 v/v/v/v; monoglyceride R_f 0.2-0.3), after visualization with 2.7 dichlorofluorescein (3).

TABLE II

Stereospecific Analysis of Phospholipids From *M. bovis* BCG

Fatty acid composition (wt %)	Cardiolipin			Phosphatidylethanolamine			Phosphatidyl-inositol-mannosides
	Total	1-1[1] position[a]	2-2[1] position[b]	Total	1 position[c]	2 position[d]	Total
14:1	---	---	---	---	---	---	---
14:0	0.4	---	0.6	0.3	---	0.6	0.2
15:1	0.1	0.2	0.2	---	---	---	---
15:0	0.6	Trace	1.0	0.3	---	0.6	0.6
16:1	7.1	1.4	11.5	1.0	0.6	1.4	1.0
16:0	26.9	5.3	50.0	39.1	15.4	62.8	41.1
17Br	0.5	0.6	0.6	0.5	0.6	0.4	0.7
17:1	2.8	2.0	3.4	0.6	0.4	0.8	---
17:0	3.2	0.6	6.6	6.0	4.0	8.0	5.0
18Br	0.7	0.7	Trace	0.8	1.2	0.4	1.2
18:1	35.3	60.1	13.7	6.6	7.4	5.8	4.7
18:0	5.3	3.7	7.5	20.5	30.9	10.1	7.3
19Br	14.0	22.3	4.0	23.4	36.6	10.2	35.9
19:1	1.1	1.8	---	Trace	0.9	---	0.5
19:0	0.1	0.1	0.1	0.2	0.8	---	0.4
20:1	Trace	---	---	---	---	---	---
20:0	0.3	1.0	0.3	0.6	1.1	0.1	0.6
21:0	---	---	---	---	---	---	Trace
22:0	Trace	---	---	---	---	---	0.3
24:0	0.2	---	---	---	---	---	0.4
27Br	Trace	---	---	---	---	---	---
26:0	1.3	---	0.1	---	---	---	---

[a]From lysocardiolipin after phospholipase A hydrolysis.
[b]Fatty acids liberated during phospholipase A hydrolysis.
[c]From lysophosphatidylethanolamine after phospholipase A hydrolysis.
[d]2(Total)-position 1-1[1].

Phospholipase A Hydrolysis

Phospholipids were hydrolyzed according to the method of Christie and Moore (3). TLC separation was performed on 0.5 mm Adsorbosil 3 plates developed with chloroform-methanol-aqueous ammonia (sp gr 0.90; 65:25:4 v/v/v) with visualization by aqueous Rhodamine 6G (0.1% w/v).

Preparation of Fatty Acid Methyl Esters

Fatty acid methyl esters were prepared from all lipid classes by transesterification in hydrochloric acid-methanol (4.2% w/w; prepared by the addition of 1.7 ml acetyl chloride, Eastman Kodak Co., Rochester, N.Y., to 20 ml of methanol) held at 60 C overnight. Excess HCl was neutralized with solid sodium bicarbonate and the methyl esters were extracted with hexane.

Gas Liquid Chromatography

Gas liquid chromatography (GLC) was performed on a Perkin-Elmer model 900 dual flame gas chromatograph equipped with 6 ft x 1/8 in. O.D. stainless steel columns packed with high performance Chromosorb G (80-100 mesh, acid washed, dimethylchlorosilane treated; Johns Manville Co., New York, N.Y.). The support was coated with 2.5% (w/w) OV-1 (Applied Science Labs., State College, Pa.). Fatty acid methyl esters were chromatographed from 180 C to 300 C at 4 deg/min 35 ml/min helium flow. These conditions were shown adequate to elute *n*-methyl triacontanoic acid (n-C_{30}).

Chromatography on Microparticulate Silica Gel

The methods as described by Ribi et al. (12) were used. Quso G-32 micro-fine precipitated silica (Philadelphia Quartz Co., Philadelphia, Pa.) slurried in isooctane-glacial acetic acid (99.5:0.5 v/v) was packed at 3420 x *g* at 20 C. Fifty to 200 μg of lipid was applied in a loading stopper (12). The columns were centrifuged at 3420 x *g* for 23 min, extruded, dried and sprayed with acid-dichromate and charred (12). Migration values (calculated as the ratio of the migration distance of the compound to the length of the column) of triglyceride standards were C_{48} (tripalmitin, 0.52), C_{54} (tristearin, 0.59), C_{60} (triarachidin, 0.67) and C_{66} (tribehenin, 0.72).

TABLE III

Stereospecific Analysis of Triglycerides From *M. smegmatis*

Fatty acid composition (wt %)		Total	Position 1[a]	Position 2[b]	Position 2[c]	Position 3[d]
1	12:1	Trace	---	---	---	---
2	12:0	0.2	0.1	---	---	0.5
3	14:1	0.6	0.3	0.5	0.6	0.9
4	14:0	3.0	1.0	6.6	6.6	1.4
5	15Br	Trace	---	---	Trace	---
6	15:1	0.1	---	Trace	0.1	0.2
7	15:0	0.1	---	0.4	0.4	---
8	16:1	9.6	8.7	12.5	13.1	7.0
9	16:0	24.4	8.9	57.6	57.3	7.0
10	17Br	0.7	0.6	0.5	0.8	0.7
11	17:1	0.8	1.5	0.6	0.8	0.1
12	17:0	0.7	0.2	1.7	1.5	0.4
13	18:1	29.2	60.4	10.4	9.3	17.9
14	18:0	9.3	6.5	6.1	5.9	15.5
15	19Br	4.9	7.4	0.7	1.2	6.1
16	20:1	0.8	0.5	0.3	0.3	1.6
17	20:0	2.8	1.1	0.6	0.4	6.9
18	21Br	Trace	---	---	---	---
19	21:1	Trace	---	---	---	---
20	21:0	Trace	0.1	0.2	0.2	Trace
21	22:1	0.6	0.3	0.2	0.1	1.4
22	22:0	2.7	0.6	0.4	0.3	7.2
23	24:1	2.5	0.6	0.3	0.4	6.5
24	24:0	6.6	1.1	0.4	0.6	18.1
25	26:1	0.2	---	---	---	0.6
26	26:0	0.2	---	---	---	0.6

[a]From lysophosphatidylphenol.
[b]Fatty acids liberated by phospholipase A from phosphatidylphenol.
[c]2-Monoglyceride from pancreatic lipase hydrolysis.
[d]3(Total-(position 1 + position 2).

RESULTS

Tables I and II summarize the results of stereospecific analyses of BCG triglycerides and phospholipids. Position 1 of the triglycerides was occupied principally by 18:1, 18:0 and 16:0 acids while position 2 was esterified with 16:0 and 18:0. By difference calculation, position 3 contained mostly C_{26} (about 48%), C_{20} (about 15%) and C_{24} (about 13%) fatty acids. The fatty acids C_{20} to C_{33} constitute a total of 76% of the fatty acids at position 3.

In the case of BCG phospholipids, position 1 is esterified principally with C_{18} related fatty acids (18:0, 18:1 and 19Br). Position 2 is occupied principally by C_{16} fatty acids (61% in cardiolipin; 64% in phosphatidylethanolamine).

Position 1 of *M. smegmatis* triglycerides is occupied principally by 18:0, 18:1 and 19Br fatty acids while position 2 is occupied by 16:0 and 16:1 acids. Position 3 (by difference calculations) is not as heavily esterified with long chain fatty acids as BCG, nevertheless, $C_{20\text{-}26}$ acids make up about 43% of the fatty acids at this position.

Position 1 of *M. smegmatis* cardiolipin and phosphatidylethanolamine is esterified principally by 18:1, 18:0 and 19Br acids. Position 2 of cardiolipin contains 80% of 16:1 plus 16:0, and in the case of phosphatidylethanolamine 70% of the fatty acids at this position are 16:0 and 16:1.

DISCUSSION

Brockerhoff's original procedure (1,2) for the sterospecific analysis of triglycerides was found to be unsatisfactory for the study of mycobacterial triglycerides, principally because of low yields of 1,3 diglyceride and the large amounts of triglyceride required. The modifications proposed by Christie and Moore (3) were found to greatly facilitate analysis. Maintenance of absolutely moisture free reaction conditions, use of ethyl-magnesium-bromide and use of α,β rather than 1,3 diglycerides were the chief innovations by these authors. As may be seen from Table I, the sum of 16:0 fatty acids of positions 1 and 2 slightly exceeds three times the total of 16:0. Many experiments were performed to determine the cause of this discrepancy. Each trial gave

TABLE IV

Stereospecific Analysis of Phospholipids From *M. smegmatis*

Fatty acid composition (wt %)	Cardiolipin			Phosphatidylethanolamine			Phosphatidylinositol	Phosphatidylinositolmannosides
	Total	1-1[1] position[a]	2-2[1] position[b]	Total	1 position[c]	2 position[d]	Total	Total
14:1	---	---	0.2	---	---	---	0.9	0.4
14:0	1.8	---	3.8	0.5	0.2	0.8	3.0	2.4
15Br	---	---	Trace	---	---	---	---	---
15:1	---	---	0.6	---	---	---	0.6	0.2
15:0	---	---	0.4	0.1	Trace	0.2	0.2	0.4
16:1	33.3	4.5	63.6	6.0	1.9	10.1	9.6	15.1
16:0	7.9	0.9	18.6	39.1	16.8	61.4	36.4	34.9
17Br	1.0	1.4	1.1	1.4	1.6	1.2	1.2	2.0
17:1	1.5	1.7	1.6	0.1	---	0.2	0.3	0.5
17:0	---	---	---	0.2	---	0.4	0.2	0.6
18Br	---	---	0.2	0.6	0.5	0.7	0.4	0.7
18:1	39.3	67.4	6.3	9.0	11.4	6.6	3.8	3.6
18:0	0.7	1.3	0.9	1.6	1.7	1.5	0.8	1.0
19Br	11.7	21.8	1.3	41.4	65.9	16.9	34.6	38.3
19:0	---	---	0.7	---	---	---	0.3	---
20:0	0.1	---	---	---	---	---	Trace	---
21:1	---	---	---	---	---	---	0.2	---
21:0	0.1	1.0	---	---	---	---	7.3[e]	3.0
22:0	0.2	---	---	---	---	---	---	---
24:1	0.2	---	---	---	---	---	---	---
24:0	2.2	---	0.6	---	---	---	---	---

[a]From lysocardiolipin after phospholipase A hydrolysis.
[b]Fatty acids liberated during phospholipase A hydrolysis.
[c]From lysophosphatidylethanolamine after phospholipase A hydrolysis.
[d]2(Total)-position 1.
[e]Unidentified C number approximately 21.3.

essentially the same results. The possible presence of very long chain fatty acids which were not elutable by GLC was explored in three ways. Free fatty acids obtained from base hydrolysis of the BCG triglycerides were extracted with boiling methanol. No insoluble residue indicative of mycolic acids was observed (13). To the above fatty acids which had been completely methylated by excess diazomethane was added methyl-tridecanoic acid as an internal standard. GLC accounted for 94% to 95% of the fatty acids. Chromatography of the triglyceride fraction on microparticulate silica gel (12) showed the presence of only trace amounts of high molecular weight triglyceride. Most of the triglycerides had migration values of compounds containing 48 to 60 fatty acid carbons.

The experimental conditions described for pancreatic lipase hydrolysis were arrived at after much experimentation. Partial hydrolysis (as described by Luddy (14) gave high yields of diglycerides and low yields of monoglyceride because of the extreme asymmetry of the molecules. Hydrolysis times had to be greatly extended to obtain satisfactory hydrolysis. Admittedly, the longer hydrolysis time may allow for acyl migration, however, the close correlation of the data for position 2 (Tables I and III) obtained by the two methods would seem to indicate that acyl migration was minimal.

A striking similarity is noted between the fatty acid contents of the 1 and the 2 positions of the phospholipids and the triglycerides of the same organism. It is intriguing to postulate the presence of a common intermediate where the 1 and 2 positions of L-glycerol are substituted by biosynthetically related fatty acids.

Kennedy (15) showed that in mammalian liver, 1,2-diacy-*sn*-glycerols react with long chain acyl CoA compounds to yield triglycerides or with CDP-choline and CDP-ethanolamine to form the respective phospholipids. The common intermediate in the biosynthesis of these two classes of lipids is the diglyceride derived from phosphatidic acid. In the mycobacteria, the biosynthetic pathways operative in triglyceride and phospholipid biosynthesis have not been determined. The data obtained in this study would seem to indicate that both types of lipids were derived from a common intermediate. The close similarities between the distribution of fatty acids at the 1 and 2 positions of both the triglycerides and the phospholipids, however, may merely reflect the specificities of separate transferase systems, one synthesizing triglycerides and the other phospholipids.

The origin of the shorter chain fatty acids on the 1 and 2 positions of the triglycerides and phospholipids and the longer chain fatty acids on the 3 position of the triglycerides is not known. Studies of the nature of the fatty acid synthesizing systems of the mycobacteria are actively being pursued. Kanemasu and Goldman (16) and Wang et al. (17) have found two fatty acid elongating systems to be operable in their studies of the avirulent *M. tuberculosis*, a condensing system where two molecules of a fatty acid (C_n) were condensed to form a C_{2n} normal fatty acid and an elongation system. The elongation system, located in the soluble, cell free extracts, adds C_2 units from acetyl-CoA to the carboxyl end of an acceptor lipid (long chain acyl-CoA).

Matsumura et al. (18,19) have characterized two fatty acid synthetase systems in *M. phlei*. System 1, which effects fatty acid biosynthesis from malonyl-CoA and acetyl-CoA, is a multienzyme, ACP containing complex. System 2 is a chain lengthening system which uses palmitoyl- or stearoyl-CoA and not acetyl- or octanoyl-CoA for chain initiation. This system requires an external source of ACP.

The differences which have been noted between the specificities of the elongation systems by Block et al. (18,19) and those studied by Goldman et al. (16,17) may reflect basic differences in the mycobacteria being studied. The first group has used the saprophytic mycobacterium, *M. phlei* for their studies, while the Goldman group has used the avirulent *M. tuberculosis* strain $H37R_a$ in their studies. As shown in this study, profound differences in the amounts and types of longer chain fatty acids produced by the saprophyte, on the one hand, and the attenuated pathogen, on the other hand, were observed.

Others have noted asymmetry in the fatty acid distribution at position 3 of naturally occurring triglycerides. Breckenridge and Kuksis (20) observed, while studying a lower molecular weight triglyceride distillate from milk fat, that 95% of the C_4 to C_8 fatty acids of this fraction were esterified at the 3 position. Akesson (21) showed that a higher proportion of polyunsaturated fatty acids were located at the 3 position than at any other position of rat liver triglycerides.

Akamatsu and Law (22), Walker and Howard (23), as well as Brennan and Ballou (9) have also found high levels of 19Br (tuberculostearic; 10-methyl stearic) acid in some mycobacterial phospholipids. Age of the cells and turnover rate of the phospholipid appear to have some relationship to the amount of this acid present in a particular lipid. Kaneshiro and Thomas (24) have observed that conditions

conducible to methyl-branched and cyclopropane synthesis were low aeration, high temperature and excess methionine. Moreover, cells aged in the stationary phase may have an excess of hydrogen donor groups as well as methyl donor groups.

The presence of small amounts of $C_{19}Br$ fatty acids in the triglyceride fraction may be due to turnover of fatty acids from the phospholipids. These fatty acids may also have been synthesized at a triglyceride-phospholipid intermediate stage or after oleate or stearate had been attached to the triglyceride. Akamatsu and Law (22), however, believe that the $C_{19}Br$ acid is synthesized after oleate has been esterified to the phospholipid molecule.

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Studies on the Glycolipids and Phospholipids of Immature Soybeans

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ABSTRACT

The lipid of immature soybeans was extracted with chloroform-methanol and fractions containing the glycolipids and phospholipids were separated by column chromatography. Phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidyl inositol (PI), phosphatidic acid (PA) phosphatidyl glycerol (PG), n-acyl phosphatidyl ethanolamine (APE) and sulfolipid (SL) were identified by thin layer chromatography (TLC). Sterol glucoside (SG), esterified sterol glucoside (ESG), digalactosyl diglyceride (DGDG) and cerebrosides (CE) were isolated by TLC and identified by color reactions, chemical degradation and spectral analysis.

INTRODUCTION

Using paper chromatography and complementary techniques, Hirayama and Hujii (1) detected phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidic acid (PA), inositol containing phospholipids and a number of unidentified compounds that contained phosphorous or sugars in the lipid of immature soybeans. Sterol glucoside (SG) and esterified sterol glucoside (ESG) are common constituents of plant lipids and have been isolated from soybean phosphatides by Lepage (2) and from soybean oil by Kiribuchi et al. (3). Galactolipids and sulfolipids are also common plant lipid constituents (4-7), although they do not appear to have been isolated from soybeans. The occurrence of cerebrosides (CE) in plants has been proven by Carter et al. (8), and compounds of this type have been detected in soybeans by van Handel (9). Recent studies in this laboratory (10,11), as well as those of Hirayama and Hujii (1), show that during maturation of soybeans, triglycerides increase from minor to major components of the lipid. Simultaneously, there is a decrease in the concentration of phospholipids and glycolipids and, except for PC, PE and phosphatidyl inositol (PI), these compounds are difficult to detect in the lipid of mature soybeans. As a prelude to studies on the biosynthesis of the lipid of soybeans during maturation, examination of the composition of the phospholipids and glycolipids of immature beans was undertaken. Results of this study are reported here.

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MATERIALS AND METHODS

Soybeans. Immature Chippewa 64 soybeans were picked at approximately 40 days after flowering. Mature beans were also picked (at approximately 88 days after flowering) for comparative analyses. The beans were frozen on dry ice in the field as they were picked.

Extraction of the Lipid. The beans were separated from the pods and extracted in batches of 10 g with 20 vol of chloroform-methanol (2:1) and once with 10 vol of a 1:2 mixture of these solvents in a VirTis model 45 homogenizer. The extracts were combined and evaporated under reduced pressure to approximately one third of the original volume precipitating denatured proteolipid (4). The precipitated material was removed by filtration and evaporation of the solvent continued until a thin slurry of lipid was obtained. The slurry of lipid was then transferred to a separatory funnel with approximately 100 ml of chloroform and 50 ml of an aqueous solution of 0.5% sodium chloride. The chloroform phase was extracted three times with the salt solution and then once with water to remove non-lipid material. The aqueous extracts were combined and re-extracted once with fresh chloroform to recover lipid that was removed by the aqueous washings.

Standards. Pure methyl esters (> 99%) and tripalmitin were purchased from the Lipids Preparation Laboratory of The Hormel Institute. Phospholipids were isolated from beef heart, CE from beef brain according to the procedure described by Rouser et al. (12). Mono- and digalactosyl diglycerides were isolated from spinach leaves as described by Allen et al. (6).

Analytical Methods. Infrared spectra were recorded with a Perkin Elmer spectrophotometer Model 237 using either KBr pellets or 10% solutions in CS_2 or $CHCl_3$. Ester content was determined by a hydroxamic acid method (13) using tripalmitin as a standard. Total sugar

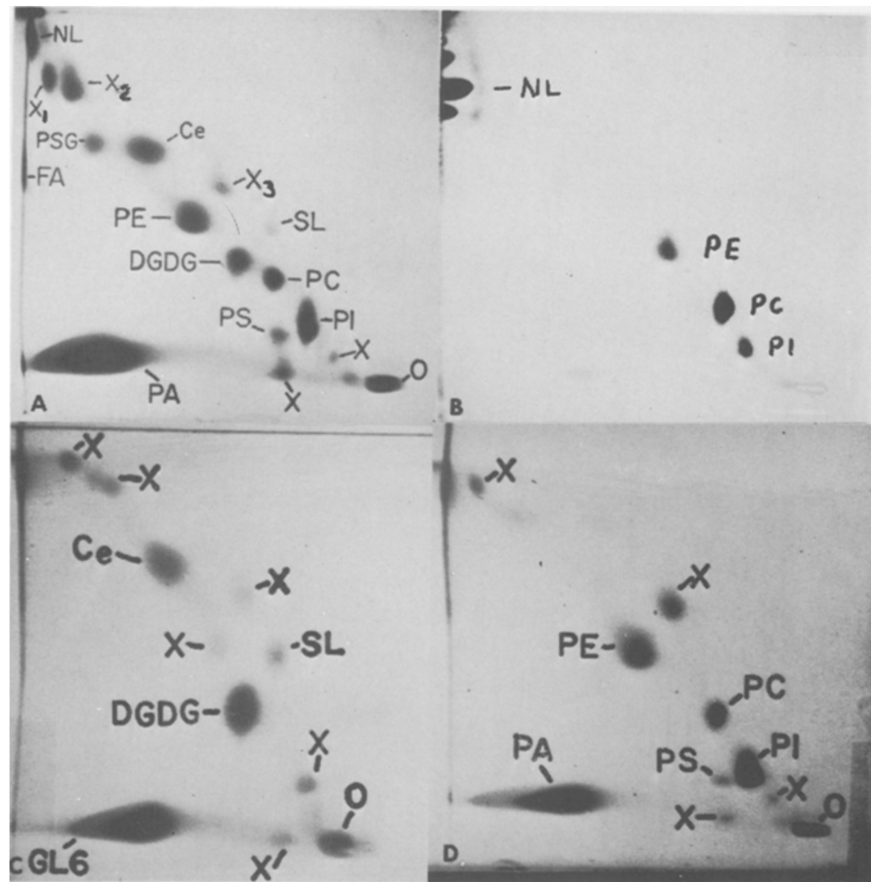

FIG. 1. Two-dimensional TLC of soybean lipids with chloroform-methanol-concentrated (28%)NH_4OH (65:35:5) in the first dimension (vertical dimension) followed by drying for 10 min in nitrogen and then development in second dimension (from right to left) with chloroform-acetone-methanol-acetic acid-water (100:40:20:20:10). Upper left (A), lipid of immature bean; upper right (B), total lipid of mature bean; lower left (C), glycolipid fraction separated by column chromatography; lower right (D), phospholipid fraction separated by column chromatography. O, origin; PA, phosphatidic acid; PS, phosphatidyl serine; PI, phosphatidyl inositol; PC, phosphatidyl choline; SL, sulfolipid; PE, phosphatidyl ethanolamine; CE, cerebrosides; PSG, sterol glucoside; NL, neutral lipid; X, unidentified compounds. GL6, plate C, is so designated because it gave a strong positive test for glycolipid (see text).

was determined colorimetrically by the phenol-sulfuric acid method (14) using galactose as a standard. The method of Hanahan and Olley (15) was used to determine glycerol. Quantitative analysis of sugar, ester and glycerol in digalactosyl diglyceride was carried out by the procedure of Sastry and Kates (16).

Column Chromatography. The total lipid extract was fractionated in batches of approximately 0.5 g on columns of 2.8 x 30 cm of HCl treated Florisil (Fisher Scientific Co., Fair Lawn, N.J.) (17), or Unisil (Clarkson Chemical Co., Inc., Williamsport, Pa.) silicic acid using the solvent systems described by Rouser et al. (12). Fractionations were monitored by thin layer chromatography (TLC) using the solvent systems described below.

TLC. Plates coated with Silica Gel H, 250 μ thick, were activated at 120 C and stored at 80 C until used. Chromatography was performed at room temperature in chambers lined with filter paper. The following solvent systems were used: (a) petroleum ether (b.p. 30-60 C)-ethyl ether-acetic acid (70:30:1 or 80:20:1) for neutral lipids; (b) two dimensional system; first dimension, chloroform-methanol-14 N NH_4OH (65:35:5); second dimension, after drying for 10 min under nitrogen, chloroform-acetone-methanol-acetic acid-water (100:40:20:20:10) (18); (c) chloroform-

TABLE I

Fractionation of Soybean Lipids on Acid Treated Florisil[a]

Fraction	Solvent	Eluate volume (ml)	Per cent of total lipid: Immature soybeans	Mature soybeans	Main constituents[a]
I	Chloroform	200	62.2	92.0	Neutral lipids
II	Chloroform-acetone (1:1)	140	18.2	---	ESG, SG
III	Acetone	600	11.2	1.9	CE, SL, DGDG
IV	Methanol	200	7.9	6.1	PA, PI, PE, PC, PS

[a]ESG, esterified sterol glucoside; SG, sterol glucoside, CE, cerebrosides; SL, sulfolipid; DGDG, digalactosyl diglyceride; PI, phosphatidyl inositol; PE, phosphatidyl ethanolamine; PC, phosphatidyl choline; PS, phosphatidyl serine; PA, phosphatidic acid.

methanol-water (65:25:4) for highly polar phospholipids; (d) chloroform-methanol-water (100:42:6) (19) or chloroform-methanol-2 N NH_4OH (100:25:2.5) (20) for sphingosine bases; (e) chloroform-methanol-7 N NH_4OH (80:20:2) for SG and ESG; (f) chloroform-methanol-14 N NH_4OH (65:35:5) (18) for CE, SL and DGDG.

Spots were made visible by staining them

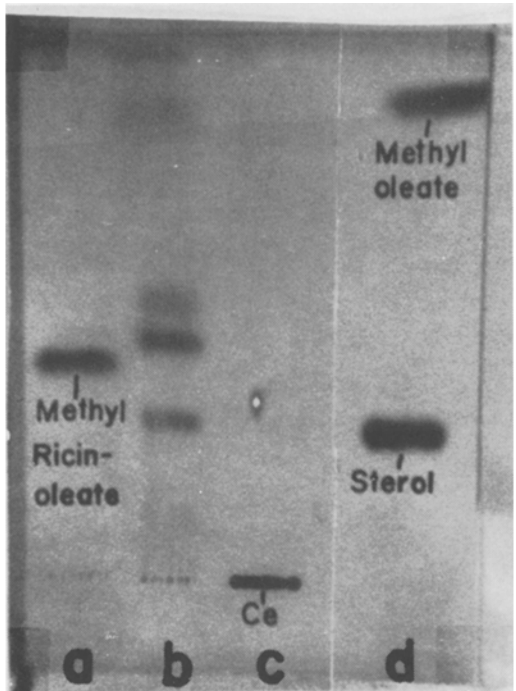

FIG. 2. TLC on Silica Gel H of: a, methyl recinoleate standard; b, methyl esters obtained from soybean CE; c, CE; and d, methyl oleate and sterol standards. Solvent system, petroleum ether-ethyl ether-acetic acid (80:20:1).

with iodine vapor (21) or by charring (22). The reagent described by Vaskovsky and Kostetsky (23) was used to detect phospholipids, α-naphthol-H_2SO_4 for glycolipids (24) and ninhydrin for compounds containing free amino groups. Spots or bands in preparative TLC were detected by spraying with water (25), scraped from the plate and extracted with chloroform-methanol (1:1).

Gas Liquid Chromatography (GLC). Methyl esters and aldehydes were analyzed on a 6 ft x 1/4 in. column of 8% EGSS-X on Gas Chrom P (Applied Science Labs., State College, Pa.) at 200 C and 125 C, respectively, with an F & M Model 1605 instrument equipped with a flame ionization detector. Sterols were analyzed by GLC on a 6 ft glass column of 3% JXR on Gas Chrom Q (Applied Science Labs., State College, Pa.) at 220 C or by temperature programming from 180 C to 260 C at 2.5 C/min using a Barber Colman Model 5000 instrument equipped with a flame ionization detector.

RESULTS

Composition of the glycolipids and phospholipids of soybeans is illustrated in Figure 1. Identification of the spots was made on the basis of comparison with authentic standards and color tests. Comparison of the two upper plates (Fig. 1) show that the concentration of glycolipids is much greater in immature than mature beans. These plates also show large differences in the composition of the phospholipids in immature and mature beans. The compounds designated by X in Figure 1 could not be positively identified. However, in the upper left plate of the total lipid X_1 appears to be ESG, X_2 n-acyl phosphatidyl ethanolamine (APE) and X_3, phosphatidyl glycerol (PG), on the basis of their Rf values, color tests and

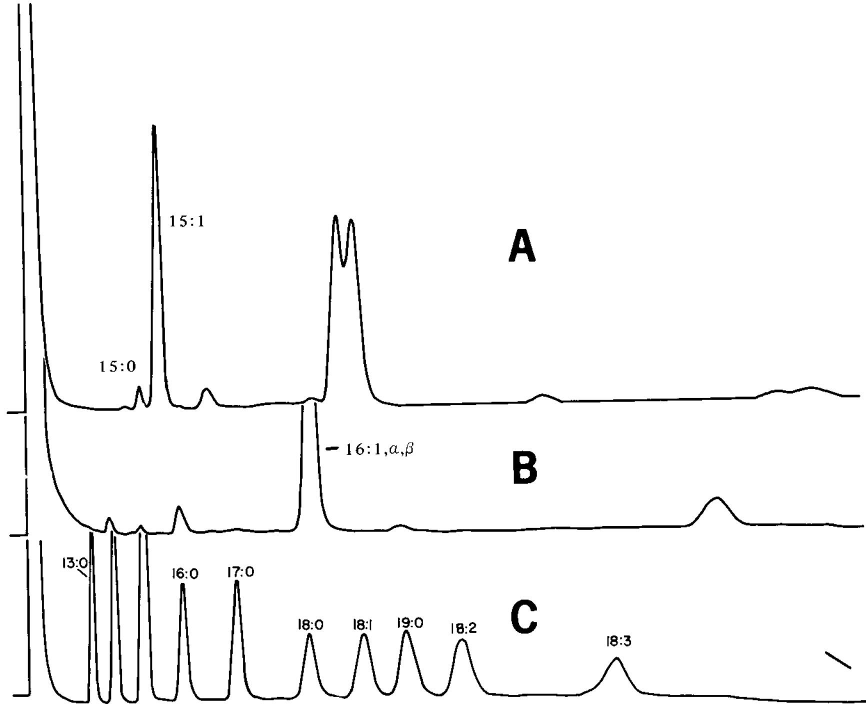

FIG. 3. GLC of aldehydes obtained by periodate cleavage of sphingosine bases of: A, soybean CE; B, beef brain CE; C, standard mixture of aldehydes. Number before colon, chain length; number after colon, number of double bonds.

recent studies by the authors on the biosynthetic labeling of these compounds (26). The large spot designated as PA in the upper left plate contained a mixture of compounds in addition to phosphatidic acid as evidenced by a positive test for glycolipid as well as phosphorous and the general staining pattern of the spot. The compounds giving this spot separated into the glycolipid and phospholipid fractions by column chromatography. Except for this fraction, the phospholipids and glycolipids separated with little overlapping of components by column chromatography as illustrated by the TLC analyses in Figure 1. Because the spot in the lower left plate with the same position as PA in the lower right plate gave a strong test for glycolipid, it was designated as GL 6 to distinguish it from PA. Analyses of the fractions separated by column chromatography also showed the presence of several compounds that were not detected in the original oil. Spots that could not be positively identified in these analyses (lower plates, Figure 1) were also designated by X. The general pattern of the fractionation of the total lipid by column chromatography is summarized in Table I. These results also show the large differences in composition between the lipids of immature and mature beans. Fractions rich in individual components were selected for preparative TLC. From these fractions employing the solvent systems described above, preparations that were homogeneous by two-dimensional TLC were obtained for ESG, SG, digalactosyl diglyceride (DGDG) and sulfolipid (SL). These compounds were characterized as follows:

Esterified Sterol Glucoside

(a) Color tests for sterols and sugars were positive. (b) Methanolysis (27) gave methyl esters, free sterol and glucose. GLC showed that the sterols consisted of β-sitosterol as a major component and campesterol and stigmasterol as minor components. (c) Steryl glucoside was identified among the products of deacylation (28) by TLC and by infrared spectral analysis (6). (d) Ester value gave one mole of fatty acid per mole of sterol glucoside.

Sterol Glucoside

(a) Color tests for sterols and sugars were positive. (b) The infrared spectrum was identical to that reported in the literature for this compound (6). (c) Acid hydrolysis (16) gave sterol and glucose. (d) GLC showed that the composition of the sterols was very similar to

TABLE II

Fatty Acid Analyses of Soybean Lipids

Fatty acid[a]	Digalactosyl diglyceride: Immature soybeans	Digalactosyl diglyceride: Mature soybeans	Esterified sterol glucoside: Immature soybeans	Esterified sterol glucoside: Mature soybeans	Esterified sterol glucoside: LePage (2)	Esterified sterol glucoside: Kiribuchi et al. (3)	Sulfolipid of immature soybeans
16:0	11.6	11.4	38.2	33.9	33.7	42.7	37.1
18:0	5.3	5.7	17.3	6.2	7.0	9.6	12.3
18:1	6.2	5.3	7.0	9.1	8.8	13.7	5.0
18:2	13.2	31.0	23.4	43.2	47.4	31.2	30.9
18:3	63.7	46.6	14.0	7.6	2.2	2.8	30.9
Others	---	---	---	---	0.9[b]	---	5.0

[a]Number before colon, chain length, number after colon, number of double bonds.
[b]16:1.

that of ESG and consisted of one major component, β-sitosterol, and two minor components, campesterol and stigmasterol.

Digalactosyl Diglyceride

(a) Color test for sugars was positive and that for phosphorous negative. (b) Methanolysis gave methyl esters, galactose and glycerol. (c) TLC properties and infrared spectrum were identical to those of an authentic sample isolated from spinach leaves.

Cerebrosides

(a) This fraction had the same TLC properties as beef brain CE except that it chromatographed as a single spot. (b) The color test for sugar was positive and that for phosphorous negative. (c) Methanolysis (29,30) gave methyl esters, sphingosine bases and sugars. (d) TLC of the methyl esters from CE on Silica Gel H separated these compounds into two minor and two major fractions as shown in Figure 2. Comparison with methyl ricinoleate and oleate (Fig. 2) indicated that the two major components and one of the minor components (which made up most of the ester fraction) were hydroxy esters. Confirmation of hydroxy esters was obtained by acetylation and TLC which gave only one spot corresponding to acetylated ricinoleate.

The sphingosine bases of soybeans also consisted of a mixture of compounds as demonstrated by GLC of the aldehydes obtained by periodate cleavage (29,30) (Fig. 3). The presence of only minor amounts of 15:0, 16:1 α-β unsaturated and 16:0 aldehydes among the products of the reaction (Fig. 3) showed that the soybean bases contained little or no phytosphingosine, sphingosine or dihydrosphingosine, respectively. The major components of the bases of soybean CE were dehydrophytosphingosine (15:1 aldehyde) and two compounds that gave the pair of unidentified aldehydes shown in Figure 3. The chain length of these aldehydes was 16 carbon atoms because after hydrogenation periodate cleavage gave only two major aldehydes, the 15:0 and the 16:0. The 15:0 was obtained from the hydrogenation of dehydrophytosphingosine whose presence in the original mixture was illustrated by the 15:1 aldehyde. The unknown aldehydes also apparently contained α-β unsaturated linkages because they had retention times greater than the 16:1 α-β unsaturated aldehyde derived from sphingosine of beef brain cerebroside as illustrated in Figure 3, Curve B. Because the unknown aldehydes had retention times greater than that given by the 16:1 α-β unsaturated aldehyde, they contained unsaturation in addition to the α-β unsaturated linkage.

It is unlikely, on the basis of the resolution obtained between 15:0 and 15:1 aldehydes, that the column used could separate positional isomers. Thus, the two unknown aldehydes apparently contained two and three double bonds, respectively, in addition to the double bond in the α-β position.

Sulfolipid

This compound was identified by comparison of its TLC properties with that of sulfolipid isolated from spinach leaves according to the procedure described by Allen et al. (6). Material was not available for structural analysis. However, color tests were positive for glycolipid and negative for phosphorous.

Fatty acid composition of ESG, DGDG and SL are shown in Table II. Compounds isolated from the immature bean had higher concentrations of linolenic acid than those from mature beans.

DISCUSSION

The complexity of the lipid of immature soybeans is well demonstrated in the present investigation. Not only does the lipid contain a large number of phospholipids and glycolipids, but a number of as yet unidentified compounds. In addition to the complexity of the lipid in general, structures of individual components also appear to be complex as evidenced by the structural analysis of the CE fraction as well as previous work on triglycerides species composition (10).

DGDG, SL and ESG contain appreciable amounts of linolenic acid especially DGDG but not as much as the galactolipids isolated from highly active photosynthesizing tissues (6,7,31-33). Galactolipids particularly those compounds containing linolenic acid are believed to be associated with photosynthesis either in a functional role (7,32) or because oxygen provided by photosynthesis is required for the conversion of oleic to linolenic acid (34,35). That DGDG, ESG and triglycerides (10) isolated from immature beans contained higher concentrations of linolenic acid than those isolated from mature beans also indicates an association of this fatty acid with photosynthesis inasmuch as during maturation the soybean undergoes a transformation from mainly a photosynthesizing tissue to one of storage. In support of this view is the recent observation by the authors (26) that although 1-^{14}C-acetate was converted readily to neutral and phospholipids none of the label appeared in the glycolipids. Apparently the synthesis of these compounds decreases as photosynthesis decreases and affords an explanation of their low concentration in the mature bean. In this regard the relatively low concentration of linolenic acid reported in ESG by Lepage (2) and Kiribuchi et al. (3) indicates that their preparations originated from mature soybeans.

The differences in the structures of the sphingosine basis and composition of the fatty acids between soybean and beef brain cerebrosides are striking and appear to represent, further, differences that exist generally between the structures of the same lipid classes from plant and animal sources.

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Dietary Induced Alterations in the Fatty Acids of Rat Bone Marrow

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ABSTRACT

Weanling rats were fed fat free diets supplemented with 10% added fatty acids so that dietary effects on bone marrow fatty acids could be determined. The addition or deletion of linoleic acid from the fatty acid supplement resulted in alterations of the fatty acid patterns of bone marrow lipids but to a lesser degree than in erythrocyte lipids. With myristic acid supplementation, increased amounts of stearic acid were found in the lipid fractions, the difference between the bone marrow and erythrocyte lipids being less marked than when linoleic acid was fed. The activities of the bone marrow lipases varied with the dietary treatment. When linoleic acid was fed, higher rates of hydrolysis were observed with saturated fatty acid substrates. The reverse occurred when saturated fatty acids were fed.

INTRODUCTION

Recent investigations have rather clearly defined the effect that dietary lipids exert on the fatty acid composition of erythrocytes and other tissues. The exchange of fatty acids between serum lipids and erythrocyte phospholipids is primarily responsible for these effects in the former case (1,2).

The rapid appearance of dietary induced changes in erythrocyte fatty acid composition generally precludes the study of the contributions of the hematopoietic bone marrow lipids to the erythrocyte lipids. This communication describes the effects of dietary fat alterations on rat bone marrow phospholipid and triglyceride fatty acid compositions. Observations are made on the similarities in the fatty acid patterns of erythrocyte and bone marrow phospholipids. The activities of the bone marrow lipases are briefly examined.

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METHODS

Groups of six weanling male rats (Sprague-Dawley Inc., Madison, Wis., average weight 45 g) were fed a fat deficient diet patterned after Wooley and Sebrell (3) to which additions of the following fats were made: (a) 10% (wt) myristic acid; (b) 10% (wt) linoleic acid; (c) 5% (wt) palmitic acid and 5% (wt) linoleic acid; (d) 5% (wt) myristic acid and 5% (wt) linoleic acid; (e) 5% (wt) myristic acid and 5% (wt) palmitic acid; or (f) 10% (wt) palmitic acid. The fatty acids, purchased from General Biochemicals Inc., contained by analysis, 95%, 95% and 75% myristic, palmitic and linoleic acids, respectively. The diets contained 10 g alpha-tocopherol per 100 lb. and were stored in a nitrogen atmosphere at -20 C prior to use. Fresh diets were prepared at five-day intervals. The rats, housed two per cage, were given the diet ad lib. each evening for 20 days.

The rats were fasted 12 hr and then killed by decapitation. The limbs were removed, stripped of muscle and connective tissue and frozen. The pooled bones from each dietary group were crushed and the lipids were extracted with 20 vol chloroform-methanol (2:1). Following chromatography on thin layer plates, the phospholipids, triglycerides, cholesterol esters and free fatty acids were analyzed for their fatty acid composition. Methyl esters were prepared following the method of Bowers et al. (4) and were separated by GLC using a column packed with 20% diethylene glycol succinate on 60-80 mesh chromosorb W. Accuracy of the gas liquid chromatography detector response was checked by linearity testing with fatty acid standards (NIH B and D). Quantitative results for these standards agreed with the stated composition data showing an error less than 1% for major components.

Blood samples were collected in heparinized tubes and centrifuged for 15 min at 3000 rpm. The plasma and upper layer of leukocytes and platelets were removed and the remaining erythrocytes were washed three times with physiological saline. The erythrocyte lipids were extracted with 20 vol chloroform-methanol 2:1. The erythrocyte phospholipids from each animal were isolated by thin

TABLE I

Major Fatty Acids of Bone Marrow Phospholipids From Rats Fed Different Fats

Dietary fat	Fatty acids[a]						
	Short chain[b]	16:0	16:1	18:0	18:1	18:2	20:4
14:0	8.0±0.5	27.9±1.2	9.5±0.2	13.0±0.7	30.4±1.9	5.6±0.4	4.2±0.6
18:2	1.6±0.1	31.5±0.6	8.2±0.5	12.8±1.1	16.9±0.7	19.4±1.1	9.2±0.8
18:2-16:0	2.7±0.8	31.9±2.3	3.0±0.2	18.7±2.0	15.1±1.8	17.1±1.2	12.7±1.4
18:2-14:0	4.6±0.9	30.1±1.7	1.4±1.2	20.8±0.9	15.8±0.8	13.9±1.0	13.2±1.4
14:0-16:0	4.3±0.2	32.5±1.2	7.6±0.4	14.5±0.8	27.3±0.8	9.5±0.6	4.4±0.8
16:0	3.1±0.2	32.5±1.2	11.0±0.8	12.0±0.5	29.9±1.7	7.1±0.4	4.8±0.3

[a]Percentage.
[b]12:0, 14:0, 14:1.

layer chromatography, methyl esters prepared and analyzed by gas chromatography.

In order to magnify the degree of variation in the stearic acid, oleic acid and linoleic acid content of lipid fractions, their relative contents are related to each other and to palmitic acid. Farquhar and Ahrens (2) suggest that these ratios are the best means for evaluating dietary effects on lipid composition. The fatty acids were identified by calculations of equivalent chain lengths (5). Precautions used to avoid autoxidation included the use of nitrogen atmospheres when possible and storage at -20 C. All reagent grade solvents were redistilled immediately prior to use.

Lipolytic enzymes were extracted from bones of rats fed the diets for 33 days. The isolation procedure described by Magee et al. (6) was followed. No attempts were made to further purify the enzymes fractions or to characterize the individual enzymes. The crude extracts were tested for activity and specificity by following the increase in free fatty acids during incubation with egg lecithin, lysolecithin (snake venom-treated egg lecithin), tripalmitin or triolein. All substrates were purchased from General Biochemicals Inc.

The substrate mixture contained 1 μM of substrate, 2 mg sodium deoxycholate and 0.5 mg fatty acid-poor albumin in 0.9 ml buffer (glycylglycine .05M pH 7.3). This volume was pipetted into teflon capped test tubes, flushed with nitrogen and immersed in a water bath at 38 C. After temperature equilibration, 0.1 ml of the enzyme fraction containing approximately 2 mg protein was added. Triplicate assays were done. Following 3 hr incubation, the reaction was stopped by the addition of 2.5 ml isopropanol-heptane-N sulfuric acid (40:10:1) followed by 1.5 ml heptane and 1 ml distilled water (7). Blanks containing standards of palmitic acid were similarly treated. One ml of the heptane layer was pipetted into a cuvette, 1.5 ml of barbitol-phenol red reagent (8) was added and the tubes were read at 560 mμ. Spleen lipase activity was assayed in a similar manner.

RESULTS AND DISCUSSION

The fatty acid compositions of the bone marrow phospholipids and triglycerides (Tables I and II) are directly influenced by dietary fatty acids. Linoleic and arachidonic acids were

TABLE II

Major Fatty Acids of Bone Marrow Triglycerides From Rats Fed Different Fats

Dietary fat	Fatty acids[a]					
	Short chain[b]	16:0	16:1	18:0	18:1	18:2
14:0	23.2±2.8	22.0±1.2	15.9±0.4	2.2±0.2	28.7±2.0	7.7±0.3
18:2	7.1±0.9	23.8±0.8	12.9±0.5	4.5±1.0	26.9±1.3	25.1±1.8
18:2-16:0	5.6±0.9	29.2±1.0	15.2±0.9	3.3±0.3	29.7±1.1	16.6±1.9
18:2-14:0	21.0±0.7	24.1±1.4	11.2±0.6	9.6±0.7	19.9±1.5	13.4±0.9
14:0-16:0	32.1±1.7	20.7±1.6	13.9±1.0	4.7±0.6	25.3±1.2	4.2±0.6
16:0	18.9±1.4	23.4±0.7	19.1±1.5	2.4±0.6	30.7±1.7	5.8±0.5

[a]Percentage.
[b]12:0, 14:0, 14:1.

TABLE III

Selected Ratios of Fatty Acids in Erythrocytes and Bone Marrow Lipids

Dietary fat	18:2-18:1			18:2-16:0			18:0-16:0		
	Erythrocyte	Bone marrow		Erythrocyte	Bone marrow		Erythrocyte	Bone marrow	
		PL[a]	TG[a]		PL	TG		PL	TG
14:0	0.05	0.18	0.27	0.03	0.20	0.35	0.67	0.47	0.10
18:2	0.51	1.15	0.93	0.17	0.62	1.05	0.22	0.41	0.19
18:2-16:0	0.57	1.13	0.54	0.14	0.54	0.57	0.12	0.59	0.11
18:2-14:0	0.58	0.88	0.67	0.19	0.46	0.56	0.31	0.69	0.40
14:0-16:0	0.04	0.35	0.17	0.01	0.29	0.20	0.33	0.45	0.23
16:0	0.06	0.24	0.19	0.03	0.22	0.25	0.28	0.37	0.10

[a]PL, Phospholipids; TG, Triglycerides.

present in much greater quantities in the phospholipids when the diet contained linoleic acid. The fatty acids of the cholesterol esters showed a similar pattern. In the absence of dietary linoleic acid, the phospholipids contained a much greater proportion of oleic acid. The linoleic acid content of the triglycerides was under a direct dietary influence. The effects of dietary myristic acid were noted primarily in the triglyceride fractions of the various groups (Table II). Trace amounts of arachidonic acid were found in the triglyceride fractions. The free fatty acids of the bone marrow also directly reflected the dietary fatty acid.

The composition of the dietary fat was directly reflected in the erythrocyte fatty acid patterns; the changes were similar to those observed in the bone marrow phospholipids, but of greater magnitude. The degree of change induced by the addition to or deletion from the diet of linoleic acid is best shown by the relative ratios of the various acids in erythrocyte and bone marrow phospholipids (Table III). The change in the linoleic acid to oleic acid ratio was 11-fold in the erythrocytes and 4-fold in the bone marrow. Linoleic acid to palmitic acid ratios changed 7-fold in the erythrocyte and 2-fold in the bone marrow. The exchange of fatty acids between the serum and erythrocytes is the major factor to be considered in relating dietary lipids to erythrocytes lipid alterations as has been shown in experiments by Mulder, de Gier and van Deenen (1) and Farquhar and Ahrens (2), among others.

The incorporation of stearic acid into the phospholipids of the erythrocyte membrane would most likely occur at the 1 position (9). Dietary myristic acid caused an increase in the relative amount of stearic acid in the erythrocyte membrane. The ratios of stearic acid to palmitic acid in the bone marrow phospholipids were 0.52 and 0.47 when the diets contained myristic acid and palmitic acid, respectively. These ratios were 0.44 and 0.24, respectively, in the erythrocytes. When examined in this manner, the changes in the ratios involving fatty acids primarily esterified at the 2 position of the phospholipids due to dietary alterations are 3-fold greater in the erythrocyte than in the bone marrow. In the ratios involving acids located at the 1 position, the ratio changes to a much lesser degree. Mulder and van Deenen (9) reported that in vitro the rate of stearic acid incorporation into rabbit erythrocytes was 0.20

TABLE IV

Lipase Activities[a] in Various Reaction Systems

Substrate	Dietary fat					
	14:0	18:2	18:2-16:0	18:2-14:0	14:0-16:0	16:0
Egg lecithin	0.74±.03	0.53±.07	0.57±.03	0.64±.04	b	0.62±.02
Lysolecithin	0.36±.03	0.50±.03	0.46±.04	0.60±.03	b	0.49±.03
Tripalmitin	0.41±.02	0.67±.03	0.56±.04	0.62±.03	b	0.35±.03
Triolein	0.31±.02	0.22±.01	0.14±.07	0.24±.02	b	0.33±.03

[a]mμEq fatty acid hydrolyzed per min per mg protein, average value of three assays, two observations per assay corrected with reaction blanks.

[b]No activity.

TABLE V

Final Weight of Rats Fed the Experimental Diets

Dietary fat	Final weight	
	1[a]	2[b]
14:0	103±26[c]	180±19
18:2	117±11	169±3
14:0-18:2	115±18	188±3
16:0-18:2	110±7	203±9
14:0-16:0	113±13	185±3
16:0	108±25	142±23

[a]Weight at 50 days of age, diet 20 days, two rats per cage.

[b]Weight at 62 days, diet 33 days, one rat per cage.

[c]Standard deviation.

the rate of linoleic acid incorporation. Their data indicated that factors other than the incorporation of serum fatty acids into circulating erythrocytes are responsible for the observed diet-induced alterations in erythrocyte fatty acids. Similarily Oliveira and Vaughan (10) reported that in erythrocyte ghosts, linoleic acid was incorporated into phospholipids at a rate 10 times greater than palmitic acid.

The bone marrow triglyceride ratios of linoleic acid to oleic acid varied 3-fold, linoleic acid to palmitic acid 3-fold and stearic acid to palmitic acid 2-fold depending on the presence or absence of linoleic acid in the diet. Supplementation with myristic acid resulted in increased stearic acid to palmitic acid ratios in all lipid fractions.

The rats used in the second experiment were individually housed and fed the respective diets for a 33-day period. As the dietary period exceeded the half-life of rat erythrocytes, we expected to find maximum changes in the bone marrow lipases. Preliminary investigations indicated that the reaction rates were constant during the first three hours. Due to the low levels of activities, we allowed the reactions to proceed for this time period in order to reduce analytical errors. Since the object of the investigation was to determine whether the dietary treatment influenced the activities of individual lipases the marrow enzymes were freed of native lipids by a three-step solvent extraction. According to Magee, et al. (6), this treatment reduced the total activity of pancreatic lipases by 50%.

The specific activities of bone marrow lipases are presented in Table IV. Although the enzymes were not purified and the data are limited, the results suggest that the diet or the tissue lipids influenced the activities of the enzymes. For the saturated fatty acid substrates, lysolecithin and tripalmitin, high specific activities were observed in the marrow enzymes of rats fed the diets containing linoleic acid. When the unsaturated fatty acid substrates, lecithin and triolein, were used, high specific activities in general were observed in the marrow enzymes of rats fed saturated fatty acids. The difference between activities of the marrow enzymes of rats fed the myristic acid and the linoleic acid supplements were significant ($P < .05$) for all substrates. Using other tissues as sources of lipolytic enzymes, Pawar and Tidwell (11) reported that higher levels of lipase activity were found in tissues of rats fed unsaturated fats. We suggest that due to its erythropoietic nature, the marrow tissues respond quite differently than tissues with storage functions. In the present study, the activities of the phospholipases in the spleen , a major site of erythropoietic activity, were higher and did not follow the patterns established for the bone marrow enzymes.

The erythrocytes taken from rats fed the myristic acid supplement were extremely fragile and extensive hemolysis occurred during the isolation procedure. The addition of palmitic acid to the myristic acid supplement reduced the extent of hemolysis. No hemolysis was observed when linoleic acid was in the lipid supplement. This dietary effect on erythrocyte fragility has previously been reported by Walker and Kummerow (12).

The final weights of the rats are shown in Table IV. Increased variability in weight gain was observed in rats fed the saturated fatty acids. The relationship between food consumption and conversion on the fatty acid patterns of the bone marrow lipids was not examined.

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Incubation of Human Fecal Homogenates With 4-^{14}C-Cholesterol[1]

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ABSTRACT

Fresh fecal homogenates from nine subjects consuming solid diets have been incubated for seven days at 37 C with 4-^{14}C-cholesterol. One series of incubations was conducted under nitrogen, another under air. The extent of bacterial modification of cholesterol to known fecal metabolites varied considerably among the subjects, as expected, but when present such bacteria were shown to be highly active in the conditions used. Production of $^{14}CO_2$ was essentially zero in all incubations. Recovery of added ^{14}C from the incubated homogenates following extraction with chloroform-methanol (2:1 v/v) and evaporation to dryness was quantitative in all cases. About 4% of the labeled cholesterol added appeared to be present in acidic components following incubation. It is concluded that in the incubation system used vigorous bacterial conversion of 4-^{14}C-cholesterol to metabolites known to be produced in the human intestine could occur readily, in either aerobic or anaerobic conditions. However, CO_2 or other small, relatively volatile fragments labeled with ^{14}C could not be detected.

INTRODUCTION

In a series of papers Grundy et al. (1-3) have reported losses of cholesterol occurring during passage through the human intestinal tract. These losses, they concluded, were due not to errors in stool collection or to technical errors, but to intestinal bacterial degradation of neutral 3β-OH,Δ5-sterols. The losses reported were as great as 60% of fed sterols, but in some subjects no losses were found.

An important aspect of cholesterol metabolism is that of the excretion rate of cholesterol via the stool, including also the excretion of bacterially produced coprostanol and steroid ketones, of a complex mixture of bile acids and probably also of various unidentified substances. All of these fecal products are derived ultimately from body pools of cholesterol, or from ingested cholesterol; they contribute to the overall loss of cholesterol from the body and must be taken into account in measuring the mean daily excretion rate of cholesterol and its metabolites. It is clear that methods designed to reliably determine total cholesterol excretion rate must be able to detect and measure all of these products.

The authors of the thin layer-gas chromatographic method for measurement of fecal steroid excretion rate (4,5) conclude from their experience that all acidic excretory products are detected and accurately measured by their procedure (2), although the in vitro microbiological degradation of bile acids to small fragments has been recently described (6). However, they find that neutral steroids cannot be reliably determined by direct measurements on stool extracts. They suggest that products are formed from cholesterol (or sitosterol) with properties so different from those of the neutral sterols themselves that they are either not detectable in the stool by their procedure, or are reabsorbed from the gut to disappear into large body pools where they are difficult to identify even when radioactive. Such a situation might result if intestinal bacteria present in certain subjects are able to break the rings of the steroid nucleus, producing small fragments such as CO_2, methane or methanol. Such volatile substances would not be retained in the fecal extracts during processing prior to gas chromatographic estimation, and would thus be lost.

The question of the possible volatility of fecal products derived from cholesterol is of considerable importance in relation to the isotope balance method for measurement of fecal steroid excretion rate in man (7). In this method, which has recently been used by a number of investigators (8,9), body cholesterol pools are labeled by means of a preliminary intravenous injection of purified 4-^{14}C-cholesterol. After a period of equilibration (10-20 days) stools are collected in pools and their ^{14}C content determined. Measurement of ^{14}C-specific activity of plasma cholesterol then permits an estimate of the total steroid content of

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a stool pool, since it has been demonstrated that fecal steroids and plasma cholesterol have approximately the same specific activity at the same point in time. In this procedure the chemical identity of the fecal products need not be known (although they are usually divided into neutral and acidic components). The major requirement with the isotope balance method is that all radioactivity (at the 4 position on the steroid nucleus) entering the gut via the bile or the intestinal mucosa should be retained on molecules that are not so volatile as to be significantly lost during processing (e.g., at 100 C).

Since it has been implied by Grundy et al. (3) that the isotope balance method cannot be generally applied unless correction for the alleged losses of neutral steroids is made (e.g., by constant feeding of sitosterol) it was of interest to examine the fate of 4-^{14}C-cholesterol added to homogenates of human feces which were incubated under conditions such that all well-known bacterial modifications of cholesterol structure could proceed vigorously. Thus, although the in vitro conditions used here were clearly not necessarily the same as those existing in the intestine, the environment provided was suitable for activity of those bacteria producing the major known intestinal transformations undergone by cholesterol. Failure to find evidence for production of small, volatile fragments derived from labeled cholesterol under these conditions is now described for feces of subjects consuming solid diets.

MATERIALS AND METHODS

Subjects

Fresh stool samples were obtained from nine subjects, five normal and not hospitalized and four hospitalized with maladies apparently not related to the gastrointestinal tract. Details of the subjects are presented in Table I. All subjects consumed typical solid diets except 1 and 2 who did not eat green vegatables.

Incubations

A fresh stool sample from each subject was homogenized with sufficient normal saline to make a fluid mixture. A 4 ml aliquot was placed in each of two 250 ml Erlenmeyer flasks and 10 μl of an ethanol solution containing an accurately known amount (about 0.14 μc) of 4-^{14}C-cholesterol was added, with thorough mixing. The resultant very dilute solution of ethanol in water (about 0.25% v/v) was considered unlikely to exert any significant influence on the activity of microorganisms and enzyme systems present in the homogenate.

TABLE I

Subjects Used for Stool Collections

Subject	Age, Year	Sex	Wt, kg	Diagnosis
1	20	M	68	Normal
2	62	F	50	Atrial fibrillation
3	52	M	84	Normal
4	69	F	50	Asthma
5	49	F	66	Normal
6	53	M	71	Normal
7	48	F	56	Normal
8	47	F	81	Fractured shoulder
9	41	F	52	Diabetes

The radioisotopic purity of the cholesterol used was at least 99.7%, as judged by thin layer chromatography (TLC) on Silica Gel G (E. Merck AG, Darmstadt, Germany) and Silica Gel G impregnated with silver nitrate. No acidic impurities could be detected by passage of the preparation through a column of ion exchange resin (Dowex AG1-X2; Bio-Rad Laboratories, Richmond, Calif.) known to completely remove acidic materials from wet diethyl ether solution.

The flasks were closed with serum caps. One set of flasks was immediately flushed with pure nitrogen (anaerobic series); the other (aerobic series) was used with an atmosphere of air in the flask. All flasks were incubated in the dark at 37 C for seven days, with thorough agitation twice daily.

Distribution of ^{14}C After Incubation

At the end of the incubation period the atmosphere within each flask was slowly displaced through Hyamine using a nitrogen stream and an aliquot of the Hyamine was counted for ^{14}C, followed by quench correction by the internal standardization method.

The fecal homogenate was then extracted with chloroform-methanol (2:1 v/v), the residue being washed several times with the boiling solvent. The combined extracts were made up to 200 ml with the same solvent and aliquots were taken for the following determinations.

Total ^{14}C. Duplicate 0.5 ml aliquots were placed in 20 ml counting vials and evaporated to dryness under nitrogen. This step involved heating the vials at 100 C in a water bath for about 10 min, and in this respect it was identical to the procedure routinely used by us (8) during determination of total ^{14}C in fecal extracts, as part of the isotope balance method for determination of fecal steroids. The residues were dissolved in 2 ml methanol and 10 ml of toluene-based scintillation fluid added. The vials were counted to a standard deviation of

TABLE II

Recovery of ^{14}C in Total Extracts and in Acid-Free Extracts of Stools Incubated With 4-^{14}C-Cholesterol

Incubation	Total ^{14}C, %	Nonacidic ^{14}C, %
Anaerobic series		
1	100.1	98.2
2	98.6	95.7
3	102.4	95.3
4	100.1	97.0
5	101.3	99.0
6	101.2	95.5
7	102.1	97.2
8	102.0	99.1
9	101.8	95.2
Mean ± SD	101.1 ± 1.24	96.9 ± 1.58
Aerobic series		
1	102.8	98.0
2	102.6	96.6
3	101.7	94.8
4	100.2	97.5
5	100.6	96.7
6	101.8	94.0
7	100.5	95.8
8	101.7	99.4
9	100.4	95.2
Mean ± SD	101.3 ± 0.89	96.4 ± 1.66

less than 1%, followed by quench correction.

Nonacidic ^{14}C. Duplicate 1 ml aliquots were evaporated to dryness under nitrogen and the residue transferred in a few milliliters of wet diethyl ether to a column of the ion exchange resin Ag1-X2 in the hydroxyl form to remove all acidic products. The eluate was evaporated to dryness in a counting vial, the residue dissolved in 2 ml methanol and 10 ml scintillation fluid added. The vials were counted, with quench correction.

Distribution of Recovered ^{14}C on TLC. Five milliliters of the extract was evaporated to dryness and applied as a streak to a thin layer plate coated with Silica Gel G, using chloroform-methanol (2:1 v/v). Cholesterol and coprostanol standards were applied to the sides of the plate. The plate was developed in diethyl ether-petroleum ether-acetic acid (60:40:3 v/v/v), and the separated bands visualized by spraying lightly with 0.01% Rhodamine 6G in ethanol, followed by viewing under UV light. After delineation of bands the silica gel was quantitatively scraped from the plate in its entirety, in four regions: (a) the cholesterol region; (b) the adjacent coprostanol region; (c) the area from coprostanol to the solvent front and (d) the area below cholesterol down to (and including) the origin. The silica gel from the four regions was transferred to elution columns and eluted with diethyl ether or, in the case of region (d), with diethyl ether-methanol (10:1 v/v). The eluates were evaporated to dryness, transferred to counting vials and ^{14}C determined, with quench correction. The ^{14}C found in each region was expressed as a percentage of the total ^{14}C recovered from the plate for each sample.

RESULTS

Production of $^{14}CO_2$

In most incubations (both the aerobic and the anerobic series) $^{14}CO_2$ production was not detectable. The highest count in the Hyamine solutions corresponded to less than 0.05% of the 4-^{14}C-cholesterol added.

Recovery of Total ^{14}C

The recovery from stool of total ^{14}C derived from added 4-^{14}C-cholesterol by the extraction method described is shown in Table II. Recovery of radioactivity added was essentially complete for all incubations in both series.

Recovery of Nonacidic ^{14}C

Recovery of nonacidic ^{14}C averaged more than 96% of added radioactivity for both series; there was no significant difference between recoveries in the aerobic and anaerobic series.

Chemical Nature of Recovered ^{14}C

The distribution of recovered ^{14}C among the four chromatographic regions studied is shown in Table III for both the aerobic and the anaerobic series. Radioactivity was considered to be present as cholesterol and as coprostanol, respectively, since the ^{14}C in these fractions showed the correct chromatographic mobility and was precipitated by digitonin.

DISCUSSION

The nine stool samples examined exhibited a wide range of activities with respect to the conversion of cholesterol to compounds with properties corresponding to those of known bacterial metabolites (Table III). The pattern of distribution of ^{14}C following incubation was fairly similar for aerobic and anaerobic incubations of the same sample. Coprostanol was often a major product. The chromatographic area above coprostanol is known to contain any steroid ketones, which are sometimes major fecal metabolic products of cholesterol (10). The area below cholesterol on the TLC would contain many of the known oxidation products of cholesterol (11); although such products were not present in the purified 4-^{14}C-choles-

TABLE III

Chromatographic Distribution of ^{14}C Recovered From Incubation Mixtures

Incubation	TLC region, % total ^{14}C recovered from plate			
	Cholesterol	Coprostanol	Above coprostanol	Below cholesterol
Anaerobic series				
1	95.4	0.2	0.4	4.0
2	90.3	6.1	2.6	1.0
3	40.3	35.2	21.7	2.8
4	9.8	77.0	12.1	1.1
5	58.1	22.5	18.3	1.1
6	48.7	35.7	11.7	4.0
7	38.7	50.0	8.0	3.3
8	97.7	0.4	0.2	1.7
9	6.9	25.8	62.9	4.4
Aerobic series				
1	97.8	0.2	0.1	1.9
2	97.7	0.6	0.6	1.1
3	61.8	17.2	17.1	3.8
4	23.6	57.0	15.5	3.9
5	51.5	26.7	20.2	1.5
6	58.9	24.1	12.2	4.7
7	46.8	45.2	5.0	2.9
8	98.7	0.3	0.3	0.7
9	3.5	58.7	35.1	2.7

terol preparation added to the homogenates, they may have been formed to the extent of a few per cent in all homogenates during the seven-day incubations. However, there was no significant difference between the aerobic and anaerobic series in this respect.

While it is not supposed that the conditions of these incubations were identical to those encountered by cholesterol passing through the length of the gastrointestinal tract of a particular subject, nevertheless it appeared that an active bacterial conversion of cholesterol to its presently known major fecal metabolic products was occurring in vitro. In this situation any bacterial degradation of neutral sterols to small, volatile fragments might perhaps be expected to occur concomitantly.

The virtual absence of any production of CO_2 from the 4-carbon of the cholesterol nucleus is consistent with the results of much previous work performed in vivo in man and animals (12,13). A brief reference (2) to unpublished studies by Salen, Grundy and Ahrens also implied that the 4-carbon of cholesterol was not converted to CO_2 during in vitro incubation of fecal homogenates.

Complete recovery of added ^{14}C by extraction with chloroform-methanol (2:1 v/v) was experienced for all incubations (aerobic and anaerobic), as shown in Table II. Since the procedure used involved evaporating the extract to dryness under nitrogen, during which operation the material was exposed to a temperature of 100 C for about 10 min, it seems most unlikely that any significant amounts of relatively volatile, small molecular weight radioactive compounds (e.g., short-chain fatty acids or aldehydes, methanol, methane) were formed from 4-^{14}C-cholesterol during the incubation conditions used. A similar process of extraction, evaporation of solvent and determination of ^{14}C is employed during estimation of neutral steroid excretion rate by the isotope balance method (3,7,8,14). The present results, which refer only to feces of subjects on solid diets, thus did not provide any in vitro evidence that the isotope balance method is in error due to the production of volatile fragments derived from cholesterol.

Quantitative removal of any acidic materials from the extracts by passage through an ion exchange column resulted in an average loss of 4-5% of ^{14}C in both series (Table II). Since the 4-^{14}C-cholesterol added initially contained insignificant amounts of acidic impurities, and since handling losses by the procedure used have been found by us to be less than 1%, it appeared that conversion of cholesterol to acidic products occurred to a minor degree. The effect of this conversion on estimation of excretion rate by the isotope balance procedure might be that a minor proportion of the steroid put into the gut as neutral sterol would be determined as part of the fecal bile acid frac-

tion, but no loss of extracted total steroid would be experienced. When processed by the thin layer-gas chromatographic method (4) which involves mild saponification of the stool followed by extraction with petroleum ether, any acidic products derived from cholesterol would presumably remain in the alkaline aqueous residue and hence be excluded from the subsequent gas chromatographic quantitation step. In fact, losses of 15-40% of incubated 4-^{14}C-cholesterol have been found in this residue in stools from certain patients, according to the very brief report of unpublished data by Salen et al. (included in Reference 2).

The precise nature of some of the less-familiar bacterial metabolites of cholesterol clearly remains to be established. Grundy et al. (2,3) are of the opinion that significant bacterial conversion of cholesterol (or plant sterols) to small fragments occurs by rupture of the steroid nucleus. However, Connor et al. (15), using plant sterols as a marker for recovery of cholesterol in studies on six healthy men, as recommended by Grundy et al. (2), concluded that until the end products of neutral sterol losses can be identified, the significance of the plant sterol losses remains uncertain.

Our limited studies using stools from subjects eating solid diets indicate that incubation in vitro, while allowing conversion of cholesterol to well-known metabolites, did not result in formation of substances of very low molecular weight derived from the 4-carbon. In these conditions conversion of cholesterol to unfamiliar and unexpected compounds apparently occurred to only a relatively small extent. These results do not preclude the possibility that stools from formula-fed subjects (especially those found to be degraders by plant sterol recovery studies) may be able to make such conversions during incubation in vitro.

The formation of cholesterol sulfate may account for some small loss of cholesterol, as discussed by Grundy et al. (2). In addition the bacterial production of polar steroid derivatives (16) by opening of one or more rings of the cyclopentanoperhydrophenanthrene nucleus may contribute to the loss, yielding compounds that are not easily volatile but which may have properties rendering them undetectable by the gas chromatographic method.

Note Added in Proof:

In a recent publication Denbesten et al. (17) have reported on six normal men, one of whom showed incomplete recovery of ingested plant sterols in the stool. When 4-^{14}C-cholesterol was incubated with bacterial cultures of stools from this subject during a diet of mixed general foods, complete recovery of ^{14}C was achieved, in agreement with our findings. However, when this subject was fed a formula diet low in lactose and cellulose, and the fecal incubation was repeated, a loss of up to 28% of added ^{14}C was experienced, notwithstanding that the missing radioactivity was sought by counting the entire liquid phase and also the CO_2 in the gas above it.

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MRA Microreactions for Lipid Analysis[1]

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ABSTRACT

Many types of reactions have been performed on a microscale with the microreactor apparatus (MRA) system, which incorporates a modified soldering gun. Specific procedures developed for some typical reactions include bromination of olive methyl esters, silylation of castor methyl esters, homogeneous reduction of oleic acid, heterogeneous reduction of soybean methyl esters, several methods of methyl ester preparation from soybean fatty acids and saponification of soybean triglycerides followed by esterification. The reaction chamber of the MRA has been redesigned to increase its versatility and to reduce explosion hazards when diazomethane is used. Advantages of the MRA over other systems are less handling of sample, minimum hazards due to small sample size and direct injection of products into analytical instruments, such as a gas chromatograph or mass spectrometer.

INTRODUCTION

The advent of gas liquid chromatography (GLC) made microanalysis of lipids possible but has required the development of microchemical techniques and microanalytical procedures. Earlier, reactions for GLC analyses were performed externally and usually on a macroscale, whereas many reactions today are performed on a microscale in an apparatus that can be made an integral part of GLC (1). Some examples of microreaction apparatus (microreactors) can be found in the literature (1-4). Most, if not all, microreactors were designed for a specific study, such as catalytic performance and hydrogenation (5,6).

The microreactor apparatus (MRA) system we developed (7), in contrast to equipment designed for specific uses, has many applications. Previous publications have already shown how the MRA may be used for ozonization-pyrolysis (7,8) and transesterification (7,9). Now, with the aid of a redesigned reaction chamber, several more microreactions for lipid analysis have been successfully completed and indicate the MRA's wider use for performing microscale chemical analyses in conjunction with a variety of analytical instruments.

EXPERIMENTAL

Apparatus Design

A description of the microreaction apparatus has already been published (7,9). Figure 1 illustrates diagrammatically the latest design of the microreactor showing its assembly and attachment to the soldering gun. The original assembly (8) has certain disadvantages, such as reactivity of metals of construction, difficulties in removal of chamber from gun and hazards when using diazomethane. A new version of the reaction assembly is shown at A. Connected to the end of the soldering gun is a small brass cylinder (B- -dotted area, approximately 4.3 cm long x 0.65 cm I.D.) into which is inserted a Luer-type glass joint (a) connected with a Teflon ferrule to a tee tubing fitting, shown at C. The reaction chamber, per se, is the Luer-type glass joint.

Conditions

Reaction chamber conditions were controlled automatically by the MRA system. Temperatures and helium flow during injection, if not listed under procedures, were 250 C and a minimum of 50 cc/min, respectively. An Aerograph Model 350 (Wilkens Instrument and Research, Inc.) GLC was used for all experiments with filament current at 200 ma, attenuation on X2, and helium flow at 60 cc/min unless otherwise stated under procedures. Detector temperature was held at 220 C and column oven was temperature programmed from 90 to 180 C. The column was 9 ft x 1/4 in. and packed with 25% stabilized DEGS on 60/80 mesh Chromosorb W. For the hydrogenation and silylation experiments, 10 ft x 1/4 in. and 4 ft x 1/4 in. columns were used, respectively, both packed with 3% EGSS-X on 100/120 mesh Chromosorb W (Applied Science Lab., State College, Pa.).

The reaction chamber was loosely packed with glass wool for all experiments except hydrogenations, for which it was filled with catalyst and terminal glass wool plugs.

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[2]No. Utiliz. Res. Dev. Div., ARS, USDA.

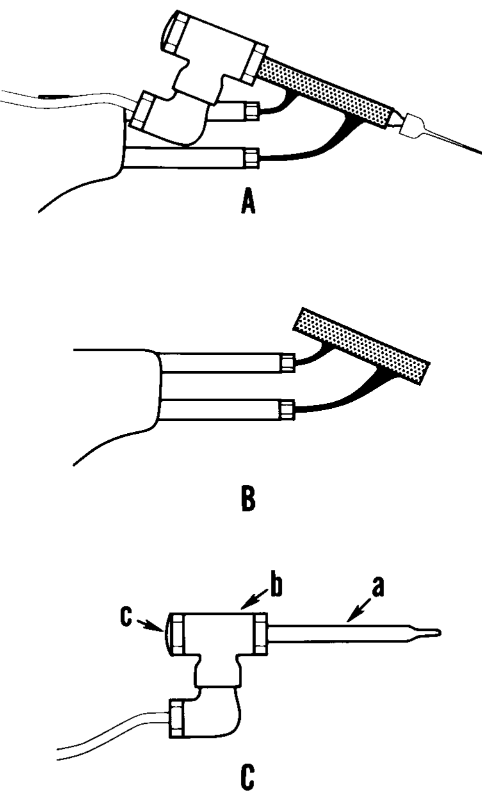

FIG. 1. Reaction chamber of microreactor apparatus (MRA). Only front portion of soldering gun diagrammed. (A) Complete reaction chamber formed by assembling B and C and attaching a needle. (B) Dotted area is brass tubing portion of new reaction chamber. (C) Luer-type glass joint (a) and tubing tee connector (b) with septum (c).

Oils were commercial grade. Methyl esters were made on a macroscale by transesterifying with sodium methoxide, and fatty acids were obtained by saponifying the refined oil and acidifying.

PROCEDURES AND RESULTS

Esterifications

Three methods were used to prepare methyl esters. The first method with BF_3-CH_3OH as the esterifying reagent consisted of the following steps: (a) Pull 2.5 μl of soybean fatty acids (SFA) and 6 μl of 10% BF_3-CH_3OH solution, in that order, into a 10 μl syringe. (b) Inject mixture into glass reaction chamber. (c) React for 1 min at 60 C with no gas flowing through chamber. (d) Hold chamber at 100 C for 3 min with 30-40 cc/min helium flow to evaporate any volatiles, including water. (e) Inject into GLC for 3 min at a chamber temperature of 250 C. The chromatographic results are shown in Figure 2. A typical soybean methyl ester chromatogram is obtained with the exception of the initial peaks, which are due to injection irregularities as noted in the figure caption. Percentage composition of the esters was calculated from areas of peaks and is listed in Table I for comparison with other esterification experiments.

Steps in the second method, which used 2,2-dimethoxypropane (DMP) as the reagent, are as follows: (a) 2 μl 9:1 methanol to concentrated HCl, 2 μl SFA and 5 μl DMP are pulled into a 10 μl syringe, consecutively. (b-d) Same as previous procedure. (e) Inject into

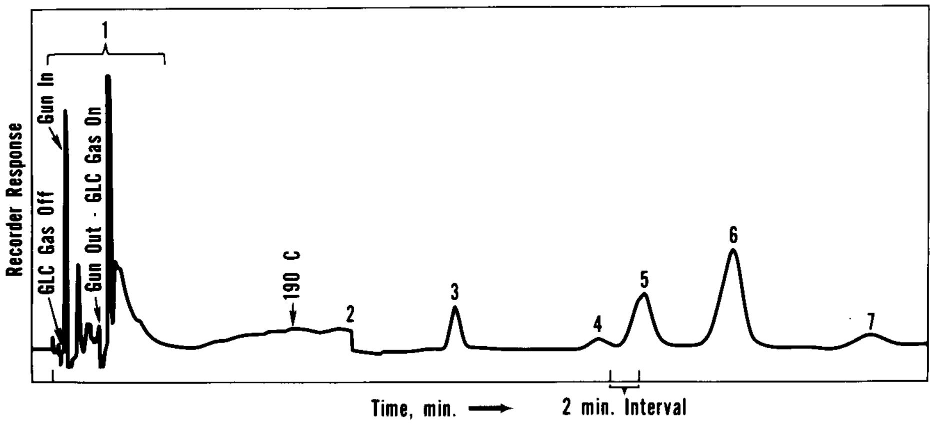

FIG. 2. Chromatographic results of BF_3CH_3OH esterification of soybean fatty acids in MRA. Gas liquid chromatographic (GLC) conditions: 25% DEGS on 60/80 mesh Chromosorb W, 9 ft x 1/4 in. column, thermoconductivity detector, 60 cc/min helium flow rate, temperature programmed from 90 to 190 C. 1, Peaks due to injection technique caused by turning gas flow off and on and changing rate of flow. 2, Fine control adjustment. 3, Palmitate. 4, Stearate. 5, Oleate. 6, Linoleate. 7, Linolenate.

TABLE I

Esterification of Soybean Fatty Acids

Method	Methyl ester from peak area, (%)[a]				
	Palmitate	Stearate	Oleate	Linoleate	Linolenate
BF_3-CH_3OH	10.9	3.6	25.3	52.8	7.4
CH_2N_2	10.2	3.0	26.6	53.1	7.1
$(CH_3)_2C(OCH_3)_2$	10.4	3.1	25.7	53.4	7.4
BF_3-CH_3OH[b]	10.6	4.4	25.1	52.3	7.6

[a]Per cent variations due to experimental error. Calculations by triangulation with slight drifting baseline.

[b]Metcalfe and Schmitz method (11).

GLC at a chamber temperature of 300 C for 1-1/2 min. All chromatographic peaks appear identical to Figure 2, and the area percentage composition is given in Table I.

In the third method of ester preparation diazomethane was used as the esterifying agent. It was prepared in a manner similar to that described in the literature (10). Nitrosomethylurea (0.25 g or less) was added to a mixture of 1 cc of 50% aq KOH and 4 cc of diethyl ether in a test tube immersed in ice. The test tube had a rubber stopper containing an inlet tube for passing nitrogen (a carrier gas for the diazomethane) through the system and an outlet tube for connecting either to the reaction chamber or to a collecting tube containing ether. All connections were made with Tygon tubing, and generation of the diazomethane was carried out in a hood for safety purposes even though very small quantities were produced. The procedure for esterifying was as follows: (a) Inject 1 to 2 μl of SFA into MRA reaction chamber, detached from gun. (b) Connect reaction chamber to line from CH_2N_2 generator. (c) Pass CH_2N_2 through chamber for 3 min. (d) Evaporate excess CH_2N_2 by purging chamber with N_2. (e) Disconnect from tubing and assemble as in Figure 1A. (f) Same as step e of second method.

The chromatographic recording was similar to Figure 2 and the composition is given in Table I. Included in Table I are results from a

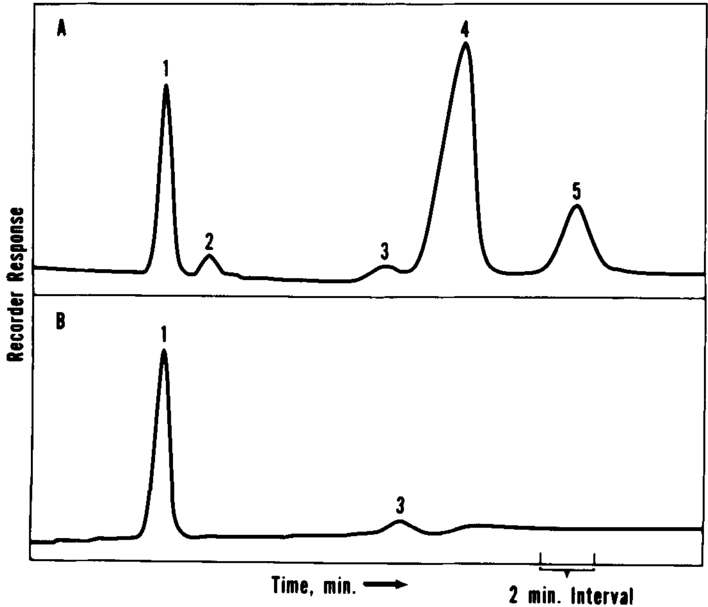

FIG. 3. Chromatographic recordings of methyl esters of olive oil before (A) and after (B) brominating in MRA. Note absence of unsaturated peaks after brominating. GLC conditions: Same as Figure 2. Portion of recording shown is after temperature had reached 190 C. Major peaks are (1) palmitate, (2) palmitoleate, (3) stearate, (4) oleate and (5) linoleate.

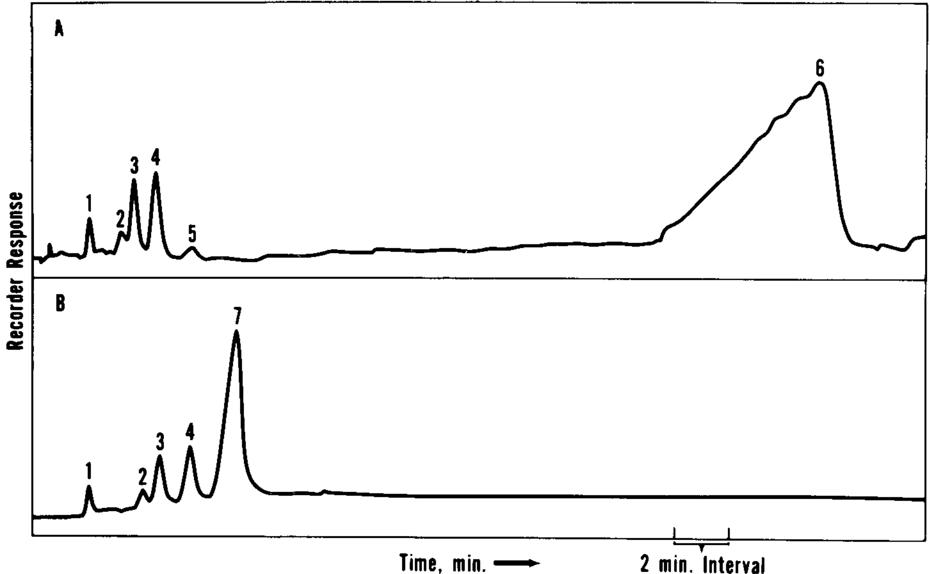

FIG. 4. Chromatographic recordings of methyl esters of castor oil before (A) and after (B) silylating in MRA with bis(trimethylsilyl)acetamide (BSA). GLC conditions: 3% EGSS-X on 100/120 mesh Chromosorb W, 4 ft x 1/4 in. column, thermoconductivity detector, 50 cc/min helium flow rate, temperature programmed for silylated sample from 100 to 190 C, isothermal at 190 C for nonsilylated sample. A 1 μl sample was used in (A) whereas a 0.5 μl sample was used in (B). Portion of silylated chromatogram shown is after reaching temperature of 180 C, extraneous peaks due to solvent and injection excluded. Significant peaks are (1) palmitate, (2) stearate, (3) oleate, (4) linoleate, (5) linolenate, (6) ricinoleate and (7) its trimethylsilyl derivative.

conventional type (11) esterification for comparative purposes.

Halogenation

Bromination can be a relatively rapid and simple procedure with the MRA system as shown by the following description: (a) Inject 10 μl of a 10% solution of olive oil methyl esters in pentane-hexane into the reaction chamber, again detached from gun. (b) Attach Tygon tubing to both ends of the reaction chamber. (c) Immerse reaction chamber in ice. (d) Allow to cool for 3 min. (e) Pass bromine gas through chamber for 5 min by bubbling nitrogen gas through a small amount of liquid bromine in a test tube at 20-40 cc/min. (f) Flush out reaction chamber with nitrogen to evaporate excess solvent and bromine. (g) Assemble as in Figure 1A. (h) Inject sample into GLC at an MRA temperature of 200 C for 3 min.

As noted in the literature (12,13), brominating a sample is an excellent method for determining which peaks are due to saturated and unsaturated compounds on a GLC recording. Bromine compounds have a much higher vaporization temperature than nonbrominated compounds and either disappear from the chromatogram or appear much later. This effect was substantiated in our experiment (Fig. 3). Chromatogram A is typical of olive methyl esters; chromatogram B is after bromination. It is obvious that the unsaturated compounds have been brominated and have disappeared from the chromatogram. The brominated compounds remain behind in the reaction chamber during sample injection into the GLC since their vaporization temperature is above 200 C.

Silylation

Silylation of relatively nonvolatile compounds to volatile derivatives to obtain a rapid GLC analysis has become increasingly important (14). Derivatives, as noted by the literature (15-18), can be prepared simply by adding a silyl compound to a sample, mixing for approximately 2 min, and taking a portion for analysis. The sample can be handled even less by using the MRA system, as shown by the following procedure for silylating methyl esters of castor oil: (a) Pull 3 μl bis(trimethylsilyl) acetamide (BSA), 1 μl purified pyridine and 1 μl castor methyl esters into a 10 μl syringe, consecutively. (b) Inject mixture into reaction chamber while ca. 20 cc/min helium is flowing through chamber to exclude moisture. (c) Allow mixture to react for 2 min at room tem-

perature. (d) Same as step e in DMP esterification procedure.

The results of silylating castor methyl esters are shown in Figure 4. Chromatogram A is of the methyl esters before silylating and B, after silylating. The long leading edge of the methyl ricinoleate peak is probably due to either partial adsorption on the DEGS column or overloading the column slightly. Comparing Figure 4A with Figure 4B shows that the trimethylsilyl (TMS) derivative elutes much faster than the methyl ester as shown in earlier literature (16).

Saponification and Esterification

The procedure for saponifying lipids from refined soybean oil and then esterifying them in the MRA for GLC analyses is as follows: (a) Draw 4 μl of 10% KOH in methanol, 0.5 μl of refined soybean oil and 4 μl of 10% KOH in methanol into a 10 μl syringe, consecutively. (b) Same as step b in first esterification procedure. (c) React for 5 min at 60 C with no gas flow through chamber. (d) Add 15 μl BF_3-CH_3OH (10% solution). (e) React for 1-1/2 min at 50 C with no gas flow through chamber (f-g) Same as steps d and e of DMP esterification procedure.

This experiment produced a chromatographic recording identical to that for the methyl ester preparations shown in Figure 2. Composition was palmitate, 10.6%; stearate, 3.3%; oleate, 26.2%; linoleate, 53.4%; and linolenate, 6.5%.

Hydrogenation

A homogeneous and a heterogeneous reduction were performed. The homogeneous hydrazine reduction of oleic acid was as follows: (a) Mix solution to a 60:1 molar ratio of N_2H_4 to sample, in this experiment, oleic acid. Sample components were: 50 μl CH_3OH, 5 μl oleic acid and 33 μl N_2H_4 (95+%). (b) Inject 18 μl of 60:1 solution into reaction chamber. (c) React for 10 min at 80 C with 5-10 cc/min oxygen flow through chamber. (d) Esterify with CH_2N_2 for 2 min. (e) Inject sample into GLC for 1 min at 300 C. The GLC recording showed only a methyl stearate peak.

Our experience has shown that many factors are important in obtaining complete reduction in the MRA with hydrazine. Essential points to remember are pre-mixing, 60:1 molar ratio, minimum of 10 min reaction time at 80 C and no more than 5 or 10 cc/min oxygen flow. Sample size was chosen as 1 μl to conform with GLC sensitivity.

The heterogeneous reduction in the MRA reaction chamber is similar to that previously reported (19). The sample is passed through a small column packed with a catalyst. In the following procedure the glass reaction chamber of the MRA was packed with platinum-on-alumina catalyst and mixed with Chromosorb P in such a way that the mixture contained 0.16% platinum. The catalyst packing measured 35 mm long by 4 mm in diameter and the sample was injected so it traversed the entire length. Procedure: (a) Mount MRA so needle from reaction chamber is through the GLC inlet septum. (b) Pass helium through chamber to flush out system. (c) Turn helium off and pass hydrogen through chamber at 60 cc/min for 2 min. (d) Inject 0.5 μl of soybean methyl esters into chamber with hydrogen flowing. (e) Raise temperature of reaction chamber to 250 C and react for 1 min. (f) Turn off hydrogen and pass helium through chamber for 3 min at 250 C to ensure transfer of sample. (g) Remove MRA needle from GLC septum.

The chromatographic results of this hydrogenation experiment were identical to those reported earlier (19). An injection of 0.5 μl of soybean methyl esters was used as a standard. The appearance of the appropriate percentage palmitate and stearate peaks in the GLC recording after hydrogenating indicated complete reduction.

DISCUSSION

The foregoing experiments, in addition to ones in previous publications (7-9,20), are indicative of the versatility of the MRA system. Its expanded capability is due to the latest design of the reaction chamber (Fig. 1). Advantages of the glass chamber over the stainless steel chamber are: easy replacement due to commercial availability; fewer hazards when using diazomethane; easy cleaning; easy removal for reactions away from MRA system, such as a hood; and ready packing with material to serve as a pre-column or catalyst.

Experience has shown that a number of points should be remembered to ensure complete transfer of sample from MRA to GLC during injection: Slide the MRA heating chamber against the back end of the needle and allow the other end to touch the inner side of the hot injector port by inserting needle completely and at a slight angle through the GLC septum to prevent sample condensation. Hold needle tightly against the Luer fitting, when removing from the septum, to prevent separation and backflow of sample. Determine that there is sufficient gas flowing from MRA to GLC, when using a thermoconductivity GLC, by noting that the recorder pen returns to

or near the base line.

Most reactions that can be performed in a test tube can be done in the MRA. These include many pre-column GLC-type reactions which can be found in the literature (1,2,4), subtraction chromatography and pyrolysis. The hydrazine-reduction technique employed with the MRA should be useful for making standard samples for analytical instruments, such as the mass spectrometer, especially when labeling compounds with deuterium by using tetra-deutero-hydrazine. Other uses of the MRA, which have already proved successful, are the analysis of oil from the crop of an ant, the location of double bonds in GLC fractions collected in the reaction chamber and the determination of conjugatable dienes with tetramethylammonium hydroxide. An untried, but interesting, use of the MRA would be the sequential analysis of blood lipids with microgram amounts as suggested by F.D. Collins of the University of Melbourne, Australia.

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Comparative Autoxidative Susceptibility of Fatty Esters With 0-6 Methylene-Interrupted Double Bonds

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ABSTRACT

Disappearance of unsaturated fatty esters due to autoxidation was estimated by gas liquid chromatography. The stability of a mixture containing from 0-6 methylene-interrupted double bonds was significantly increased by chromatography over silver nitrate-silicic acid. The relative stability of the esters is a linear function of the number of double bonds.

INTRODUCTION

Available information indicates that the stability of long chain fatty acids and their esters varies considerably with the extent of their unsaturation. Commonly quoted figures for relative autoxidation rates are 1:12:24 for the methyl esters of oleic, linoleic and linolenic acids (1), and 1:100:1000 for methyl stearate, oleate and linoleate (2). Data for materials containing more than three double bonds are considerably more sparse. For fatty acids with more than two double bonds, it has been suggested that the autoxidation rate doubles for each additional double bond (3,4), or that there is an exponential increase in autoxidation rate with increasing unsaturation (5).

The susceptibility of unsaturated fatty acids to deterioration has elicited many recommendations for precautions to limit the degradation of materials containing highly unsaturated fatty acids. These include storage and handling at low temperature in the absence of oxygen, use of antioxidant (6-8), and storage in solvent solution (9) because of the deterioration of neat lipid at -20 C (4).

In the course of separating a mixture of methyl esters containing from 0-6 double bonds by silicic acid-silver nitrate chromatography (10), recoveries of unsaturated esters were not less than 80% despite the fact that the fractionation, analysis and recombination were made during a month's time. Subsequent measurements have indicated that fractions high in penta- and hexaenoic methyl esters did not suffer measureable degradation after four weeks of storage in air at -20 C even though distribution of 0.5 to 5 mg quantities over the bottom surface of a 125 ml Erlenmeyer flask might be considered conductive to rapid autoxidation. Possible explanations for this degree of stability are that, (a) a high purity ester is significantly more stable than one ordinarily encountered (11,12), (b) relative molecular immobility imparted by the adsorbed state of the molecules in a thin film so that chain propagation is slowed down, (c) presence of an antioxidant derived from the chromatography, by chemical reaction of the reagents or by use of a reagent containing undeclared antioxidant (13).

To obtain insight into the nature and extent of stability that chromatography imparts to unsaturated esters against autoxidation, a standard mixture of esters was chromatographed and stored under conditions described below. The standard mixture included methyl palmitate as an internal reference, although it is recognized that even saturated esters may autoxidize under relatively mild conditions when in the presence of impurities or autoxidation products of more labile esters (14).

MATERIALS AND METHODS

Reagents

Silica gel (Baker 3405), silicic acid powder (Baker 0324), silver nitrate (reagent grade) and diethyl ether (Baker 9244) were used as received. Pentane and cyclohexane were distilled; cyclohexene (Aldrich C 10,230) was freshly distilled from sodium. Methyl palmitate (16:0), oleate (18:1), linoleate (18:2) and linolenate (18:3) were >99% pure; methyl arachidonate (20:4), eicosapentaenoate (20:5) and docosahexaenoate (22:6) were >90% pure (Hormel Institute, Austin, Minn.). Antioxidants were not added in preparation of the fatty esters.

Gas Chromatography

A Barber-Colman Model 10 with a flame ionization detector was used for analysis of the

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TABLE I

Composition of Primary Standard and Silica-Chromatographed Esters

Methyl ester	Percent by weight	Percent of total area[a] Unchromatographed 0.210 g	Fraction 4 0.1521 g	Fraction 5 0.0415 g
C_{16}	2.64	2.89	3.03	0.69
$C_{18:1}$	7.05	8.25	9.47	2.42
$C_{18:2}$	8.93	10.0	12.1	4.71
$C_{18:3}$	11.8	13.0	14.5	9.11
$C_{20:4}$	15.9	15.4	17.4	12.2
$C_{20:5}$	24.2	21.4	19.1	27.7
$C_{22:6}$	29.5	29.0	24.4	43.2

[a]Average of two determinations. GLC on ethylene glycol succinate.

methyl ester mixtures. The column was 12.2% (w/w) ethylene glycol succinate on acid-washed, siliconized Chromosorb G, 6 mm x 41", 80-100 mesh. At 185-200 C and 20-30 psig nitrogen, methyl palmitate eluted in 2-3 min. Detector area response to a mixture containing equal weights of methyl palmitate, stearate, arachidate and behenate was within ±3% (relative) of the known weight percent. Area response to NIH standard F was within ±5% (relative) for components greater than 3% of total mixture, and 8% (relative) for components comprising less than 3% of total.

Sample Handling

Methyl esters were weighed to give a mixture containing some commonly encountered esters (Table I).

The mixture was diluted with distilled cyclohexane. Aliquots were used as control, a load on a silver nitrate-silicic acid column, and on a silica gel column. The $AgNO_3$-silicic acid column (10) was activated with 50 ml Et_2O, 500 ml 25% cyclohexane - 75% Et_2O, and 50 ml pentane; then 0.210 g of standard mixture was applied to the column in pentane. A 25 ml pentane eluate and a 50 ml 25% cyclohexane-75% Et_2O eluate contained 0.0005 and 0.0145 g, respectively. The bulk of the sample was eluted by 200 ml 25% cyclohexene-75% Et_2O and weighed 0.2029 g. This last fraction was similar in composition to the unchromatographed esters, and was used for the stability survey. A third aliquot was eluted from a solvent-activated silica gel column with 100 ml of 4% (v/v) diethyl ether-pentane.

Solvent containing from 4 to 6 mg of the control and chromatographed samples was pipetted into 125 ml Erlenmeyer flasks. The solvent was evaporated with nitrogen and inverted beakers were placed over the necks of the flasks. Sets of flasks were kept at approximately 26 C (range 23-29 C), 2-5 C and -17 to -22 C. Humidity and exposure to volatile laboratory chemicals was not controlled. At intervals, distilled pentane was added to each flask, and a sample was withdrawn for gas liquid chromatographic (GLC) analysis. The solvent evaporated spontaneously from the flask after sampling, and the flask was returned to the appropriate environment. To prevent

TABLE II

Stability of Control and Chromatographed Methyl Esters at Various Temperatures

Unchromatographed mixture[a]				Silver Nitrate - Chromatographed mixture[a]				Silica gel - Chromatographed mixture[a]			
Day (temp.)	1 (26 C)	21 (4 C)	46 (-20 C)	Day (temp.)	1 (26 C)	21 (4 C)	46 (-20 C)	Day (temp.)	1 (26 C)	21 (4 C)	46 (-20 C)
C_{16}	100	100	100	C_{16}	100	100	100	C_{16}	100	100	100
$C_{18:1}$	94	76	153[b]	$C_{18:1}$	84	97	101	$C_{18:1}$	84	84	90
$C_{18:2}$	58	36	104	$C_{18:2}$	82	92	102	$C_{18:2}$	57	46	52
$C_{18:3}$	37	3.9	65	$C_{18:3}$	81	94	101	$C_{18:3}$	42	29	32
$C_{20:4}$	23	1.8	43	$C_{20:4}$	80	92	101	$C_{20:4}$	28	17	16
$C_{20:5}$	10	Trace	24	$C_{20:5}$	77	90	100	$C_{20:5}$	17	9.5	5.9
$C_{22:6}$	8	0	16	$C_{22:6}$	81	94	105	$C_{22:6}$	11	5.8	6.1

[a]Per cent of original remaining.

[b]It is probable that accidental contamination with me $C_{18:1}$ occurred.

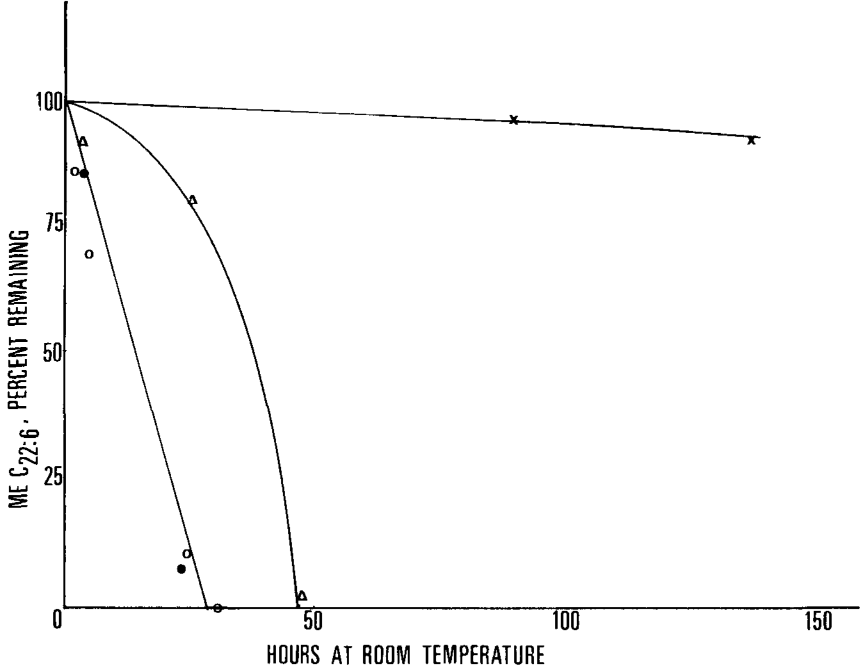

FIG. 1. Effect of silver nitrate-silicic acid chromatography on room temperature stability of methyl docosahexenoate. Mixture contained less than 2 mg of me $C_{22:6}$. X——X $AgNO_3$, Experiment 1. Δ——Δ $AgNO_3$, Experiment 2. ●——● unchrom., Experiment 2. ○——○ silica, Experiment 2.

moisture condensation in the -20 C flasks, they were warmed to room temperature while being flushed with nitrogen.

In connection with the chromatography of the esters, a considerable degree of fractionation occurred even on silica gel. Partial separation by unsaturation has been found on silica (15-18) but its possible extent is not usually stressed. Consideration should be made of the possible importance of these effects during purification of natural mixtures of unsaturated lipids by column or thin layer chromatography (TLC). Table I indicates the composition of the two succeeding fractions of methyl esters eluted by 4% diethyl ether in pentane from 27 g of silica gel. The two fractions account for 92% of the weight taken for chromatography.

Fraction 5 is grossly different from the unchromatographed mixture. Since these differences can exceed differences due to biological variation and GLC precision, it seems obligatory to have relatively complete recoveries in order to obtain the representative sampling necessary for valid interpretation of data from biochemical experiments.

RESULTS AND DISCUSSION

Autoxidation of fatty acids is usually measured by following oxygen uptake (19), weight change (20), hydroperoxide content (21), or UV absorption (22). The use of several of these methods (23) will give a more accurate depiction of the reaction. Autoxidation in the present experiment was evaluated by following the disappearance of unsaturated esters. In the early stages of autoxidation, the disappearance of unsaturated fatty ester should follow the oxygen uptake closely. In later stages, divergences is expected, not necessarily in a predictable manner. In any event, measurement of the disappearance of starting materials should be qualitatively consistent with oxygen uptake studies, although there may be quantitative differences. GLC evaluation of autoxidation is (probably) more appropriate to following changes in highly autoxidized fatty acid mixtures than any of the more common methods used individually, because the rate of disappearance of starting material can be followed until late in the reaction process.

The foregoing conclusion is based on the fact that in autoxidation studies, UV spectrophotometry and peroxide determinations measure compounds that are formed which are also substrates for autoxidation processes and are therefore reliable indicators of the disappearance of starting material only very early

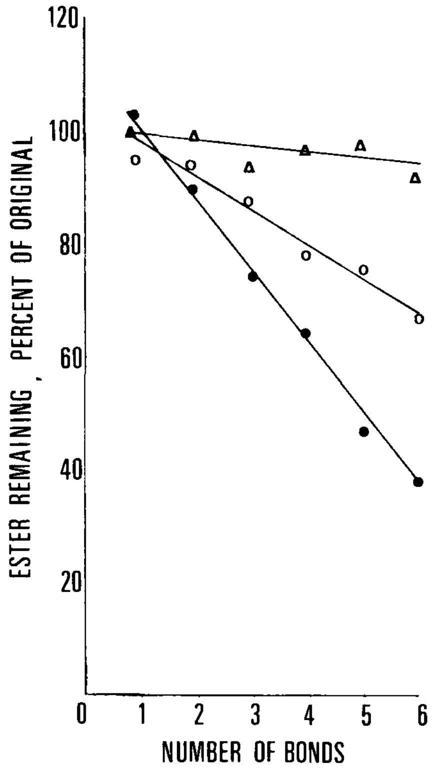

FIG. 2. Changes measured after 11 days at 4 C. These curves show the proportionality of the number of double bonds in the substrate to the amount not changed during autoxidation. Type of chromatography used in sample preparation: Δ——Δ $AgNO_3$. ○——○ silica. ●——● unchrom.

in the autoxidation process. Oxygen is a nonspecific reagent and uptake measurements represent oxidation of both starting material and products. On the other hand, the disappearance of starting material is caused by the combined processes of autoxidation, other oxidations which are not a part of a radical chain, and by nonoxidative charge (e.g., isomerization and polymerization). On balance, even though we have not evaluated the rates of the alternation pathways, it seems likely to us that the deviations from the actual course in measuring the disappearance of starting material are smaller than the errors in measuring formation of products on oxygen consumption.

Deviations of repeat chromatograms were less than 15% for any component. These differences were caused by variation in measurement of the methyl palmitate reference peak. The palmitate peaks were approximately 0.2 cm wide at half height, and errors were ±0.02 cm. So there may be moderate error in the value of a component in any instance, but these errors do not obscure the general trend.

Table II shows representative values for the percent of the individual ester remaining in the mixture stored at room temperature (4 C and -20 C).

The greater stability of the silica gel-chromatographed sample over that of the control at 4 C, and the reverse of this order at -20 C, is probably of no significance. These differences are small compared to those between the silver nitrate-chromatographed samples and others, and may be a consequence of unknown and uncontrolled variables in the methods that were used.

The *stability* of the silver nitrate-chromatographed esters suggests that the esters are protected by an antioxidant or by removal of a prooxidant. It is possible that an antioxidant was formed during chromatography from the acetonitrile and cyclohexane that were used exclusively in the silver columns. However, if an unknown protective agent had been introduced with these solvents, it would probably have been removed by subsequent solvent washing. Formation of an antioxidant from cyclohexene is also doubtful, since cyclohexene readily forms a hydroperoxide which would be considered capable of accelerating the autoxidation of polyene fatty esters. Even though the possibility of an antioxidant arising from decomposition of a cyclohexene hydroperoxide cannot be completely excluded, it seems improbable.

We are attracted to the hypothesis that the increased stability of silver nitrate-chromatographed esters results from removal of a prooxidant. The effectiveness of purification as a means of increasing the stability of polyunsaturated esters has been demonstrated (12,24). Extremely small concentrations of metal salts (25) or porphin complexes of heavy metals (26) can have catalytic effects on autoxidation and *metal* catalytic effects may also be enhanced by complexing, as with protein (27). Even highly purified esters prepared by conventional methods may have metal concentrations sufficient for catalysis (25,26). Removal of small amounts of metal could be beneficial. Further, from consideration of the half cell voltages of metals and their ions, it is evident that the Ag-Ag^+ system is capable of substituting metallic silver for metals such as copper and iron. The silver content of the silver nitrate-chromatographed sample was in fact found to be higher than that of the control by emission spectrometry. It is also possible that the silver nitrate columns effectively separate organic prooxidants from methyl esters, where as only poor separation is obtained of the compounds on silicic acid (12).

A comparison of the stability of me $C_{22:6}$ in the mixture of esters is made in Figure 1. Although the results of two experiments were quantitatively different, the silver nitrate-chromatographed material was considerably more stable than the others. The difference shown between these experiments points out clearly the need for a better understanding of the many factors that influence autoxidation. In Experiment 1, me $C_{22:6}$ had disappeared from the other samples by 90 hr, the first storage point analyzed. Experiment 2, started after 19 days of bulk storage at -20 C, was similar, but not nearly as dramatic. As a crude approximation of stability, one may read the time at which 50% of the original ester remained. This was 34 hr for silver nitrate-chromatographed esters, and 14 hr for the others. The time ratio is 2.4; this is a significant difference in stability.

The susceptibility of polyenoic esters to attack by oxygen is a function of the number of activated methylene groups. One might expect a linear relationship between the number of potential sites for attack and the relative rate of substrate disappearance. Such a relationship is shown in Figure 2, which is a plot of ester remaining as a function of the number of double bonds. This linearity holds for the early stages of autoxidation. As the amount of hexaenoic ester decreases the rate of autoxidation slows; the break point between linear and nonlinear relationship is in the vicinity of 40% me $C_{22:6}$ remaining. This change is undoubtedly due to the increasing concentration of autoxidation products which serve as substrates for autoxidation reactions.

Relative autoxidation of unsaturated esters was estimated by the percent decrease of the components of a mixture spread on glass. The extent of autoxidation under given conditions was a roughly linear function of the number of methylene-interrupted double bonds. The stability of silver nitrate-chromatographed esters was extended considerably over that of silica gel-chromatographed or unchromatographed esters. This increased stability is presumed to be associated with the removal of a prooxidant of unknown type by silver nitrate-silicic acid chromatography.

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SHORT COMMUNICATIONS

Sphingolipid Metabolism II. The Biosynthesis of 3-Keto-Dihydrosphingosine by a Partially-Purified Enzyme From Rat Brain

ABSTRACT

A condensation reaction between serine and palmitoyl CoA by a partially-purified enzyme from rat brain is described. The product of the reaction, 3-keto-dihydrosphingosine is characterized by the conversion to several derivatives. The addition of EDTA to the incubation mixture results in inhibition of the conversion of serine to phospholipid, with the result that 3-keto-dihydrosphingosine is the sole product.

3-Keto-dihydrosphingosine formed in the condensation between palmitoyl CoA and serine has been implicated as the immediate precursor of dihydrosphingosine. The sequence postulated is: (1) L-serine+palmitoyl CoA → 3-keto-dihydrosphingosine+CO_2+CoA (2) 3-keto-dihydrosphingosine+NADPH → dihydrosphingosine+NADP.

Reaction 1 has been demonstrated to occur in particulate fractions from yeast (1-3), rat liver (4) and rat brain (5) employing either labeled serine or labeled palmitoyl CoA as substrate. In addition to 3-keto-dihydrosphingosine several other radioactive products were routinely obtained. Previous studies from this laboratory documented the ability of a rat brain particulate system to incorporate L-serine-^{14}C into ceramide, cerebroside and dihydrosphingosine (6). The present communication reports: (a) the liberation from these particles, and partial purification, of an enzyme which catalyzes the palmitoyl CoA dependent incorporation of L-serine-^{14}C into 3-keto-dihydrosphingosine; (b) conditions under which the major radioactive product detected is 3-keto-dihydrosphingosine; (c) certain properties of this system.

Palmitoyl CoA was obtained from PL Biochemicals. Myristoyl and stearoyl CoA were synthesized enzymatically (7). Erythro DL sphingosine and dihydrosphingosine were purchased from Miles-Yedda. 3-Keto-dihydrosphingosine, N-acetyl 3-keto-dihydrosphingosine, N-acetyl dihydrosphingosine, N-acetyl 3-keto-sphingosine and N-acetyl sphingosine were synthesized by published procedures (8,9). Silica Gel G thin layer plates were obtained from Analtech, Wilmington, Del.

The preparation of the rat brain particulate system, employed as the starting enzyme source, has been described (6). A suspension in 0.25 M sucrose was placed in an ice-salt bath and sonified for 4 thirty-second intervals with a Branson Model 175 sonifier. The sonicate was centrifuged at 40,000 x *g* for 1 hr. The supernate was fractionated with ammonium sulfate to give three fractions: 0-30%, 30-60% and 60-90% of saturation. The activity was usually distributed between the first and last fraction, with an overall purification of 5-10 fold.

In order to isolate and identify the product of the reaction, 10 tubes each containing 20 μmoles phosphate buffer pH 8.0, 100 millimicromoles palmitoyl CoA, L-serine-^{14}C (1×10^6 total counts, S.A. = 7.5×10^7 cpm/μmole from New England Nuclear); 0.5-1.0 mg protein in a total volume 0.5 ml were incubated at 37 C for 30 min. The method of obtaining the labeled lipid product was identical to that previously described (10), except that each water-containing extracting solution was 0.05 N with respect to sodium hydroxide. Aliquots of the final lower phase were counted in toluene-POPO-PPO mixture in a Packard Scintillation Counter. The radioactive product was characterized as 3-keto-dihydrosphingosine by the following parameters: (a) the product was found to co-chromatogram with an authentic standard of 3-keto-dihydrosphingosine on thin layer plates (TLP) with chloroform-methanol-water (65:25:4); (b) the sample was reduced with sodium borohydride (2) and the material migrated with dihydrosphingosine on TLP with chloroform-methanol-2N NH_4OH as solvent (11); (c) the borohydride reduced sample was treated with fluorodinitrobenzene and the derivative was found to co-chromatogram with

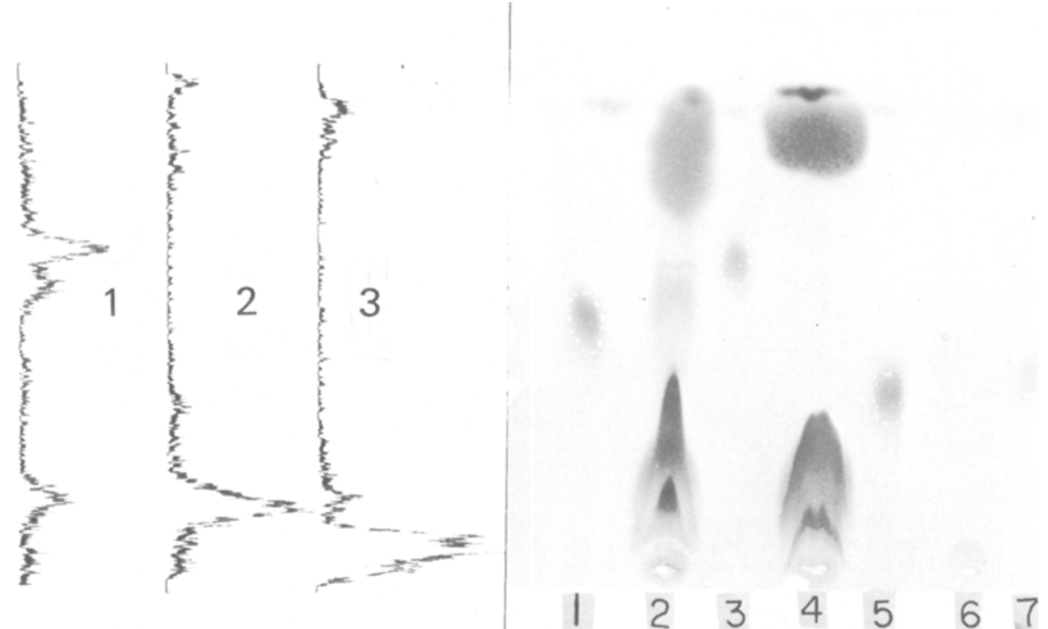

FIG. 1. TLC of dinitrophenyl (DNP) derivatives of metabolic sample and standards: Lane 1, standard DNP *erythro* sphingosine; Lane 2, metabolic sample reduced with $NaBH_4$ and converted to DNP derivative; Lane 3, standard DNP *erythro* DNP dihydrosphingosine; Lane 4, metabolic sample not reduced with $NaBH_4$ but converted to DNP derivative; Lane 5, standard DNP *threo* sphingosine; Lane 6, untreated metabolic sample; Lane 7, standard DNP *threo* dihydrosphingosine; tracing 1, scan of Lane 2; tracing 2, scan of Lane 4; tracing 3, scan of Lane 6.

the dinitrophenyl derivative of dihydrosphingosine (12) (Fig. 1); (d) a portion of the borohydride treated sample was N-acetylated and the product co-chromatogrammed with N-acetyl dihydrosphingosine using a published procedure (8); (e) the product was destroyed by incubation in alkalai at 37 C. This chemical instability is characteristic of 3-keto-dihydrosphingosine (3,5,9); (f) radioactivity from L-serine-^{14}C was found in this compound only when palmitoyl CoA was present in the incubation mixture.

Initial studies indicated that in addition to 3-keto-dihydrosphingosine, L-serine-^{14}C was incorporated into a material which: (a) had chromatographic properties similar to phosphatidyl serine; (b) was labile to mild alkaline hydrolysis; (c) was formed independent of the presence of palmitoyl CoA. The amount of radioactivity in this material, presumed to be phosphatidyl serine, could be significantly reduced by the presence of non-radioactive ethanolamine (3 μmole/ml) or completely eliminated by addition of EDTA (50 μmoles/ml) giving rise to only one radioactive product, 3-keto-dihydrosphingosine as shown in Figure 2.

This observation provided an opportunity to study certain properties of this enzymatic reaction and should provide a convenient assay procedure for further enzyme purification. Preliminary experiments have indicated that neither Mg^{+2} nor Mn^{+2} are required, sulfhydryl compounds such as BAL, cysteine, dithiothreitol, as well as PCMB and NEM inhibited the incorporation of serine into 3-keto-sphingosine. It was found that neither myristoyl nor stearoyl CoA were effective in replacing palmitoyl CoA in the formation of 3-keto-sphingosine base. The presence of a TPNH generating system consisting of glucose-6-phosphate, TPN and glucose-6-phosphate dehydrogenase did not give rise to dihydrosphingosine under thsese assay conditions.

These experiments describe for the first time the partial purification of an enzyme from a rat brain particle system which catalyzes the condensation reaction between L-serine and palmitoyl CoA to yield 3-keto-dihydrosphingosine. The use of EDTA to inhibit the incorporation of L-serine into phospholipid results in the synthesis of one principal radioactive product in this system. This is in contrast to previous investigation where several radioactive

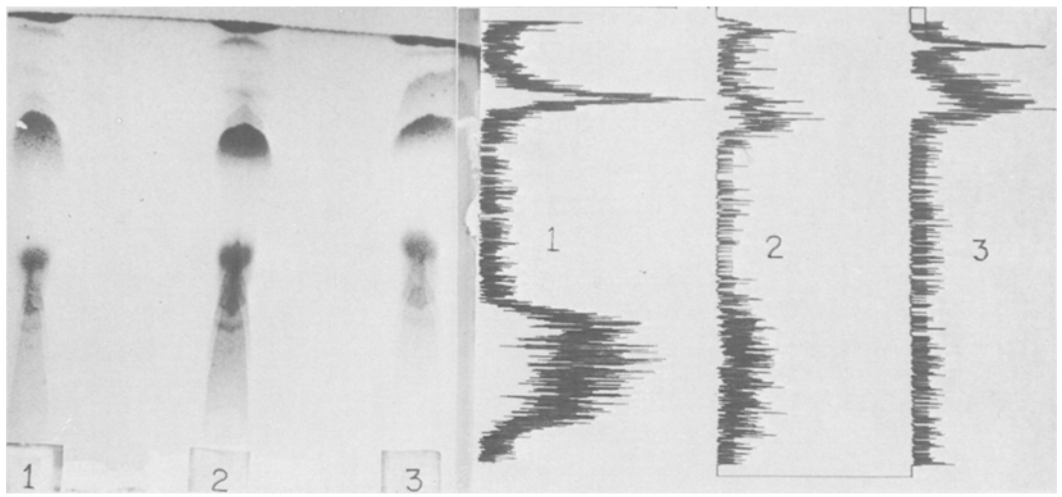

FIG. 2. Effect of ethanolamine and EDTA on serine incorporation into 3-keto-sphingosine: Lane 1 and tracing 1, products of basic incubation; Lane 2 and tracing 2, ethanolamine added to incubation; Lane 3 and tracing 3, EDTA added to incubations.

products were formed. This observation should provide a simple assay system without resorting to TLC resolution of a mixture of radioactive products and subsequent counting of the gel co-chromatography with 3-keto-dihydro-sphingosine. The inability of stearoyl CoA to support the incorporation of serine into sphingosine base would suggest that the synthesis of eicososphingosine (the C_{20} homologue) which is found in gangliosides (13) may arise by a different pathway.

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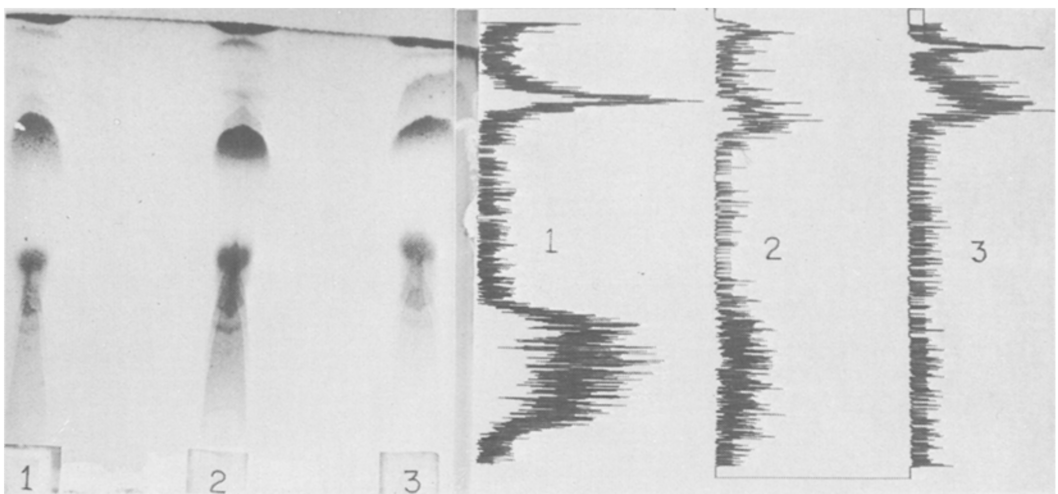
Unsaturated Fatty Acids of Mycobacteria

ABSTRACT

The double bond locations have been determined for the mono-unsaturated fatty acids, C_{14} to C_{26} of *M. smegmatis* and *M. bovis* BCG. The 14:1 and 16:1 fatty acids from *M. smegmatis* are principally Δ^{10}, while the 17:1, 18:1 and 19:1 fatty acids from both organisms are Δ^{9}. In the case of *M. smegmatis*, the 20:1, 22:1 and 24:1 fatty acids are principally Δ^{11}, Δ^{13} and Δ^{15}, respectively, while the 22:1, 24:1 and 26:1 fatty acids of BCG are principally Δ^{13}, Δ^{15} and Δ^{17}, respectively.



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products were formed. This observation should provide a simple assay system without resorting to TLC resolution of a mixture of radioactive products and subsequent counting of the gel co-chromatography with 3-keto-dihydrosphingosine. The inability of stearoyl CoA to support the incorporation of serine into sphingosine base would suggest that the synthesis of eicososphingosine (the C_{20} homologue) which is found in gangliosides (13) may arise by a different pathway.

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Unsaturated Fatty Acids of Mycobacteria

ABSTRACT

The double bond locations have been determined for the mono-unsaturated fatty acids, C_{14} to C_{26} of *M. smegmatis* and *M. bovis* BCG. The 14:1 and 16:1 fatty acids from *M. smegmatis* are principally Δ^{10}, while the 17:1, 18:1 and 19:1 fatty acids from both organisms are Δ^{9}. In the case of *M. smegmatis*, the 20:1, 22:1 and 24:1 fatty acids are principally Δ^{11}, Δ^{13} and Δ^{15}, respectively, while the 22:1, 24:1 and 26:1 fatty acids of BCG are principally Δ^{13}, Δ^{15} and Δ^{17}, respectively.

The double bond locations of mycobacterial mono-unsaturated fatty acids have been determined for only a few fatty acids. Scheuerbrandt and Bloch (1) showed that the C_{16} fatty acid consisted principally of the Δ^{10} isomer and the C_{18} fatty acid the Δ^9 isomer. Burke (2), using the avirulent *Mycobacterium tuberculosis* strain $H37R_A$, found that C_{16} monoene consisted of a mixture of 10-, 6- and 9-hexadecenoic acids in a proportion of 65, 15 and 10. The C_{17} and C_{18} fatty acids were mostly the Δ^9 isomer. The C_{20} acid appeared to be *trans*-2-eicosenoate.

This report describes the double bond location of the series of mono-unsaturated fatty acids from C_{14} to C_{26} in *M. smegmatis* ATCC 19420 and *M. bovis* BCG (Glaxo strain).

The organisms were grown and harvested as described previously (3). Cells were hydrolyzed in 95% methanol containing 20% potassium hydroxide (4). Fatty acid methyl esters were prepared by refluxing the crude fatty acid mixture in methanol-benzene-concentrated sulfuric acid 20:10:1 for 2 hr. Mercuric acetate adducts were prepared (5) and the esters were separated by column chromatography on Florisil [Fisher Scientific Co., Medford, Mass.; 100-200 mesh; deactivated with 7% water, w/w] (6). The solvent sequence petroleum ether-diethyl ether 95:5, petroleum ether-diethyl ether 85:15 and methanol-concentrated hydrochloric acid 90:10 was used. The last fraction, which contained the unsaturated fatty acids, was further purified by thin layer chromatography on Adsorbosil 1 (Applied Science Labs., Inc., State College, Pa.) impregnated with 12% silver nitrate (developing solvent petroleum ether-diethyl ether 90:10). The unsaturated fatty acid esters were purified by repeated preparative gas liquid chromatography (GLC) and were then oxidized by periodate-permanganate (7,8,9).

The periodate-permanganate cleavage products were analyzed by GLC on a Perkin-Elmer 900 gas chromatograph using three sets of columns: 5 ft x 1/8 in., 2.5% OV-225 on 80-100 mesh AW Chromosorb G; 6 ft x 1/8 in., 15% degs on 60-80 mesh AW Celite; and 6 ft x 1/8 in., 2.5% OV-1 on 80-100 mesh AW, DMCS Chromosorb G (high performance). Double bond location was assigned principally on the basis of the identification of the dicarboxylic acid oxidation product.

Table I lists the relative yields of dicarboxylic acids obtained from periodate-permanganate oxidation. The C_{14} and C_{15} fatty acids consist of a wide range of isomers and may reflect diverse biosynthetic origins. The C_{16} fatty acids consist principally of the Δ^9, and Δ^{10} isomers, while the C_{17}, $_{18}$ and $_{19}$ acids are mostly the Δ^9 isomers, an indication of a common desaturase system.

The $C_{20,22,24}$ fatty acids of *M. smegmatis* and $C_{22,24,26}$ acids of BCG are a related series reflecting a common origin from the Δ^9 C_{18} fatty acid with C-2 elongation. The C_{20} fatty acid of BCG is a mixture of 9, 10 and 11 isomers and may have originated by a combination

TABLE I

Relative Proportions of Dicarboxylic Acids Obtained From Periodate-Permanganate Oxidation of *M. bovis* BCG and *M. smegmatis* Unsaturated Fatty Acids

Proportions	Dicarboxylic acid	BCG	*M. smegmatis*
14:1	C_4	10	---
	C_5	20	---
	C_6	5	---
	C_7	25	10
	C_8	20	15
	C_9	5	5
	C_{10}	15	70
15:1	C_5	10	---
	C_6	---	---
	C_7	20	---
	C_8	15	---
	C_9	30	---
	C_{10}	25	---
16:1	C_7	10	7
	C_8	5	3
	C_9	40	30
	C_{10}	45	60
17:1	C_8		10
	C_9	70	70
	C_{10}	30	20
18:1	C_8	5	5
	C_9	90	90
	C_{10}	5	5
19:1	C_9	90	60
	C_{10}	10	40
20:1	C_9	35	15
	C_{10}	20	10
	C_{11}	25	60
	C_{12}	10	10
	C_{13}	10	5
22:1	C_{12}	---	10
	C_{13}	55	80
	C_{14}	25	5
	C_{15}	20	5
24:1	C_{15}	75	85
	C_{16}	10	10
	C_{17}	15	5
26:1	C_{16}	10	---
	C_{17}	60	---
	C_{18}	10	---
	C_{19}	20	---

of desaturation-elongation and direct desaturation.

The long chain fatty acids (C_{20} and up) of the mycobacteria are located principally in the triglyceride fraction. These fatty acids are located mostly on the 3 position of the triglyceride (3). Previously, a scheme was hypothesized whereby the long chain fatty acids were suggested to have originated from a biosynthetic system separate from the de novo pathway, being elongated and desaturated in effectively a compartmentilized system (3). The studies reported here would seem to support this hypothesis.

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Free Fatty Acids in the Protective Coats of *Spongilla Wagneri* Gemmules

ABSTRACT

A gas chromatographic and mass spectrometric analysis of the free fatty acids found in the resistant coat of *Spongilla wagneri* gemmules was performed. The coats were found to contain 8.79% extractable lipids with 18.53% free fatty acids, ranging in chain length from C_{10} to C_{24}. The unsaturated acids were in relatively low concentrations with the preponderant saturated being palmitic and behenic. Distinct differences in distribution were observed when compared to the total gemmule fatty acids.

It has been reported that both silicious marine (1) and fresh-water sponges of the genus *Spongilla* contain significant amounts of free fatty acids (2). As part of a continuing study of the surface lipids present in natural resistant structures (3-5), we are investigating the lipids found in the outer coat of the gemmules produced by fresh-water sponges. These protective structures enable the sponge to survive adverse environmental conditions. A feature which makes these gemmules of experimental significance is the ease with which the outer protective coats can be removed for study. Of additional interest is that only a small body of knowledge exists on the lipids of sponges, while none exists on the gemmules or other highly adaptive structures these sessile animals produce. *Spongilla wagneri* (6) was chosen for this work since abundant gemmules were available in local waters during the fall of 1969. The mature gemmules were carefully removed from the parent structure and passed through a No. 30 sieve (U.S. standard sieve 595-μ openings) and then collected and washed with water in a No. 60 sieve (U.S. standard with 250-μ openings). The gemmules were examined with a light microscope to establish purity and the type present. Great care was taken to ensure complete separation from the parent body materials. All samples were stored at 4 C.

To obtain the total free fatty acids, approximately 5.0 g of intact gemmules were first broken by intensive grinding in a mortar with two 50 ml portions of *n*-heptane at room temperature. The residual material was then reextracted with 50 ml of benzene-chloroform (1:3) for 1 hr at 50 C followed by extraction with 50 ml of chloroform-methanol (1:1) at 50 C. The combined extracts were taken to

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The long chain fatty acids (C_{20} and up) of the mycobacteria are located principally in the triglyceride fraction. These fatty acids are located mostly on the 3 position of the triglyceride (3). Previously, a scheme was hypothesized whereby the long chain fatty acids were suggested to have originated from a biosynthetic system separate from the de novo pathway, being elongated and desaturated in effectively a compartmentilized system (3). The studies reported here would seem to support this hypothesis.

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TABLE I

Free Fatty Acid Composition of the Outer Protective Coat as Compared to the Entire Gemmule of *Spongilla wagneri*

Fatty acid Carbon No.	Relative percent distribution	
	Entire gemmule	Gemmule outer coat
10:0	Trace[a]	0.10
12:0	Trace	0.41
13:0	Trace	0.36
14:0	0.90	1.35
14:1	Trace	Trace
15:0	0.26	1.35
16:0	10.30	19.20
16:1	2.25	0.20
17:0	0.80	2.04
17:1	0.85	1.04
18:0	5.68	7.84
18:1	19.70	1.00
18:2	7.20	0.50
18:3	9.02	0.11
19:0	3.15	1.26
19:1	0.85	0.30
20:0	15.45	9.39
20:1	1.70	1.30
21:0	0.71	6.80
21:1	0.57	1.09
22:0	15.05	23.30
22:1	2.06	1.49
23:0	1.70	9.31
23:1	Trace	1.35
24:0	1.80	5.70
24:1	Trace	3.16

[a]Less than 0.1%.

dryness under a stream of prepurified nitrogen. The residue was taken up in *n*-heptane and transferred to the top of an activated silica gel column (1 x 20 cm). The silica gel had been preconditioned by heating to 425 C for 10 hr followed by cooling and washing with 3 vol of *n*-heptane. The various lipid fractions were eluted with *n*-heptane, followed by benzene and a final polar fraction was eluted with 100 ml of absolute methanol. The latter fraction was methylated by adding a mixture of boron trifluoride-methanol and heating as described by Morrison and Smith (7). For comparison, esters were also prepared by use of a diazomethane reagent. Our experience has been that the milder methylation agent will not open the epoxide ring known to be present in several natural lipid sources.

Removal of the gemmule coat was accomplished by gently crushing gemmules in a mortar with a small amount of water until all of the outer coats were broken open. They were then washed with water in a No. 325 sieve (U.S. standard sieve with 44-μ openings) until microscopic examination did not reveal any trace of the inner cellular material. The extraction and isolation of the free fatty acids from the gemmule coats and subsequent conversion to the corresponding esters followed the same procedure as described for the entire gemmule. Fatty esters were resolved by gas chromatography on a 30 m x 0.025 cm stainless steel capillary column coated with Apiezon-L (purchased from Applied Science Laboratory, Inc.) and a 200 m x 0.067 cm stainless steel capillary column coated with Igepal CO-880 (purchased from General Aniline and Film Corp.). Details of gas chromatographic operation were as described previously (3). Characterization of individual components was carried out by allowing approximately 85% of the injected sample to be eluted from a Perkin-Elmer 900 gas chromatograph into a Perkin-Elmer Hitachi RMU-6E single focusing mass spectrometer.

On a dry weight basis the gemmule structure contains 7.15% total extractable lipids of which 28.53% occur as free acids. By comparison, the outer coat contained a total of 8.79% lipids comprised of 18.53% free fatty acids. The extracts were a light yellow color from the coats and a dark orange from the total gemmule. Thin layer chromatographic data indicate that these colors originated from 10-12 chromatographically distinct pigments.

Table I illustrates the relative distribution of free fatty acids found in the outer coat as compared to the entire gemmule calculated from gas chromatographic peak areas. The bimodal distribution was most apparent in gemmule coats having major peaks at palmitic and behenic acids representing 19.20% and 23.30%, respectively. The entire gemmule, which includes fatty acid contributions from the outer coat, demonstrated a preference for a palmitic and an arachidic acid. The bimodal distribution in fatty acid chain length is not unique to gemmules but has also recently been observed in surface wax of resistant uredospores produced by several rust fungi (unpublished data, J.L. Laseter). Over one third of the free fatty acids in the entire gemmule belong to the C_{18} unsaturated series whereas the contribution of these acids to the outer coat represents only 1.61%. It is possible that the synthesis and accumulation in the coat of saturated chains longer than C_{20} and the correspondingly low population of unsaturated acids may be a response to the needs of the organism to develop a more resistant barrier to the environment.

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The Incorporation of Phosphorylethanolamine Into the Phospholipids of Brain Microsomes in Vitro

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ABSTRACT

Synthesis of ethanolamine phosphoglycerides (EPG) from labeled ^{14}C-phosphorylethanolamine (PE) and cytidine triphosphate (CTP) has been studied in vitro in particles and in soluble fractions of chicken brain. The microsomal membranes can carry out this conversion, but supplementing the microsomes with an enzymic fraction derived from the particle-free supernatant results in a noticeable increase of the rate of EPG synthesis. Mitochondria are almost inactive, in this connection. The conversion of PE to lipid is very low, in no case exceeding 0.5-0.6%, even in the presence of diacyl glycerols or 1-alkenyl 2-acyl glycerol. No stimulation occurs by supplementing the incubation system with natural lipid acceptors, intermediates of lipid synthesis, energy-producing cofactors · or monoacyl *sn*-glycero-3-phosphorylethanolamine (GPE), monoacyl *sn*-glycero-3-phosphorylcholine (GPC) and other lipid material. From these and other results, the conclusion is made that the PE:CTP cytidylyltransferase (E.C. 2.7.7.14) must display very low activity in vitro, thus limiting the overall rate of synthesis from PE. Diacyl GPE is the only lipid which has been found labeled after incubation with PE of the brain preparations. A small synthesis of alkenyl acyl GPE takes place, only when PE is incubated with suitable concentrations of a plasmalogenic diglyceride.

INTRODUCTION

In the last few years a series of contributions have appeared concerning the synthesis of ethanolamine phosphoglycerides (EPG) in brain tissue in vitro (1-4), and the role played by the cytidine coenzymes in this metabolic process (1-7). Only a few studies have examined the in vitro incorporation of labeled precursors into EPG by isolated brain microsomes (3,8), although results obtained both in vitro (3,8) and in vivo (9) pointed to the microsomal fraction of brain tissue as the main site for this process.

The purpose of the present contribution is to investigate further the synthesis of EPG from labeled phosphorylethanolamine (PE) by particulate fractions of avian brain in vitro, and to obtain more information on the properties of the synthetic pathway.

EXPERIMENTAL PROCEDURES

Biological Materials

Procedures for the Isolation of Subcellular Fractions. Chickens, six weeks old, all females were obtained from the same source. The brains of two chickens were weighed, suspended in 7 vol of ice-cold 0.32 M sucrose solution (in 5 mM phosphate buffer, pH 7.6) and homogenized in a Potter homogenizer with six excursions of the teflon pestle for 40 sec. The clearance of the glass homogenizer was about 0.15 mm and the average speed 1300 rev/min. The homogenate was centrifuged at 1500 *g* for 10 min, and 80% of the supernatant fraction (S_1) carefully separated from the precipitate of large myelin, nuclei and cell debris. Mitochondria were sedimented by centrifuging S_1 at 7000 *g* for 10 min, its supernatant fraction being recentrifuged at 18,000 *g* for an additional 10 min. The supernatant (S_2) was saved, and the combined precipitates, referred to respectively as mitochondrial and submitochondrial crude preparations, were further separated into fractions containing myelin, nerve endings and purified mitochondria (10). The purified mitochondrial fraction was washed with 0.32 M sucrose, in about one fourth of the volume of the original homogenate, to remove the Ficoll, and centrifuged at 18,000 *g* for 15 min (M_1). The supernatant S_2 was then centrifuged for 60 min at 105,000 *g* to bring down the microsomal fraction and to obtain a particle-free supernatant (S_3). Microsomal pellets were washed once, in about one fifth of the volume of the original homogenate, in the above mentioned 0.32 M sucrose and recentrifuged for 60 min at 105,000 *g* (M_2). The supernatant fraction was combined with the corresponding original supernatant (S_3) and processed as described later. Each of the M_1 or M_2

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fractions was taken up in small volumes of 0.32 M sucrose and stored at 0 C for a few minutes, if not immediately used.

The M_1 and M_2 were analyzed for the presence of enzymic markers. No evident cytochrome oxidase (11) and succinic oxidase (12) activities [(ferro cytochrome *c*:O_2 oxidoreductase, E.C. 1.9.3.1 and succinate: (acceptor) oxidoreductase, E.C. 1.3.99.1 activities, respectively)] were present in the microsomal particles. A glucose-6-phosphate phosphohydrolase (E.C. 3.1.3.9) activity has been predominantly found in the microsomes (80% of the activity of the whole homogenate), by analyzing the release of inorganic phosphate (P_i) according to Harper (13) and Ellingson and Lands (14). It is not known whether this activity represents a specific enzyme or an unspecific enzyme with low substrate specificity. As reported previously (15,16), the microsomal NADH:cytochrome *c* oxidoreductase (E.C. 1.6.99.1) activity is insensitive to antimycin A, whereas the mitochondrial enzyme is inhibited; the results obtained here have confirmed this finding, indicating that contamination of microsomes with mitochondria had occurred only to the extent of about 5%.

In some instances membrane components were separated from ribosomes and vesicular content, and in others microsomal RNA was removed from original microsomes. In the first case, microsomes (about 0.8 mg of protein N per milliliter of 0.32 M sucrose) were treated with 0.25% sodium deoxycholate and the suspension centrifuged for 3 hr at 105,000 *g*. Tightly packed pellets of ribosomes and membranes, and supernatant fraction constituting microsomal contents and the solubilized part of the membranes, were thus separated, the pellets rinsed two times with 0.32 M sucrose, and both fractions made up to known volumes with ice-cold 0.32 M sucrose. In the second instance, the isolated microsomes were suspended in 0.25 M sucrose containing sodium pyrophosphate (0.05 M), pH 7.5 (17), reisolated by centrifugation and resuspended in 0.32 M sucrose, as above. Determination of the RNA (18) and protein content (19) showed that at least 80% of the total microsomal RNA (27 μg P/mg protein ± 2.8) had been removed by this treatment, whereas the protein content (0.70-0.75 mg/100 mg of fresh brain tissue) had only slightly decreased.

Preparation of the Supernatant Fraction. The original S_3 (about 45-50 mg protein) was further treated with ammonium sulfate. Before fractionation it was passed through a column (1.8 x 22 cm) of Sephadex G-25, particle size 30-70 μ, and eluted with phosphate buffer (0.01 M), pH 7.6 (20). The protein-containing fractions (about 6-7 ml) were pooled and precipitated at 0-4 C by adding saturated ammonium sulfate solution; the active fraction, precipitating between 25% and 38% of saturation, was collected by centrifugation at 9000 *g* for 15 min, taken up in 1 ml of cold 0.32 M sucrose, dialyzed against tris-hydrochloric acid buffer (0.1 M), pH 7.4, for 2 hr at +2 C, and used for incubation without further treatment.

Chemicals and Labeled Substrates

Materials. Cytochrome *c* (Fe, 0.33%), NADH, NAD, sodium phosphoenolpyruvate (PEP) and sodium 3-phosphoglycerate (PGA) were from Biochemica Boehringer (Milan, Italy). Sodium malate, AMP, ATP, cytidine-5'-triphosphate (CTP), cytidine-5'-monophosphate (CMP) and antimycin A were from Sigma Chemical Co. (St. Louis, Mo.). Phospholipase C (E.C. 3.1.4.3) from *Clostridium perfrigens* was donated by R. E. McCaman, Duarte, Calif. Activated charcoal was prepared according to Threlfall (21).

Almost pure diacyl *sn*-glycero-3-phosphorylethanolamine (GPE) was obtained by silicic acid chromatography of egg lipids, which rendered the lipid free from small amounts of other GPE lipids. Synthetic 1,2-dipalmitoyl GPE and 1,2-distearoyl GPE, from Applied Sciences Labs. (State College, Pa.), were purified on a silicic acid column by elution with chloroform-methanol (4:1). A preparation of acyl alkenyl GPE was obtained from brain EPG lipids by selective alkaline deacylation procedures (22). The plasmalogen was freed from small unexpected amounts of diacyl *sn*-glycero-3-phosphorylcholine (GPC) by chromatography on alumina column (23). The final product still contained a small amount of diacyl GPE, as a minor entity, and possessed a P-ethanolamine-acyl ester-vinyl ether molar ratio of 1.02:1:0.98:1.

1,2-Diacyl glycerols were prepared from yeast, egg and soybean lecithins by the action of phospholipase C (24). After hydrolysis any unreacted lecithin was removed by adsorption on silicic acid, while the diglycerides were eluted from the same columns with 20% ether in *n*-hexane. The purity of the diglycerides was checked by thin layer chromatography (TLC) of alternate fractions in a diethyl ether-chloroform-*n*-hexane-acetic acid solvent (55:5:40:0.2 v/v/v/v); single spots with R_f identical to those of synthetic diglycerides were obtained. The fractions were pooled, evaporated to dryness and stored at -10 C in known volumes of diethyl ether. DL-1,2-diolein, D-1,2-dimyristin, D-1,2-dipalmitin and D-1,2-distearin were either

gifts of E. Baer (Toronto, Canada) or products of Sigma. Measured aliquots of the natural and synthetic diglycerides were freed from the ether by gentle evaporation under nitrogen and resuspended, at 40 mM and 20 mM concentration, respectively, in water containing bovine plasma albumin (0.05%) and Tween 20 (0.1%). Stable solutions were obtained when kept in the refrigerator.

The 1-alkenyl 2-acyl glycerol (plasmalogenic diglyceride) was prepared by reaction of phospholipase C with a beef heart lecithin fraction (25), as reported by McMurray (26), followed by silicic acid chromatography in a benzene-chloroform gradient (25). Emulsions were prepared as above.

Labeled Compounds. 1,2-^{14}C-Ethanolamine hydrochloride (S. A. of 9.8 mc/mmole) were purchased from International Chemical and Nuclear Corp. (City of Industry, Calif.). Labeled PE was prepared, as described elsewhere (27). Its purity was checked by paper chromatography, followed by radioautography (27). Uniformly-labeled ^{14}C-diacyl GPE, obtained from Applied Sciences Labs., was purified by chromatography on a short DEAE cellulose column, before using as a reference standard. The lysoderivatives of the ^{14}C-labeled diacyl GPE, as well as that of unlabeled diacyl GPC and diacyl GPE, were obtained by the method of Long and Penny (28), with a phospholipase A_2 preparation. Scintillation chemicals were products of Packard S.A. (Zürich, Switzerland).

Incubation

Experiments were carried out in heavy wall pointed tubes with a standard incubation mixture, as follows: diglyceride (2 mM), dissolved in Tween-20 (0.005-0.01% final concentration) and albumin (0.005%); phosphate buffer, pH 7.50 (50 mM); ATP (6 mM); CTP (4 mM); cysteine (12 mM); labeled PE (2 mM), at the specific activity (SA) of 400 μc/mmole; microsomes (mitochondria), corresponding to 0.15-0.20 mg of protein N (about 120-150 mg of original fresh tissue); magnesium chloride (20 mM). Enzymes and other components were added at +4 C at the indicated order. The total volume was 0.7 ml, and the pH (measured at the end of the incubation period) was 7.4-7.5. The purified preparations of diacyl GPC, diacyl GPE, 1-acyl GPC and 1-acyl GPE were added either as emulsions or in micellar form, exactly as described by Fiscus and Schneider (29). The tubes were stoppered and shaken at about 80 strokes/min in a water bath at 37 C, for 30 min. The reaction was then stopped, and the mixture treated as described in the following section.

Isolation and Assay

Total Lipid. After incubation the reaction mixture was inactivated by adding ice cold 50% trichloroacetic acid (TCA) to give a final concentration of 5%. After 10 min at 2 C the suspension was centrifuged, the supernatant removed and the precipitated material washed twice with a few milliliters of unlabeled, ice cold PE (2 mM), and three times with ice cold H_2O. The lipid material was dispersed in water to a volume of 0.5 ml, mixed briefly at room temperature with 18 ml of chloroform-methanol (2:1 v/v), extracted and washed (30,31). The last lower phase was dried under nitrogen and dissolved in a known volume of chloroform-methanol (2:1 v/v). (No serious loss of the vinyl ether linkage of plasmalogen occurred following this treatment, provided that ice cold diluted TCA was used and long contact with the precipitated material was avoided). The lipid P was determined after digestion according to Ernster et al. (32). A known portion of the radioactive lipid solution was dried directly in the counting vial before the addition of 10 ml of the scintillation liquid (3,16), and then counted. Eighty-four per cent efficiency was obtained.

Hydrolytic Procedures. Hydrolytic procedures (33), modified for plasmalogen determination (9), were carried out on the lipid extract of incubations performed on twice the scale indicated (see Incubation). Small portions of the first and second upper phases were taken for determination of P (32) and of total radioactivity, and the remainder was used in duplicate for radioactivity assay and P determination (34) of the separated water-soluble components. For radioactivity measurements, the spots were removed, cut into small pieces into the counting vials and dissolved in 0.35 ml of water plus 10 ml of Buhler's scintillation solution (35). Recovery was 75-80%. Counting efficiency was over 80%, as checked with calibration curves. Radioactivity counts and P determinations on the last lower phase (33,36) were carried out on the whole dried residue, as described previously (see Total Lipid).

Separation of Phospholipids. Phospholipid classes were separated, in duplicate, from the lipid solution (about 1.3 μmoles of lipid P), according to Horrocks procedure (37). The thin layer chromatograms were used for P determination (34) and for radioautography. The radioactive spots of the radioautogram were then assayed for ^{14}C content (3,16). Recovery was 90-95%.

Analytical Methods. Vinyl ether groups in the total lipid extract were determined according to Rapport and Norton (38). Car-

TABLE I

The Transfer of Radioactivity From ^{14}C-Labeled Phosphorylethanolamine to the Ethanolamine Phosphoglycerides of Chicken Brain Particulate Fractions in Vitro[a]

Tissue fraction	Activity[b]
Mitochondria	< 5
Mitochondria + supernatant	< 5
Microsomes	15
Microsomes + supernatant[c]	121
Microsomes + supernatant	93
Microsomes[d] + supernatant	107
Microsomes[e] + supernatant	22
Microsomes + supernatant[e]	91
Microsomes + supernatant[f]	116
Supernatant	0
Supernatant[c]	13

[a]Microsomes (0.8-1.1 mg protein), mitochondria (1.2 mg protein) and supernatant fraction (S_3), precipitated at 25-38% of ammonium sulfate saturation (0.38 mg protein), were incubated for 30 min at 37 C in the standard incubation mixture described in the text. The diglyceride was DL-1,2-diolein. After incubation, radioactivity into the total lipid was estimated as described in the text.

[b]Activity expressed as μμmoles of labeled phospholipid per milligram protein per minute.

[c]Unfractionated supernatant fraction (S_3):2.5 mg protein.

[d]Stored at -20 C for 4 hr.

[e]Stored at 0-4 C for 24 hr.

[f]Stored at 0-4 C for 96 hr.

TABLE II

The Effect of Adding Different Diglycerides on the Synthesis of Ethanolamine Phosphoglycerides From Labeled Phosphorylethanolamine[a]

Diglyceride	Final concentration (mM)	Activity[b]
DL-1,2-Diolein	2	101
	6	70
	10	106
D-1,2-Dimyristin	2	57
	4	69
	10	96
D-1,2-Distearin	4	93
	10	102
From yeast lecithin	2	138
	4	140
From soybean lecithin	2	162
	10	198
From egg lecithin	2	151

[a]Microsomal protein (1.15 mg), supplemented with the fractionated supernatant fraction described in Table I (0.40 mg protein), was incubated in the medium reported in the text. Incubations were carried out at 37 C for 30 min, and the lipids extracted, as described in the text.

[b]Activity expressed as μμmoles of labeled lipid per milligram protein per minute.

boxyl esters were determined according to Stern and Shapiro (39). Protein of the membranous structures was assayed according to Lowry et al. (19) after deoxycholate treatment (40).

RESULTS

Intracellular Distribution

The results tabulated in Table I show that the enzymic system which converts PE into EPG is present in the microsomal fraction of chicken brain and requires the addition of the supernatant for maximal activity. In some experiments, however, microsomes alone had the same or higher activity than microsomes plus the supernatant; this discrepancy was observed only when synthetic diglycerides were used, and was not seen when natural diglycerides were used. Mitochondria are inactive, either alone or supplemented with the supernatant protein. The original supernatant material (S_3) possesses very low activity, but significant incorporation of label into the lipid can still be obtainable with this fraction.

The overall results may be explained by the fact that the PE:CTP cytidylyltransferase activity (E.C. 2.7.7.14) is partially released by the microsomes into the supernatant fraction, when homogenates are prepared in 0.32 M sucrose or in other hypertonic media (3,41-43), whereas the PE:diacyl glycerol phosphotransferase (E.C. 2.7.8.2) is a microsomal enzyme (3,8,42).

A slight loss of activity occurs during the preparation of an active fraction of the supernatant by ammonium sulfate precipitation at 25-38% of saturation (Table I). While storage of the microsomes at -20 C for a few hours does not alter the incorporation rate, incorporation markedly decreases after 24 hr at 0-4 C. To the contrary, storage of the supernatant up to four days at 0-4 C does not lead to decreased incorporation rates. These results suggest that the phosphotransferase, a microsomal enzyme (3,8,42), has a labile protein component.

When membrane components of microsomes are separated from their content by deoxycholate treatment, no activity is found either in the membrane and ribosome pellets or in the solubilized part of the membranes present in the supernatant fraction, probably because of inactivation produced by the deoxycholate treatment. Removal of RNA was without effect on the rate of PE incorporation into lipid.

From the results reported in this section it

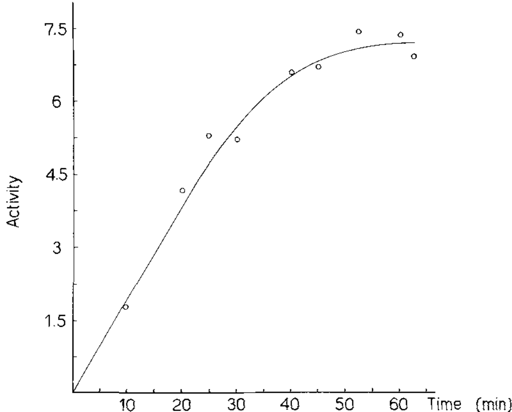

FIG. 1. The effect of the time of incubation on the synthesis of ethanolamine phosphoglycerides from labeled phosphorylethanolamine by chicken brain microsomes, supplemented by the supernatant fraction. Incubation with 10 mM diglyceride from soybean lecithin was carried out as reported in Table II. Activity expressed in mμmoles labeled lipid per milligram protein.

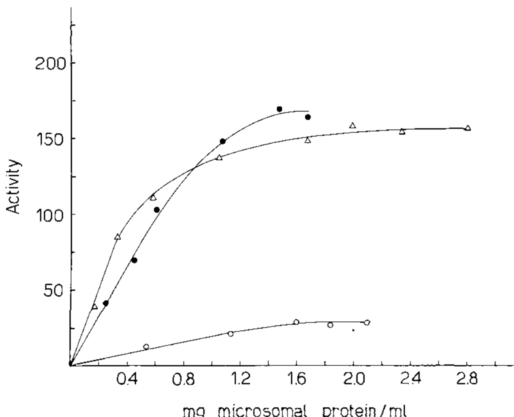

FIG. 2. The effect of protein concentration on the enzymic conversion of phosphorylethanolamine to ethanolamine phosphoglycerides by chicken brain particles in vitro. Incubation with 6 mM diglyceride from yeast lecithin was carried out for 30 min at 37 C, as described in the text. Fractionated supernatant fraction and pure microsomal pellets were used. Total lipid was extracted and counted as described in the text. Activity expressed in μμmoles labeled phospholipid per minute. O-O-O-O-, microsomes only; ●-●-●-●-, microsomes supplemented with the fractionated supernatant protein (1 mg of microsomal protein per milliliter equals to 0.35 mg of fractionated supernatant protein per milliliter; Δ-Δ-Δ-Δ-Δ-Δ-, increasing concentrations of the microsomal protein. Fractionated supernatant protein corresponded to 0.6 mg protein per milliliter of the incubation mixture.

appears that the rate of PE incorporation into lipid by the microsomal fraction supplemented with its supernatant results in only 0.25% incorporation after 30 min of incubation, and that incorporation displayed by microsomes alone is very feeble (0.03-0.05%). These results are comparable to previous data obtained with brain homogenates and slices (2,7,44,45) and with particulate brain fractions (3).

Properties of the Enzymic System

Similar enzymic activities are obtained when different diglyceride preparations are tested at various concentrations (Table II). Diglycerides prepared from natural sources are more active. Increased concentrations of 1,2-diacyl glycerol greater than 2 mM do not result in an increased formation of lipid.

The reaction proceeds linearly with time up to 20-30 min of incubation at 37 C (Fig. 1), either with microsomes alone or with microsomes supplemented with the fractionated supernatant material. Synthesis of lipid is proportional to the amount of enzymic protein up to about 1.6 mg of microsomal protein per milliliter, when microsomes alone are used (Fig. 2). By using microsomes supplemented with the fractionated supernatant, and by concurrently increasing their concentrations, a good correlation is apparently evident only up to 50 mg of correspondent fresh brain tissue, i.e., up to 0.5-0.6 mg of microsomal protein per milliliter and up to 0.20 mg of supernatant protein per milliliter. Increasing the concentration of microsomal protein, without varying the amounts of the added supernatant fraction, does not result in linear increase of activity (Fig. 2). The incorporation rate is satisfactorily proportional to the amounts of the supernatant protein, when the concentration of microsomes is kept constant (not shown in the Figure).

Better correlation between enzyme concentration and activity has been consistently found when natural diglycerides are used in place of the synthetic acceptors.

It has been impossible to calculate Michaelis constant for PE or CTP, or both, since the incorporation of PE into EPG results from the combined action of two activities both present in the enzymic system. However, though keeping in mind these limitations, it is possible to obtain values of increased incorporation of PE into EPG at increasing concentrations of PE, by using a high concentration of CTP (4 mM), and an initial level of 0.57 mM PE (CTP/PE ratio of 7:1); under these conditions, the apparent K_m of PE with the use of microsomes and supernatant fraction is 8×10^{-4} M.

The yield of PE incorporation into EPG increases slightly if the levels of CTP are raised

TABLE III

The Effect of Adding Various Concentrations of CTP and PE on the Rate of Conversion of PE to Ethanolamine Phosphoglycerides by Brain Tissue in Vitro[a]

Ratio of			Per cent of activity[a]	
CTP	to	PE	b	c
10		1	107	66
5		1	101	88
3		1	130	120
2		1	100	100
1		1	80	76
1		2.5	32	0
1		6	17	0
-		2[d]	0	0

[a]Incubation was carried out for 30 min at 37 C as described in the text, by using soybean diglyceride (6 mM), as lipid acceptor. Lipids were extracted and counted as reported in the text. The per cent of activity is referred to values obtained with the standard incubation mixture (PE, 2 mM; CTP, 4 mM).

[b]Incubation with microsomes + fractionated supernatant protein.

[c]Incubation with microsomes only.

[d]Millimolar concentration of PE.

(Table III). By decreasing the molar ratio of CTP/PE from 1:1 to 1:2.5 or less, the conversion of PE to EPG drops to lower values, and no longer takes place when only microsomes are used. The best CTP/PE ratio to obtain maximal values of PE incorporation into EPG is apparently 3:1, but in no case does the conversion of PE to EPG appear to be higher than 0.32-0.33% with the combined microsomal and supernatant enzymic system. The incorporation into EPG decreases by 40-50% by incubating CTP and PE (3:1) in the presence of unlabeled PC, this reduction probably being due to the removal of CTP.

A broad optimum pH, lying between 6.5 and 7.8, has been found for the conversion of PE to EPG. Very low or undetectable conversion occurs at lower and higher pH values.

No incorporation of PE into EPG occurs in the absence of magnesium chloride; the optimum concentration of the Mg^{2+} is 20 mM. Mn^{2+} can replace Mg^{2+}, although less efficiently at all concentrations. When Mn^{2+} is added to a Mg^{2+} -containing incubation mixture, a slight inhibition (20-30%) occurs. Calcium ions (0.5 mM), incubated with a tris-hydrochloric acid buffer (30 mM), pH 7.5, exerts a noticeable inhibitory effect (80% of inhibition), also in the presence of magnesium ions. Incubating with tris buffer (30 mM), in place of the phosphate solution, is without effect upon the enzymic conversion, as well as adding EDTA (from 0.5 to 3 mM) or sodium fluoride (up to 20 mM).

The effect of omitting from the incubation mixture various components related to the PE-dependent EPG synthesis gave the following results: (a) diglyceride, reduction by 30-40%; (b) ATP, no variations up to 30 min of incubation, with depression of rate of synthesis after that time; (c) CTP, complete reduction (Table III); (d) cysteine, no change. Supplementing, either separately or together, the standard incubation system with 2 to 10 mM PEP, sodium malate (2 mM), NAD (0.5 mM), cytochrome *c* (10 μM), AMP (2 mM) and CMP (from 1 to 3 mM) does not increase the enzymic activity. By replacing PEP with PGA (16 mM), a higher activity occurs in the presence of suitable concentrations of CTP, PE and ATP, but in no case does this activity convert PE to EPG in amounts greater than 0.35%. Cytidine 5′-diphosphate choline (0.3 to 1 mM) produces a 25% inhibition.

Identification of the Labeled Lipid

Determinations have been made of the EPG which occur in isolated mitochondrial and microsomal fractions of the six weeks old chicken brain. It was impossible to directly measure the amounts of the separated glyceryl ether form of ethanolamine lipid (alkyl acyl GPE), owing to the very low content of this lipid, but quantitative evidence for the presence of this lipid in the brain particles was obtained by determining the total P content of the last lower phase lipids after alkaline and acid hydrolysis (33,36), and by subtracting from this value the sphingomyelin P (determined by analysis of the intact phospholipid classes). The corrected levels of the glyceryl ether P, which is almost totally representative in brain of alkyl acyl GPE (36,46), are reported in Table IV, together with the levels of diacyl GPE and alkenyl acyl GPE. The absolute amounts of each EPG agree with those reported elsewhere (9,47).

When microsomal EPG were analyzed after a standard incubation with labeled PE (Table V), nearly 85% of the total radioactivity of the lipid extract was shown to possess an R_f value identical to authentic diacyl GPE. Treatment of such extract with methanolic potassium hydroxide (33,36) rendered water-soluble more than 95% of this radioactivity. Small amounts of labeled monoacyl-GPE were often detected after incubation. This lipid was identified by use of chromatography and cochromatography with labeled and unlabeled marker; a spot, of R_f value of 0.31, identical with that of the reference sample, was found in a chloroform-methanol-15 N ammonia (100:50:12 v/v/v) solvent.

TABLE IV

The Distribution of Ethanolamine Phosphoglycerides in Microsomal and Mitochondrial Fractions of Six Weeks Old Chicken Brains[a]

Lipid	Mitochondria	Microsomes
Diacyl GPE	0.17 ± 0.05 (7)	0.20 ± 0.06 (7)
Alkenyl acyl GPE	0.13 ± 0.04 (7)	0.25 ± 0.10 (7), 0.28[b] (6)
Alkyl acyl GPE[c]	0.022 (4)	0.030 (4)
Total EPG	0.32	0.48

[a]Values are reported as μmoles P/150 mg of original brain wet weight ± S.D. The protein content of the correspondent mitochondrial and microsomal fractions is 1.1-1.2 and 1.0-1.1 mg/150 mg original fresh tissue, respectively. Number of experiments in brackets.

[b]Spectrophotometric iodine addition method (41,42).

[c]The value of the alkyl acyl GPE content may not be accurate (see text). Owing to the small number of estimations carried out, no standard deviation values are reported.

No labeled alkenyl acyl GPE is formed in brain microsomes upon incubation in the standard incubation mixture (Table V). However, when the enzymic sources were incubated in the presence of suitable amounts of 1-alkenyl 2-acyl glycerol, an additional labeled faint spot, separated from that of diacyl GPE (37), was observed. R_f values of 0.30 and 0.38, identical with those of reference markers, were obtained respectively with the solvent systems chloroform-methanol-15 N ammonia (100:50:12 v/v/v) and chloroform-methanol-15 N ammonia (130:5:8 v/v/v). By adding 2 μmoles of cold ethanolamine plasmalogen, prior to the TLC, and by processing the sample as usual (37), the SA of the isolated plasmalogen spot decreased to less than 0.01 mμmoles/μg of P. Following mild acid hydrolysis with TCA-mercuric chloride solution (33,36), a feeble but definite spot was detected following radioautography, which had similar chromatographic properties to that of authentic GPE. The labeled spot might therefore be that of ethanolamine plasmalogen, which is formed following incubation of the brain microsomes with labeled PE, if the proper diglyceride is added. The radioactivity of the plasmalogen is roughly one sixth of that of the diacyl GPE (Table V), either in the presence or in the absence of DL-1,2-diolein. Conversely, the same radioactivity is present in the diacyl GPE, in the presence or in the absence of the plasmalogenic diglyceride.

Labeling has never been observed in the acid-stable fraction of the lipid extract, after incubation of microsomes with PE.

TABLE V

The Distribution of Radioactivity Into Ethanolamine Lipids of Hen Brain Microsomes After Incubation With Labeled ^{14}C-PE in Vitro[a]

Lipid	mμmoles/mg protein	mμmoles/μg P
Total lipid	3.24	---
Diacyl GPE[b]	2.76	0.24
Monoacyl GPE[b,c]	0.36	---
Diacyl GPE[d]	3.10	0.25
Alkenyl acyl GPE[b]	0	---
Alkenyl acyl GPE[b,e]	0.48	0.04
Alkenyl acyl GPE[b,f,e]	0.45	---
Diacyl GPE[b,e]	2.94	0.24
Alkyl acyl GPE[g]	0	---

[a]Microsomes (2.05 mg protein) were incubated for 30 min in the presence of the fractionated supernatant (0.75 mg protein) in the standard incubation system reported in the text and in Table I. DL-1,2-diolein (2 mM) was used as diglyceride.

[b]Labeling was determined following separation of the intact lipid classes.

[c]Not consistently found. The radioactivity assay is only indicative.

[d]Labeling was determined after hydrolytic procedures.

[e]Addition has been made to the incubation mixture of 1-alkenyl 2-acyl glycerol (2 mM).

[f]DL-1,2-diolein omitted.

[g]See text and Table IV.

Studies on the Activation of the Enzymic System

Experiments have been performed in order to find out whether the small production of EPG from PE could be stimulated by adding separately to the standard incubation system the following materials: a preincubated and boiled (O_2, 37 C, from 1 to 8 hr) brain microsomal or mitochondrial suspension, a similarly treated supernatant fraction, and various preparations of diacyl GPC, monoacyl GPC and monoacyl GPE. In no case has activation been observed. On the contrary, the addition of a preincubated mitochondrial fraction consistently lowers the rate of the incorporation of PE into EPG by the combined microsomal fraction. The addition of diacyl GPE (from 0.02 to 0.20 μmoles P) also results in decreased incorporation rates.

DISCUSSION

The main purpose of the present contribution was to find out whether brain particles or particle-free supernatant fractions might carry out efficient synthesis of EPG directly from PE, when supplemented with the appropriate cofactors and lipid acceptors. The microsomal membranes of chicken brain can carry out this synthesis, although at an extremely low rate (0.03-0.05% based on PE). Supplementing the microsomes with an enzymic fraction derived from a fractionated supernatant results in an increased rate of synthesis, which under standard conditions is however still low (0.25% conversion). When these results are compared with the high yield of EPG formed from cytidine-5'-diphosphate ethanolamine (CDPE) and lipid acceptors by brain and liver tissues in vitro (8,44,48,49), it appears that the PE/CTP cytidylyltransferase, which converts PE to its cytidine derivative, must display a very low activity in vitro, thus limiting the overall rate of synthesis of EPG from PE. Low incorporation of PE into EPG has also been found in experiments with brain homogenates and slices (2,7,44,45).

This reaction, which appears to be rate limiting, may control the biosynthetic pathway in vivo, since the great potentiality of the next successive step, catalyzed by the PE:diacyl glycerol phosphotransferase which transfers the CDPE to the lipid acceptor, cannot be expressed. These conditions are supported by the findings of the present contribution. Thus, no increase occurs in the rate of EPG synthesis, if the concentration of the diglyceride is increased up to fivefold in the standard incubation mixture, or if use is made of natural diglycerides, which are known to be better acceptors of the cytidine derivatives than the synthetic analogues (49,50). Clearly the lack of exogenous diglycerides to increase the rate of synthesis of EPG from PE is due to poor formation of CDPE. Similarly, supplementing the incubation mixture with cofactors known to constitute an external energy-producing system (oxidative or glycolytic phosphorylation reactions), or with intermediates of lipid synthesis, is without effect in stimulating the overall rate of synthesis. The demonstration first made by Fiscus and Schneider (29) on the activation of CDPC synthesis by phospholipid does not apply to the CDPE synthesis by brain microsomes. This result contrasts with the known stimulation which operates on the CTP/PC cytidylyltransferase and which presumably may influence and control the ultimate formation of lecithin. A detailed study of the possible stimulation and regulation of the synthesis of CDPE from PE seems therefore an important problem to investigate, in relation to the overall rate of synthesis of EPG.

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The Labeling of Brain Ethanolamine Phosphoglycerides From Cytidine Diphosphate Ethanolamine in Vitro

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ABSTRACT

Chicken brain microsomes convert ^{14}C-ethanolamine-labeled cytidine diphosphate ethanolamine (CDPE) to ethanolamine lipids (EPG) at a measurable rate. Mitochondria and particle-free supernatant fractions are almost inactive. On adding saturating amounts of diacyl glycerol to the microsomes, formation of EPG increases 10-fold, and is almost totally confined to the diacyl *sn*-glycero-3-phosphorylethanolamine (GPE), whose synthesis increases 20-fold (from 13.1 to 249 mμmoles/mg protein/hr). The addition of the 1-alkenyl 2-acyl glycerol also produces a noticeable increase in the rate of EPG labeling, which is almost exclusively confined to the alkenyl acyl GPE, whose synthesis is stimulated 30-fold (from 3.1 to 90 mμmoles/mg protein/hr). Synthesis of alkyl acyl GPE from CDPE was not detected in the brain microsomes. Synthesis of EPG from CDPE by chicken brain homogenates has also been examined and results similar to those reported for the brain microsomes were obtained. In addition, small amounts of labeled alkyl acyl GPE are produced from CDPE in the homogenates, but in no case does this synthesis increase on adding various lipid acceptors to the incubation system. Various properties of the phosphorylethanolamine-diacyl glycerol phosphotransferase (E.C. 2.7.8.1) of brain microsomes were examined. It is concluded that the enzymic activities which convert CDPE to diacyl GPE and alkenyl acyl GPE, respectively, are similar.

INTRODUCTION

It is known that following the intracerebral injection of ^{14}C-ethanolamine into rats, the base is rapidly converted to phosphorylethanolamine (PE), transformed into cytidine 5′-diphosphate ethanolamine (CDPE), and then incorporated into the ethanolamine phosphoglycerides (EPG) of all subcellular fractions (1). This mechanism operates in vitro, both in liver (2) and brain (3-5), being presumably involved for the production of both diacyl *sn*-glycero-3-phosphorylethanolamine (GPE) and alkenyl acyl GPE (1,3,5). While the precursor-product relationship of CDPE and diacyl GPE has been demonstrated (3,5), the exact nature of the immediate precursor of alkenyl acyl GPE is not known (6,7).

It is the purpose of the present investigation to produce experimental evidence on the enzymic reaction which transfers labeled CDPE to lipid acceptors in brain, to examine the possible relationships among the syntheses of different EPG, and the subcellular distribution of the transferase enzyme. Preliminary data have been provided recently regarding this last problem (8).

EXPERIMENTAL PROCEDURES

Biological Materials

The preparation of purified mitochondria and microsomes from chicken brain, the separation of the microsomal components and of ribosome-free microsomes, and the analyses performed on some enzymic markers were carried out as described in the previous work (9).

Chemicals and Labeled Substrates

Materials. 1,2-Diacyl glycerols and 1-alkenyl 2-acyl glycerol were prepared and successively emulsified, as described elsewhere (9). The 1-alkenyl 2-acyl glycerol was assayed for the aldehyde-ester ratio (9), which was shown to be 0.85. No attempt was made to calculate its fatty acid composition. Synthetic glyceryl diether compounds (α,β-dihexadecyl glycerol and α,β-dioctadecyl glycerol) were prepared according to Kates et al. (10). Very unstable preparations were obtained on shaking these compounds, at the liquid state, with Tween-20 (0.1%). However, more stable preparations were attained, immediately before addition to the incubation mixture, when the diethers were emulsified in Tween-20 and bovine plasma albumin (0.05%), and stirred at top speed for 1 min in a VirTis microhomogenizer.

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The samples of diacyl GPE, 1-acyl GPE, 1-acyl *sn*-glycero-3-phosphorylcholine (GPC) and alkenyl acyl GPE were obtained as described elsewhere (9). Unlabeled CDPE was a product of Sigma Chemical Co. (St. Louis, Mo), and aluminum oxide of British Drug Houses, Ltd. (Poole, Dorset, England).

Labeled Substrates. 1,2-^{14}C-Ethanolamine-labeled-CDPE was prepared enzymically from labeled PE (9) and suitable amounts of cytidine-5′-triphosphate (CTP) (11), separated and purified by chromatography on Dowex-1 (formate) column. No labeled free ethanolamine or PE were found in the radioactive product. 1,2-^{14}C-Ethanolamine-labeled-deoxycytidine diphosphate ethanolamine (*d*CDPE) specific activity (SA) between 50 and 80 μc/mmole was chemically synthesized (2); the yield was of 12%. Other radioactive compounds were prepared as reported previously (9).

Incubation

For investigation of the PE-diacyl glycerol phosphotransferase (E.C.2.7.8.1), standard incubation mixtures normally contained the following components, at the indicated final concentrations: diacyl glycerol (12 mM), dissolved in Tween-20 (0.01%) and bovine plasma albumin (0.005%); tris-hydrochloric acid buffer (50 mM), pH 7.90; cysteine (12 mM); CDPE (1.0 mM) of SA between 180 and 240 μc/mmole; brain microsomes (mitochondria), corresponding to 0.15-0.20 mg of protein N (about 120-150 mg of original fresh tissue); magnesium chloride (12 mM). The final volume was 0.8 ml and the pH (calculated at the end of the incubation) was 7.8-7.9. Operations were carried out at +4 C, enzymes and other components being added at the indicated order. When specified, 1-alkenyl 2-acyl glycerol or glycerol diethers were used in place of the diacyl glycerols. The incubation mixtures were shaken into heavy wall pointed and stoppered tubes for 30 min at 38 C in a thermo-regulated water bath, at about 80 strokes per minute. The reaction was then stopped (9), and the mixture treated as described in the sections which follow.

Analytical Methods

Total Lipid. Radioactivity and P content of the total lipid fraction was determined as reported elsewhere (9).

Phospholipid Analyses. Hydrolytic procedures were carried out, as described elsewhere (9), on the lipid extract of the incubation mixture performed on twice the scale previously indicated.

Phospholipid classes were analyzed as previously described (9,12); both acid-labile and acid-stable EPG were found to occur in brain mitochondria and microsomes (9). Free GPE was never detected following the thin layer chromatography (TLC) procedure (12), indicating that no dialk-1-enyl GPE presumably occurs in the brain particles.

Alkyl acyl GPE was quantitatively determined by two procedures. In the first case, radioactivity counts and P determinations were carried out on the last lower phase lipids obtained after alkaline and acid hydrolyses, as explained previously (9). From the P content of this phase, sphingomyelin P (determined by analysis of the intact phospholipid classes) was subtracted, in order to have the corrected values of the glyceryl ether P content. No radioactivity was found in this sphingomyelin fraction (Alternatively the alkyl acyl GPE content was determined by treating the mild acid- and alkali-stable lipids with water-free methanolic 2 N hydrochloric acid for 4 hr at 100 C (13), and by determining the P and the radioactivity in the purified, glyceryl ether-containing ether extract (13). Comparable results were obtained with this procedure). In the second instance, alkyl acyl GPE was determined following separation of the phospholipid classes (9), as suggested by Horrocks (12,14). More precisely, the original lipid extract (about 2.5 μmoles of lipid P) was chromatographed in duplicate using about 1 μmole of lipid P for each assay; the labeled areas of acid-stable EPG and acid-labile EPG were then scraped off from the plates, saponified with a mild alkaline hydrolysis procedure (13) and equilibrated with Dawson's solvent (15). The lower phase was then assayed for radioactivity and P content. The acid-labile EPG spots were found to be fully saponified, indicating the absence of alkenyl alkyl GPE lipids in the brain particles.

Column Chromatography. As an alternate way to identify and isolate EPG from the incubation mixture, a column chromatographic method was adopted. After incubation carried out on three times the scale previously indicated, the lipid extract (about 4 μmoles of P) was dissolved in chloroform-methanol (1:1 v/v), and applied to a small column (0.8 x 5.0 cm) of aluminum oxide. The EPG were separated from the bulk of the other lipids with chloroform-methanol (1:1 v/v), containing increasing amounts of water (16). Radioactive EPG were eluted between 7% and 13% of water, and found free from other lipids, as judged by the TLC of the fractions. Only small amounts of unlabeled inositol-containing lipids were occasionally present. The SA of the various

TABLE I

The PE Diglyceride Phosphotransferase Activity in Subcellular Fractions of Chicken Brain[a]

Fraction	Protein, mg/g	Activity b	Activity c	Activity d
Homogenate	87	449	5.2	(1.00)
Mitochondrial	9.0	17	1.9	0.37
Microsomal	8.0	251	31.3	6.02
Microsomal[e]	7.8	22	2.8	---
Supernatant	19	34	1.8	0.34
Microsomal[f]	8.0	267	33.4	6.42
Microsomal[g]	8.0	40	5.0	---
Microsomal[h]	6.5	214	32.0	6.15
Soluble microsomal supernatant[i]	3.1	0	---	---
Insoluble microsomal pellets[i]	5.2	21	4.0	---

[a]Microsomes (0.9-1.2 mg protein), mitochondria (1.0-1.4 mg protein) and supernatant (3.0 mg protein) were incubated for 30 min at 38 C in the standard incubation mixture described in the text. Lipid acceptors were omitted. After incubation, radioactivity into the total lipid fraction was estimated as described in the text.

[b]Mμmoles of labeled phospholipid/gram fresh tissue weight/hour.

[c]Mμmoles/milligram protein/hour.

[d]SA of each fraction relative to the SA of the original homogenate.

[e]*d*CDPE (1.0 mM), SA of 67 μc/mmole, was incubated in place of CDPE.

[f]Stored at -20 C for 4 hr.

[g]Stored at 0-4 C for 24 hr.

[h]Treated with 0.05 M sodium pyrophosphate. For details, see Reference 9.

[i]Treated with 0.26% sodium deoxycholate. For details, see Reference 9.

EPG was then directly determined by the procedures reported previously. No other phosphorous containing component was present among the different types of EPG.

RESULTS

Intracellular Distribution

The determination of the PE-diacyl glycerol phosphotransferase activity has been carried out in the mitochondrial, microsomal and soluble fractions of chicken brain (Table I). Microsomes contained by far the highest portion of the enzymic activity of the homogenate (about 55%), both in terms of fresh tissue and protein content, whereas only about 4% was detected in the mitochondria. The soluble fraction also contained some activity. In terms of SA, the microsomes displayed a sixfold increase of activity as compared to that of the original homogenate, whereas values of 0.37 and 0.34 were obtained for mitochondrial and soluble fractions, respectively. About 30% of the original activity was not accounted for, following fractionation. This may be due to residual activity of the nuclear, synaptosomal and myelin fractions, which have not been considered in this study.

As shown in Table I, storage of the microsomes at -20 C for a few hours did not change the enzymic activity, which nearly disappears (16% of the original activity retained) after 24 hr at 0-4 C. Removal of RNA from the isolated fraction (9) was without effect on the rate of CDPE incorporation into lipid, whereas treating with deoxycholate (9), for separation of the membrane components, resulted in decreased enzymic activities both in the membrane and ribosome pellets and in the solubilized part of the membranes. When the microsomes were incubated with *d*CDPE in place of CDPE, much lower conversion of this nucleotide into total lipid occurred. This last result confirms previous findings (17,18).

Comparative Incorporation of PE and CDPE Into Microsomal Lipid

The percentage of conversion of PE into microsomal EPG is small (9), when compared to that of CDPE (this study). Under optimal conditions, i.e., with natural lipid acceptors added to the incubation system, a maximum of 0.40-0.45% of PE is incorporated into the microsomal EPG of 100 mg of brain tissue per hour, whereas about 3% of the CDPE is incorporated into the same amount of microsomal EPG/hr, without the addition of exogenous diacyl glycerols. When expressed in terms of unitary figures, i.e., as μmoles EPG/microsomes/gram wet weight/hour, these percentages

become 0.06% and 0.25%, respectively. If the brain microsomes are incubated with CDPE in the presence of suitable concentrations of diacyl glycerols, these differences become even larger, as reported in the following sections.

Identification of the Labeled Lipids

Identification has been made of the lipids of brain homogenates and microsomes, which became labeled in vitro from CDPE without the addition of lipid acceptors.

Table II shows that lipid labeling from CDPE in brain microsomes is not restricted to diacyl GPE and that in the absence of exogenous lipid acceptors labeling of alkenyl acyl GPE also takes place. More precisely, about 40-42% of the incorporated radioactivity (4% of the label of CDPE) was in the diacyl GPE, 10% in the alkenyl acyl GPE and about 3% in the lysoderivative of diacyl GPE. This last lipid was identified by use of chromatography and cochromatography with labeled and unlabeled marker, as reported elsewhere (9). No radioactivity was detected in the alkyl acyl GPE. No microsomal lipids other than EPG were found labeled after incubation with CDPE.

Incorporation of CDPE into individual EPG has been followed in brain homogenates (80 mg wet weight) incubated for 30 min in the absence of lipid acceptors (Table III,b). About 5-6% of the radioactivity was incorporated into the phospholipid of 100 mg tissue (45 mμmoles/100 mg tissue/hour). Of this radioactivity, 45% (21 mμmoles) was in the diacyl GPE, 31% (14 mμmoles) in the alkenyl acyl GPE, 9% (4 mμmoles) in the lysoderivative form of EPG, and about 4% (2 mμmoles) in the alkyl acyl GPE. These results confirm previous data (5,19), but are at variance with others (3). Identification of the alkyl acyl GPE, in the experiments carried out with the brain homogenates, has been obtained by means of reported procedures (12,14). More precisely, the lower phase, obtained after the saponification of the separated acid-stable and acid-labile EPG, was analyzed for P and radioactivity; a total amount of about 1.1 μmole P and of about 600 cpm was found. In another experiment the total P content and radioactivity of the last lower phase lipids were determined after alkaline and acid hydrolyses (13,15), and the sphingomyelin P value subtracted (see Phospholipid Analyses); levels of 0.96 μmole and 641 cpm were found for the alkyl acyl P fraction. By adding to the incubation mixture 5 μmoles of cold 1-hexadecyl 2-stearoyl GPE, prior to the TLC procedure, and by processing the sample as usual (12,14), the SA of the alkyl acyl GPE dropped from a value of 0.32 μc/mmole to about 0.05. From these results it is concluded that the acid-stable and alkali-stable lipid, labeled in the brain homogenate from CDPE, is probably alkyl acyl GPE. It is not excluded, however, that a dialkyl form could also become labeled in these conditions. No labeling in alkenyl alkyl forms of EPG was detected (see Experimental Procedures).

TABLE II

The Distribution of Radioactivity Into the Ethanolamine Lipids of Hen Brain Microsomes Following Incubation With Labeled Cytidine Diphosphate Ethanolamine in Vitro[a]

Lipid	mμmoles/mg protein	mμmoles/μg P
Total lipid	30.9	---
Diacyl GPE[b]	13.1	1.08
Monoacyl GPE	< 1	---
Diacyl GPE[c]	12.2	0.96
Alkenyl acyl GPE	3.1	0.20
Alkyl acyl GPE	0	---

[a]Microsomes (about 2.0 mg protein), corresponding to about 230-240 mg of original fresh tissue, were incubated for 30 min at 38 C in the standard incubation mixture reported in the text. Lipid acceptors were omitted. After incubation, the lipid classes were separated, identified and determined, as explained in a previous work (9), and in the text.

[b]Labeling of this lipid was determined following separation of the intact lipid classes (9).

[c]Labeling of this lipid was determined after hydrolytic procedures (9).

The Incorporation of CDPE Into the Lipid of Brain Homogenates in the Presence of Lipid Acceptors

Experiments have been performed to examine in brain homogenates what the quantitative and qualitative labeling of EPG would be in the presence of different acyl glycerols. Table III shows that CDPE incorporation is stimulated fivefold by the addition of 12 mM diacyl glycerol (diacyl GPC, diacyl 3-glycerophosphate, triacyl glycerol, Tween-20 and octanol cannot be substituted for the diacyl glycerol in this action; in addition, these compounds were unable to increase the activity of the enzyme in the presence of diacyl glycerol); and about threefold by the addition of 12 mM 1-alkenyl 2-acyl glycerol, whereas it is unaffected by adding synthetic glyceryl diethers. The stimulation produced by the diacyl glycerol is confined to diacyl GPE, whose synthesis is stimulated about eightfold (78% of the incorporated radioactivity). Conversely, the addition of the 1-alkenyl 2-acyl glycerol brings about a sixfold stimulation only of the ethanolamine plasmalogen synthesis (68% of the incorporated radioactivity). Under

TABLE III

The Effect of Adding Diglycerides on Enzyme Activity of Chicken Brain Homogenates[a]

Synthesized lipid	Lipid acceptor added			
	b	c	d	e
Total EPG lipid	45	220	130	48
Diacyl GPE	21 (45)	171 (78)	26	23
Alkenyl acyl GPE	14 (31)	16 (7)	88 (68)	15
Alkyl acyl GPE	2 (4)	2 (1)	2	2 2[f]
Monoacyl GPE	4 (9)	12 (6)	6 (5)	3

[a]Brain homogenates (80 mg wet weight) were incubated for 30 min at 38 C in the standard incubation mixture reported in the text (see Incubation). Lipid acceptors were added, as indicated. After incubation, the lipid classes were separated, identified and determined, as reported in Table II. Values are reported as mμmoles lipid/100 mg wet weight tissue/hour. Per cent of the total radioactivity incorporated is shown in brackets.

[b]No lipid acceptor added.

[c]Diacyl glycerol (12 mM) from soybean lecithin, dissolved in Tween-20 and bovine serum albumin, was added to the incubation mixture.

[d]1-Alkenyl 2-acyl glycerol (12 mM) from beef heart lecithin was added, as above.

[e]Synthetic α,β-dioctadecyl glycerol (4 mM) added.

[f]Synthetic α,β-dihexadecyl glycerol (3 mM) added.

optimal diglyceride concentrations, the ratio of synthesized diacyl GPE to alkenyl acyl GPE is 2:1 (Table III). No increase of alkyl acyl GPE synthesis takes place on adding synthetic glyceryl diethers.

The Incorporation of CDPE Into the Lipid of Brain Subcellular Fractions in the Presence of Lipid Acceptors

The effect of adding saturating concentrations of diacyl glycerols has been examined in brain subcellular fractions and particle-free supernatant (Table IV), in order to compare the results with those obtained in the absence of lipid acceptors (Tables I and II). Activity increases considerably in the whole homogenate (Table III), with a parallel fivefold increase in the mitochondrial fraction (from 1.9 mμmoles to 9.2 mμmoles/milligram protein/hour. Activity of the supernatant fraction is unchanged. The greatest increase is shown by the microsomes. Labeling in the total lipid fraction (306 mμmoles/milligram protein/hour) is in fact 10-fold increased from the basal value of 31.3 mμmoles (Table I).

The increase of labeling of the microsomal lipid is confined chiefly to diacyl GPE, whose synthesis reaches a 20-fold stimulation on adding the diacyl glycerol (from 13.1 to 249 mμmoles/milligram protein/hour), whereas the rate of synthesis of the ethanolamine plasmalogen is not changed (Tables II and IV). Formation of the alkyl acyl GPE does not occur in the microsomes, either in the presence or in the absence of lipid acceptors (Tables II and IV).

When CDPE is substituted by equal amounts of the *d*CDPE, little labeling of the total lipid occurs, even in the presence of added diglycerides.

Natural diglycerides, such as those prepared from yeast or soybean lecithins, are the most suitable acceptors for diacyl GPE synthesis, when compared with the synthetic diglycerides (Table IV).

When the brain particles and supernatant fraction were incubated in the presence of 12 mM 1-alkenyl 2-acyl glycerol, the distribution of activity was comparable to the results obtained when diacyl glycerol was added (Table V). Again the microsomes showed a large increase in the rate of lipid labeling, as compared to other fractions. The increase was confined to the plasmalogen, while incorporation into diacyl GPE did not increase. The formation of the alkenyl acyl GPE in the microsomes raised in fact from values of 3.1 (Table II) to 90 mμmoles/milligram protein/hour (5.5 mμmoles/μg P), with a 30-fold increase. Labeling of alkyl acyl GPE did not occur in these conditions.

Therefore, under optimal diglyceride concentrations, the ratio of synthesized diacyl GPE to alkenyl acyl GPE within microsomes reaches a value of 249:90, i.e., of about 3:1, whereas in the absence of exogenous acceptors, this ratio is of 13.1:3.1 (Table II), i.e., of about 4:1.

To test whether synthesis of the alkyl acyl GPE would occur in the brain microsomes in the presence of other suitable lipid acceptors, incubation has been carried out of these particles with 2.0-8.0 mM of the synthetic glyceryl diethers. EPG were separated on the alumina

TABLE IV

The Effect of Adding Diacyl Glycerol on Enzyme Activity of Chicken Brain Subcellular Fractions[a]

Fraction	Lipid	Activity b	c	d	e
Homogenate	Total lipid	2,120	25.2	(1.00)	---
Mitochondrial	Total lipid	84	9.2	0.36	---
Microsomal	Total lipid	2,480	306	12.1	=-
Supernatant	Total lipid	42	2.2	0.09	---
Microsomal	Diacyl GPE	2,079	249	---	20.7
Microsomal	Alkenyl acyl GPE	31	3.7	---	0.23
Microsomal	Alkyl acyl GPE	0	---	---	---
Microsomal[f]	Diacyl GPE	1,791	224	---	18.1
Microsomal[g]	Diacyl GPE	821	101	---	8.0
Microsomal[h]	Diacyl GPE	613	76	---	6.0
Microsomal[i]	Diacyl GPE	660	82	---	6.5
Microsomal[j]	Total lipid	301	35.8	---	---

[a]Microsomes (0.8-1.2 mg protein), mitochondria (1.0-1.4 mg) and supernatant (3.0 mg protein) were incubated for 30 min at 38 C in the standard incubation mixture, supplemented with diacyl glycerol of soybean lecithin (12 mM), added as reported in Table III. After incubation, total lipid or lipid classes were determined as described in text and in Table II.

[b]Mμmoles of labeled phospholipid/gram fresh weight tissue/hour.

[c]Mμmoles/milligram protein/hour.

[d]SA of each fraction relative to the SA of the original homogenate (1.00).

[e]Mμmoles/μg of P.

[f]Diacyl glycerol from yeast lecithin (10 mM).

[g]DL-1,2-diolein (12 mM).

[h]D-1,2-dimyristin (12 mM).

[i]D-1,2-distearin (12 mM).

[j]dCDPE (1.0 mM), SA of 67 μc/mmole, was incubated in place of CDPE.

columns and hydrolytic procedures and TLC were carried out on the pooled peaks. In no case was synthesis of this lipid evident, even at higher concentrations of the diethers.

Characteristics of the Lipid Labeling

The radioactivity of any labeled EPG was found almost completely in the base moiety of the phospholipid, after 20-30 min of incubation. By hydrolyzing the total washed lipid extract in 6 N hydrochloric acid for 3 hr at 105 C and by analyzing this extract by chromatography on a column of Dowex 1x8 (OH-) (100-200 mesh), nearly 90% of the lipid radioactivity was recovered in the aqueous effluent of the column.

TABLE V

The Effect of Adding 1-Alkenyl 2-Acyl Glycerol on Enzyme Activity of Chicken Brain Subcellular Fractions[a]

Fraction	Lipid	Activity b	c	d
Homogenate	Total lipid	11.4	(1.00)	---
Mitochondrial	Total lipid	4.5	0.39	---
Microsomal	Total lipid	136.0	11.9	---
Supernatant	Total lipid	1.9	0.17	---
Microsomal	Diacyl GPE	14.2	---	1.12
Microsomal	Alkenyl acyl GPE	90.0	---	5.5
Microsomal	Alkyl acyl GPE	0	---	---

[a]Conditions were similar to those reported in Table IV, except that 1-alkenyl 2-acyl glycerol (12 mM) was incubated at 38 C for 30 min in the standard incubation mixture, in place of diacyl glycerol.

[b]Mμmoles/milligram protein/hour.

[c]SA of each fraction relative to the SA of the original homogenate (1.00).

[d]Mμmoles/μg of P.

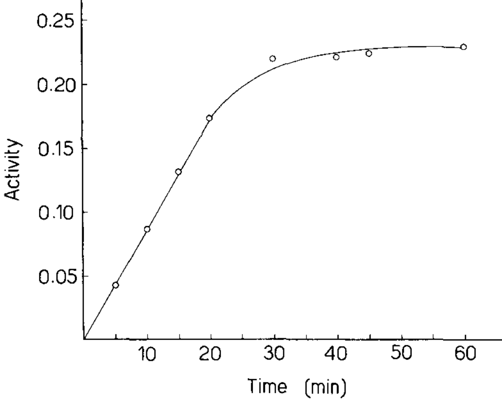

FIG. 1. The effect of the time of incubation on the synthesis of diacyl GPE from labeled cytidine diphosphate ethanolamine by chicken brain microsomes. Incubation was carried out at 38 C with the standard incubation mixture (see Incubation) at 12 mM diacyl glycerol (prepared from yeast lecithin) concentration, and with purified brain microsomes (1.07 mg of protein). The time of incubation was varied as shown. Activity expressed as μmoles diacyl GPE/milligram protein.

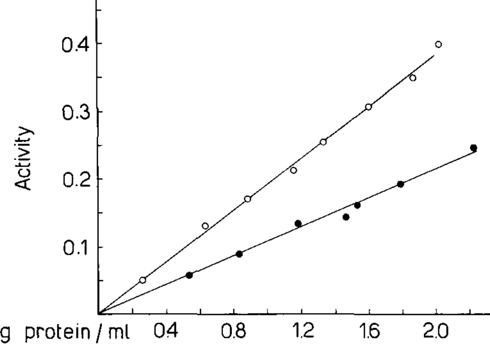

FIG. 2. The effect of protein concentrations on the enzymic conversion of cytidine diphosphate ethanolamine to diacyl GPE and alkenyl acyl GPE by chicken brain microsomes in vitro. Incubation was carried out for 30 min at 38 C with the standard incubation mixture (see incubation) at 12 mM diacyl glycerol (prepared from yeast lecithin) concentration or at 8 mM alkenyl acyl glycerol concentration, and with microsomal protein, as indicated. Activity expressed as μmoles diacyl GPE/30 min or as μmoles alkenyl acyl GPE/30 min. -○-○-○-○- incubation with diacyl glycerol; -●-●-●-●- incubation with 1-alkenyl 2-acyl glycerol.

Properties of the Microsomal Activity

Increasing the concentration of diacyl glycerol (prepared from soybean lecithin) and alkenyl acyl glycerol in the incubation mixture results in an increased formation of their respective lipids, maximum reaction rates being reached at saturating concentrations of 12 mM and 8 mM, respectively. The apparent K_m values for this type of diacyl glycerol and for the 1-alkenyl 2-acyl glycerol, with the use of microsomes, were very similar, i.e., 2.1×10^{-3} M and 2.4×10^{-3} M, respectively. Values of K_m were not different with the use of diacyl glycerol from yeast or soybean lecithins.

Synthesis of diacyl GPE from CDPE in brain microsomes was proportional to time only during the first 20 min of incubation (Fig. 1), when assayed with a protein concentration of about 1 mg/0.8 ml of final volume. During that period, formation of the product was proportional to the amount of microsomal protein over the range from 0.2 to 2.0 mg/ml, when assayed in the presence of saturating concentrations of CDPE and diacyl glycerol (Fig. 2). Comparable results were obtained by replacing diacyl glycerol with 1-alkenyl 2-acyl glycerol (Fig. 2).

When the concentration of CDPE was varied over more than a 10-fold range, the enzymic system for the microsomal synthesis of the diacyl GPE was found to be saturated at about 0.9 mM of CDPE, while concentrations of about 2.1×10^{-4} M led to synthesis of the lipid at half the maximal rate (Fig. 3). Slightly different values were obtained when synthesis of ethanolamine plasmalogen was studied in similar experimental conditions.

The optimum pH was a broad region between 7.6 and 8.3, and was similar for both diacyl and 1-alkenyl 2-acyl glycerol. The activity fell off rapidly at pH values below 7 and above 8.6.

No activity was detected in the absence of Mg^{2+}. The optimum concentration of the Mg^{2+} was 18-20 mM, with no other changes at higher values. Mn^{2+} were also effective, though less efficiently. Ca^{2+} inhibited the transferase activity by nearly 100% at 1 mM, and by 60-70% at 0.4 mM, even in the presence of 12 mM Mg^{2+}.

The omission of cysteine from the incubation system did not change the standard reaction rates. Similar effect had the addition of ATP (from 1 to 4 mM), NAD (2 mM), PE (1 mM), cytidine-5'-monophosphate (CMP) (2 mM) and CTP (2 mM). Adding cytidine diphosphate choline (CDPC) (1 mM) produced a consistent 25-30% inhibition.

DISCUSSION

The results of the present contribution show that synthesis of lipid in brain particles from CDPE is much more efficient than from labeled PE (9). The same difference between synthesis

of choline phosphoglycerides (CPG) from phosphorylcholine (PC) or directly from CDPC has been reported (2,18,21), for brain and liver tissues. Probably the enzymic conversion of PE to CDPE by the PE/CTP cytidylyltransferase activity (E.C.2.7.7.14) represents a limiting step throughout the overall synthesis of EPG in animal tissues.

The high conversion of cytidine-derivatives to lipid in vitro (2,3,5,17-19,21,22) has been confirmed. The considerable per cent of conversion of CDPE to EPG by brain homogenates, which takes place in the absence of added diglycerides, and which has been reported to range between 20-30% (5,18,19), is only apparently higher than that reported in this paper (about 6%), because in our experiments saturating CDPE concentrations have been used. Conversely, higher absolute levels of synthesized lipid have been obtained at saturating CDPE concentrations (45 mμmoles/100 mg tissue/hour) as compared to values of 10-23 mμmoles (3,5,11,18,19,21).

The pattern of labeling of the various EPG from CDPE in brain homogenates is similar to previous data (5,19), but is at variance from others reported by McMurray (3), who found a large conversion of CDPE into alkyl acyl GPE (but see 7). No other lipid was found labeled from the EPG, thus suggesting that the ethanolamine moiety does not find a way to enter in other lipid moieties, at least after 30 min of incubation.

The rate of synthesis of EPG from CDPE increases fivefold in the brain homogenates (30% of conversion) on adding optimum concentrations of diacyl glycerols (Table III). Only diacyl GPE synthesis is increased (eightfold stimulation). It is worth mentioning that synthesis of diacyl GPC in brain homogenate is much more stimulated by adding diacyl glycerol, reaching values of about 1,300-1,400 mμmoles/100 mg wet weight tissue/hour (22) from starting levels of 50 mμmoles (3,22). Therefore, under both optimal diacyl glycerol concentrations, maximal synthesis of diacyl GPE and diacyl GPC is given by 2.2 and 14.0 μmoles/gram/hour, respectively.

When 1-alkenyl 2-acyl glycerol is added to brain homogenates, in place of the diacyl glycerol, synthesis of EPG is threefold stimulated (Table III), the increase being confined only to the alkenyl acyl GPE (sixfold stimulation). Previous authors have found a twofold (3) and sevenfold (8) stimulation, under similar conditions.

Therefore, under both optimal diglyceride concentrations, the synthesis of diacyl GPE and alkenyl acyl GPE in brain homogenates reaches

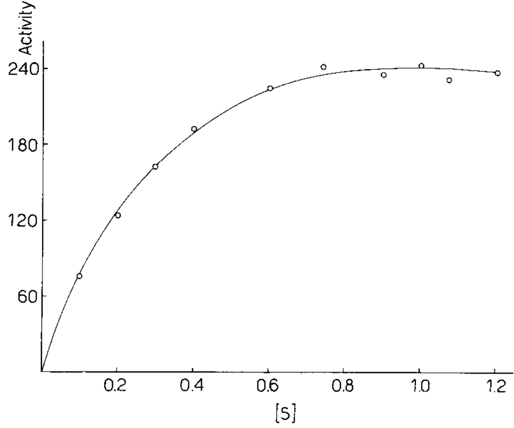

FIG. 3. The effect of cytidine diphosphate ethanolamine concentration on the synthesis of diacyl GPE by chicken brain microsomes in vitro. Each tube contained 40 μmoles of tris-hydrochloric acid buffer, pH 7.90, 9.6 μmoles of magnesium chloride, 9.6 μmoles of diacyl glycerol (from soybean lecithin), 0.08 mg of Tween-20, 9.6 μmoles of cysteine and 1.04 mg of microsomal protein; final volume of 0.8 ml. The concentration of the cytidine diphosphate ethanolamine (232 μc/mmole) was varied as shown. The tubes were incubated for 30 min at 38 C. The activity is expressed as mμmoles/milligram protein/30 min. [S] = μmoles of substrate added per 0.8 ml of reaction mixture.

SA values of 7.7 and 1.8 mμmoles/μg P, respectively (calculated from Table III). These apparently large differences become smaller, in terms of total radioactivity, if we consider that the amount of ethanolamine plasmalogen is, at least in rat brain, approximately 2.5 times that of diacyl GPE (1,5).

The subcellular distribution of the enzymic activity, tested in the absence of added diglyceride, is rather similar to that reported by Schneider (23), and that examined after the addition of diglyceride also is similar to previous data (8,22,23). In our experiments the incorporation of CDPE into total EPG of brain microsomes increases about 10-fold on adding the diacyl glycerols. Although levels of incorporations of CDPE into microsomal diacyl GPE and alkenyl acyl GPE in the absence of added diglycerides were higher than others (8), they became strictly comparable after the addition of the diglycerides. Thus, the incorporation of CDPE into microsomal diacyl GPE and alkenyl acyl GPE is stimulated 20- and 30-fold, respectively, by the addition of the lipid acceptors, reaching values of 249 and 90 mμmoles/milligram protein/hour, which compare well with the levels of 269 and 70, respectively, reported elsewhere (8). Under optimal conditions, the original microsomal amounts of

diacyl GPE (9) can therefore be more than doubled after 1 hr of incubation (from 160 to 410 mμmoles/milligram protein), and those of alkenyl acyl GPE (9) can be increased by 45% (from 215 to 305 mμmoles/milligram protein).

The enzymic activity which transfers the PE from CDPE to the plasmalogenic diglyceride seems to possess the same properties of that which catalyzes the transfer to the diacyl glycerol. Thus, both have similar distribution patterns in the subcellular fractions, either in the presence or in the absence of the lipid acceptors. In addition, the two activities possess similar K_m and pH optimum values. The transfer may therefore probably be catalyzed by the same enzyme protein, with a different degree of specificity towards the two diglycerides. It follows that probably the factor which regulates the overall rate of synthesis of each EPG in vivo might be the cellular concentration of the endogenous diglyceride acceptors. Unfortunately, an insufficient number of studies have been carried out concerning the cellular distribution of the natural diglycerides, and no plasmalogenic diglyceride has, as yet, been detected in brain. The absence of an increase in the synthesis of alkenyl acyl GPE on adding the diacyl glycerol (Tables II and IV), and vice versa (see text), also indicates that a plasmalogenic diglyceride cannot be converted into a diacyl glycerol form, and vice versa at least in vitro.

The pathway for the synthesis of the alkenyl acyl lipids is still under investigation (24). According to Kiyasu and Kennedy (25) the enzyme which catalyzes the synthesis of diacyl GPC from the diglyceride in the liver may also act on the plasmalogenic diglyceride, as we have suggested for brain tissue. Therefore, the conversion of CDPE to acyl alkenyl GPE in vitro through the participation of the plasmalogenic diglyceride might not be operative in vivo, and may represent only the result of interactions among the externally added plasmalogenic diglyceride, the CDPE and the sufficiently unspecific action of the enzyme which converts CDPE to lipid material. Synthesis of alkenyl acyl GPE probably has to occur by other mechanisms.

The results of the present work show that little labeling occurs in the alkyl acyl GPE upon incubation of brain homogenates with CDPE (about 2 mμmoles/100 mg tissue/hour), and that synthesis does not increase upon addition of either α,β-dioctadecyl or α,β-dihexadecyl glycerols, thus confirming previous findings (3). Further, brain microsomes do not seem to catalyze the transfer of PE from CDPE to alkyl acyl GPE, even in the presence of considerable amounts of the above mentioned compounds. The failure of brain tissue and brain microsomes to utilize the diether compounds for the synthesis of the glyceryl ether form of EPG opens the question as to how this lipid can be synthesized. The very recent finding (26) of a new metabolic pathway for biosynthesis of alkyl ether bonds from glyceraldehyde-3-phosphate and fatty alcohols by microsomal enzymes could be of interest, in this connection.

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Fatty Acid Composition of Human and Rat Adrenal Lipids: Occurrence of ω6 Docosatrienoic Acid in Human Adrenal Cholesterol Ester

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ABSTRACT

Fatty acids in human and rat adrenal lipids were analyzed by A_gNO_3-impregnated silica gel, thin layer chromatography and gas liquid chromatography (GLC). In human adrenal cholesterol ester, 26 kinds of fatty acids were estimated. The percentage of 18:1 was highest, and 20:3ω6 and 22:4ω6 represented high percentages in polyenoic acids. Docosatrienoate was found in all the five men, representing from 1.0% to 2.8%. Its retention time on GLC was different from that of 22:3ω9 found in the adrenal cholesterol ester of fat deficient rats. The methyl esters of dicarboxylic acids produced by KM_nO_4-oxidation of the docosatrienoate had a chain of 10 carbon atoms. These results elucidate that the docosatrienoate from human adrenal cholesterol ester belongs to the linoleate family. In the adrenal cholesterol ester of 10-week fat free rats, the percentages of 22:4ω6 and 22:5ω6 did not decrease, compared with control rats, though arachidonate apparently decreased. The adrenal phospholipid contained about 20% of arachidonate in four of five men and about 40% of arachidonate in rats. Much more polyenoic acids were found in the triglyceride of an adrenal adenoma of primary aldosteronism than in the adjacent adrenal tissue, whereas the fatty acid compositions of phospholipid and cholesterol ester in the adenoma resembled those in the adjacent tissue.

INTRODUCTION

It is well known that the adrenal cortex is rich in cholesterol, especially cholesterol ester (1), and that the content of cholesterol easily varies by stress. In rats, adrenal cholesterol decreases by injection of ACTH (2-4), injury (5) and infection (3,6), and also in humans, it presumably decreases owing to severe infection (7) or administration of ACTH (8). The experiments in vivo (9,10) and in vitro (11,12) showed that adrenal cholesterol could serve as a precursor of adrenal steroid hormones in various animals. The adrenocortical hormones may be also synthesized from cholesterol esters in the adrenal gland (13). The relationship between the fatty acid composition in adrenal cholesterol esters and the synthesis of adrenocortical hormones has been examined but it is not yet clarified. Essential fatty acid deficiency appears to involve the function of adrenal gland (14-16). It was reported that cholesteryl arachidonate decreased in concentration more than any other ester in rat adrenal gland after unilateral adrenalectomy (17).

Many kinds of long chain polyenoic fatty acids were found in adrenal cholesterol esters and phospholipids of various animals. It was reported that 20:3 (number of carbon atoms: number of double bonds), 20:4 and 22:4 of the linoleate family and 20:5 and 22:5 of the linolenate family were found in bovine adrenal glycerophosphatides (18), and that 20:3, 20:4 and 22:4 of the linoleate family were identified in canine adrenal lipids (19). In rat adrenal cholesterol ester, 20:4 and 22:4 of the linoleate family represented the major part (20-22), and when given a fat free diet, 20:3 and 22:3 of the oleate family increased in proportion to the decrease of fatty acids of the linoleate family (23).

Since the fatty acid composition of human adrenal lipids has not been precisely clarified, it was analyzed by argentation thin layer chromatography (TLC) and gas liquid chromatography (GLC), and was compared with that in rat adrenal lipids, in the present report.

MATERIALS AND METHODS

Human adrenal glands from four cases were obtained at autopsy and that of one case during an operation for an adrenal adenoma. Until autopsies were performed, the bodies had been kept at a temperature of about 10 C for 3-7 hr. I.T., a 69-year-old man, was a slightly obese

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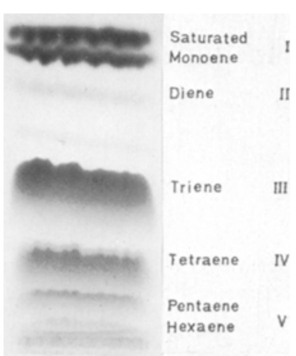

FIG. 1. Fractionation of the methylated fatty acids in the adrenal cholesterol ester of 14-week fat free rats according to the degree of unsaturation by A_gNO_3-impregnated silicic acid TLC. The plate of the chromatogram was sprayed with 15% H_2SO_4 and charred at 140 C for 1 hr. The Roman numerals at the right side agree with those in Figure 2.

patient having diabetes mellitus, disturbance of renal function and hyertension. He died of acute heart failure. K.S., a 50-year-old man, died of cachexia due to hepatoma. T.H., a 23-year-old man, died of acute myelitis. K.M., a 39-year-old man, died of renal failure due to chronic nephritis and gout. Y.S., a 25-year-old man, was a case of primary aldosteronism with symptoms of hypertension, periodic paralysis and hypokalemia. A globular adenoma of about 1 cm diameter was found in the left adrenal gland at operation. The adenoma and the left adrenal gland were removed. Histologically the adenoma was a typical one like Zona reticularis of adrenal cortex, and the left adrenal tissue was normal. The symptoms diminished completely after the operation.

Five male albino rats of 150 g body weight were given a fat free diet, consisting mainly of 70% sucrose and 20% vitamin free casein, for 10 weeks before being killed. Another five rats were fed on a commercial solid diet as controls. Six other rats were given a fat free diet for 15 weeks and three of them were given the same diet plus 40 mg methyl linoleate per day for one more week. Adrenal glands were removed from all the rats and were pooled separately in each group.

Periadrenal adipose and connective tissues were removed as much as possible. Since no attempt was made to separate the medulla and cortex, all the analyses refer to the whole gland. Fatty acids of periadrenal adipose triglyceride were analyzed in two cases by the same method as for adrenal glands.

Adrenal glands were homogenized in methanol as quickly as possible, and their lipids were extracted by Folch's method (24) and were fractionated into major classes by TLC on silicic acid impregnated with Rhodamin 6G. The developing solvent was petroleum ether-diethyl ether-glacial acetic acid (85:14:1). The bands of cholesterol ester and of triglyceride were scraped off and eluted with diethyl ether. The phospholipid fraction was eluted with methanol. Each fraction was transmethylated with dry methanol containing 5% HCl for 3 hr at 65 C. The petroleum ether extracts, the

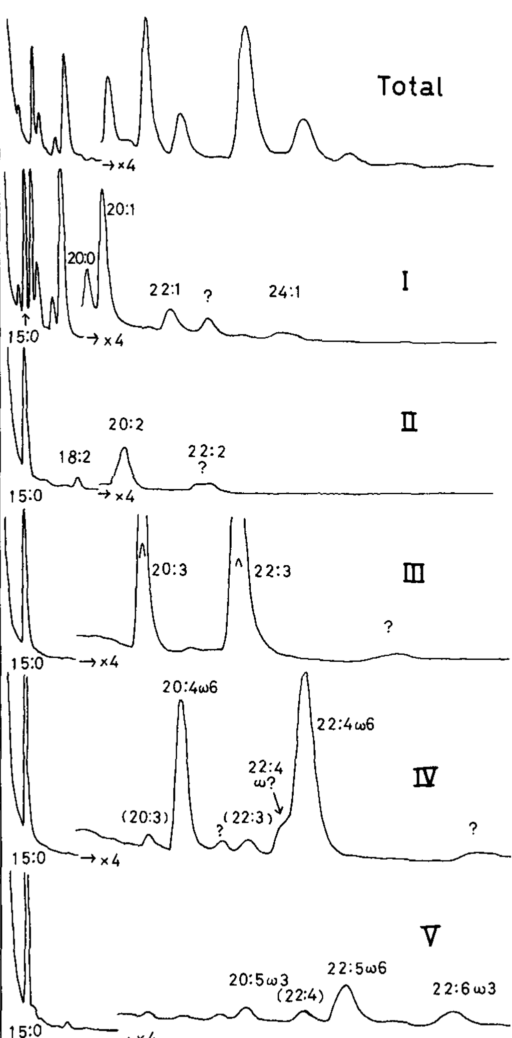

FIG. 2. Gas liquid chromatograms of the total methylated fatty acids and of each fraction on the argentation thin layer chromatogram of the adrenal cholesterol ester of 14-week fat free rats shown in Figure 1. Pentadecylic acid was added for an internal standard.

TABLE I

Lipid Contents in the Adrenal Glands of Five Men (mg/g, Adrenal Wet Weight)

Lipid	I.T.	K.S.	T.H.	K.M.	Y.S.	Y.S. Adenoma
Total cholesterol	59.0	4.0	14.5	37.6	39.4	86.4
Phospholipid	52.0	33.9	27.4	23.3	17.7	16.4
Triglyceride	N.D.[a]	29.8	13.7	13.7	29.5	12.2

[a]Not determined.

methyl esters of fatty acids, were separated by silicic acid TLC using toluene as the developing solvent. A portion of the methylated fatty acids was analyzed by GLC, and the remaining 4 mg to 5 mg was fractionated according to the number of double bonds in a fatty acid by argentation TLC (25). The chromatography was carried out on a 23 x 8 cm glass plate coated with Silica Gel G (E. Merck Ltd.) impregnated with 25% A_gNO_3 (26), 300 μm in thickness, with chloroform-methanol (95:5) as solvent, for 45 min at room temperature of about 20 C. Fractions were visualized under the fluorlamp by spraying with dichlorofluorescein. To each fraction, 20 ml of diethyl ether and the same volume of 15% methanol in distilled water were added, and the mixture was shaken vigorously. The extraction was repeated once more. After the diethyl ether was evaporated, the methylated fatty acids were eluted with acetone and analyzed by GLC with 100 μg of methyl pentadecylic acid as an internal standard. The recovery rate of fatty acids through the argentation TLC was from 95% to 100%, and no difference was found between the recovery rate of saturated acids and that of unsaturated ones.

All these procedures were carried out under nitrogen gas as much as possible. For example, solvents were evaporated under nitrogen gas

TABLE II

Fatty Acid Composition of Cholesterol Ester in Human Adrenal Glands

Fatty acids	Retention time	Average of five men (%)	Adenoma[a] (%)
14:0	0.39[b]	0.7 (0.0 - 1.2)	0.8
16:0	0.62	6.7 (5.0 - 8.8)	7.2
16:1	0.73	4.2 (3.1 - 6.7)	3.3
18:0	1.00	2.7 (1.6 - 4.1)	3.1
18:1	1.17	37.6 (33.1 - 41.6)	40.6
18:2	1.45	7.0 (5.6 - 7.9)	10.5
18:3ω6	1.72	0.3 (0.1 - 0.4)	0.5
18:3ω3	1.92	0.1 (0.0 - 0.3)	0.2
20:0	1.64	0.2 (0.1 - 0.3)	0.3
20:1ω9	1.90	3.0 (2.7 - 3.5)	2.3
20:2ω9	2.28	1.5 (0.9 - 2.2)	1.3
20:2ω6	2.36	1.1 (1.0 - 1.4)	1.6
20:3ω9	2.58	0.3 (0.3 - 0.4)	---
20:3ω6	2.83	8.3 (4.4 - 10.0)	9.9
20:4ω6	3.20	4.7 (3.2 - 5.5)	3.4
20:5ω3	4.27	0.8 (0.3 - 1.3)	0.6
22:0	2.70	0.1 (0.0 - 0.3)	0.2
22:1ω9	3.13	1.6 (1.2 - 2.1)	1.3
22:2ω9	3.73	0.2 (0.0 - 0.8)	---
22:3ω6	4.65	1.8 (1.0 - 2.8)	2.0
22:4ω6	5.42	7.5 (7.1 - 8.1)	3.8
22:5ω6	6.26	1.2 (0.7 - 1.9)	0.6
22:5ω3	7.16	2.3 (1.3 - 3.9)	1.5
22:6ω3	8.26	4.3 (2.9 - 7.1)	3.3
24:0	4.59	0.2 (0.0 - 0.8)	0.2
24:1ω9	5.22	1.7 (1.1 - 3.2)	1.5

[a]From a patient with primary aldosteronism.

[b]Retention time on the gaschromatogram (that of stearate = 1.00).

TABLE III

Fatty Acid Composition of Phospholipid in Human Adrenal Glands

Fatty acids	Average of five men (%)	Adenoma[a] (%)
14:0	0.5 (0.0 - 1.8)	---
16:0	18.1 (15.8 - 21.3)	22.9
16:1	2.5 (2.3 - 2.8)	2.4
18:0	16.2 (12.0 - 23.4)	24.6
18:1	22.3 (17.7 - 28.3)	17.4
18:2	7.3 (5.5 - 10.0)	8.7
18:3ω6	0.1 (0.0 - 0.2)	0.1
18:3ω3	0.0 (0.0 - 0.1)	0.1
20:0	0.1 (0.0 - 0.3)	0.5
20:1ω9	0.9 (0.7 - 1.2)	0.8
20:2ω9	0.2 (0.0 - 0.4)	---
20:2ω6	0.6 (0.2 - 0.9)	0.8
20:3ω9	0.2 (0.0 - 0.3)	---
20:3ω6	2.9 (1.6 - 4.1)	3.1
20:4ω6	20.1 (9.5 - 25.8)	9.8
20:5ω3	0.2 (0.0 - 0.7)	0.7
22:0	0.3 (0.0 - 0.8)	1.2
22:1ω9	0.1 (0.0 - 0.2)	0.6
22:4ω6	2.3 (1.3 - 3.8)	0.7
22:5ω6	0.1 (0.0 - 0.3)	---
22:5ω3	0.9 (0.5 - 1.5)	0.4
22:6ω3	2.1 (0.7 - 4.2)	0.7
24:0	0.5 (0.3 - 0.8)	1.4
24:1	1.5 (0.3 - 2.5)	2.8

[a]From a patient with primary aldosteronism.

and methylated fatty acids in dry benzol were preserved in ampoules filled with nitrogen gas until the next procedure. The analyses employing a large quantity of 2,6-ditert-*p*-butyl cresol (BHT) in solvents as an anti-oxidant (27) were not significantly different from the analyses without BHT.

GLC was performed on the Yanagimoto Gas-chromatograph, model 550F, equipped with a 220 cm glass column packed with 20% diethylene glycol-succinate on celite, and a hydrogen flame detector. Analyses were carried out at a column temperature of 206 C, an injection temperature of 240 C and a nitrogen flow of 20 ml/min. The peak areas were calculated by a triangulation method. As the peaks of fatty acids not separated on gas liquid chromatogram, such as 20:3ω6 (number of carbon atoms from the methyl end to the double bond) and 22:0, could be separated by the argentation chromatography, their areas were calculated from their ratios to the area of internal standard in each fraction. Technical error (coefficient of variation) for fatty acid composition through all the procedures mentioned above was less than 5% for a major component (5% or more of total fatty acids) and was less than 10% for a minor component (from 1% to 5% of total fatty acids).

Fatty acids were identified by their retention time on gas liquid chromatograms (28) and by the fractionation on argentation chromatograms (Fig. 1 and Fig. 2). Fatty acids used as standards in the both methods were as follows: 16:0 (the purity, 98%), 18:0 (99%), 20:0 (96%), 22:0 (95%), 18:1ω9, 18:2ω6 (99%), 18:3ω6 (95%), 18:3ω3 (95%), 20:3ω6 (96%) and 20:4ω6 (95%) (obtained from Nakarai Chemicals Co. Ltd. and Ono Pharmaceutical Co. Ltd.). Though fatty acids having less than two double bonds were not partitioned clearly in Figure 1, argentation chromatography with petroleum ether-diethyl ether (75:25) could easily fractionate them into three bands: saturated acids, 24:1, and the other monoenoic acids.

Some of the fatty acids separated by argentation chromatography were oxidized with potassium permanganate (KM_nO_4) according to the methods of Tinoco (29). The methyl esters of dicarboxylic acids and of monocarboxylic acids obtained as oxidation products were analyzed by GLC at a column temperature of 184 C and 98 C, respectively. Dimethylated acids of 6,8,9 and 10 carbon atoms and monomethylated acids of 6,8 and 10 carbon atoms (obtained from K&K Laboratories Inc. and Nakarai Chemicals Co. Ltd.) were used as the standard substances for identification. The recovery rate of dimethylated acids was about 90% and that of monomethylated acids was more than 70%, if the pentadecylic acid recovery was assumed to be 100%.

From the adrenal lipid extracted by Folch's method, the triglyceride (30), phospholipid (31) and cholesterol (32) contents were measured by the methods of previous reports.

RESULTS

Fatty Acid Composition of Human Adrenal Lipids

Lipid contents of the adrenal glands from five patients are shown in Table I. Total cholesterol concentration was low in K.S. and T.H., who died of liver cancer and of acute infection, respectively, and was highest in the adrenal adenoma of Y.S.

Twenty six fatty acids were estimated in the adrenal cholesterol esters (Table II). The percentage of 18:1 was highest. The existence of monoenoic acids having carbon numbers of 20, 22 and 24 were characteristic. The monoenoic acid of 24 carbon atoms could be isolated by argentation chromatography and was identified to be 24:1ω9 by the KM_nO_4-oxidation method, since the monomethylated acid produced had a chain of nine carbon atoms. Eicosatrienoate and docosatetraenoate

TABLE IV

Fatty Acid Composition of Triglyceride in Adrenal Glands and Peritoneal Fat of Humans

	T.H.		Y.S.		Y.S.
Fatty acids	Adrenal (%)	Adipose (%)	Adrenal (%)	Adipose (%)	Adenoma[a] (%)
14:0	1.6	2.4	1.2	0.9	0.4
16:0	19.7	20.3	20.0	19.4	23.4
16:1	7.4	7.0	4.7	4.5	4.0
18:0	3.4	3.2	6.8	6.5	9.4
18:1	43.7	43.3	42.5	40.5	22.3
18:2	15.5	15.5	15.3	20.5	10.3
18:3ω6	0.1	0.1	Trace	Trace	0.1
18:3ω3	0.3	0.2	0.7	1.2	0.1
20:0	Trace	Trace	0.3	0.3	0.1
20:1	2.7	3.4	1.7	3.0	1.6
20:2ω9	0.3	0.3	Trace	Trace	0.4
20:2ω6	0.4	0.3	0.6	0.2	1.2
20:3ω6	0.5	0.2	1.4	0.2	8.1
20:4ω6	0.9	0.5	1.3	0.3	6.3
20:5ω3	Trace	0.1	Trace	Trace	2.2
22:1ω9	1.0	1.4	1.0	1.6	0.6
22:4ω6	0.5	0.2	0.6	Trace	1.8
22:5ω6	Trace	Trace	Trace	Trace	2.0
22:5ω3	0.6	0.5	0.7	Trace	1.2
22:6ω3	1.4	1.1	1.2	1.1	2.6
24:0	---	---	---	---	0.8
24:1ω9	---	---	---	---	1.1

[a]From a patient (Y.S.) with primary aldosteronism.

represented a relatively large percentage. The percentages of polyunsaturated acids of the linolenate family varied in each of the five cases. Docosatrienoate was found in all cases.

In the phospholipid fraction of adrenal glands (Table III), 18:1 and 20:4ω6 represented about 20% or more except the arachidonate in Y.S.

Fatty acid composition of the adrenal triglyceride was similar to that of the adipose triglyceride in two cases (Table IV), but the adrenal triglyceride was somewhat richer in the long chain polyenoic acids than the adipose triglyceride.

The fatty acid composition in an adenoma of primary aldosteronism is shown in Tables II-IV. The percentages of 22:4 and 22:5 in cholesterol esters were lower in the adenoma than in the adjacent adrenal tissue of Y.S. However, there was little difference in the percentages of other fatty acids in cholesterol ester and in the fatty acid composition in phospholipid between the two types of tissues. Eicosatrienoate and 20:4 were found more in the triglyceride of the adenoma than in that of the adjacent adrenal tissue.

Docosatrienoic Acid in Adrenal Cholesterol Ester

In the adrenal cholesterol ester of fat deficient rats, docosatrienoate appeared as in previous reports (23). An apparent difference was found in the retention time on GLC under the same conditions between the docosatrienoate obtained from fat deficient rats and the docosatrienoate, at least its major portion, obtained from humans. Ratios of the retention times of both docosatrienoates to that of stearate (1.00) were 4.81 (from rats) and 5.18 (from humans). The KM_nO_4-oxidation products of the trienoates from a human were methylated and analyzed by GLC as described. The analysis of monomethylated acids showed a major component of 6 carbon atoms and a minor component of 9 carbon atoms. The dimethylated acids consisted of one large component and some small components (Fig. 3). The retention time of peaks A,D,E and G in Figure 3 agreed with that of the standard dimethylated acids with chains of 6,8,9 and 10 carbon atoms, respectively. Peak B was probably the dimethylated acid with a chain of 7 carbon atoms, while peaks C and F did not correspond to any dimethylated acid. The ratios of peak areas of A, B and G to that of D (D=100.0) in Figure 3 nearly agreed with the ratios of peak areas of 18:3, 22:3ω9 (?) and 22:3ω6 (?) to that of 20:3ω6 (20:3ω6=100.0) in the original trienoates (Table V).

Furthermore, about half of ω6 (?) docosatrienoate could be separated from the other

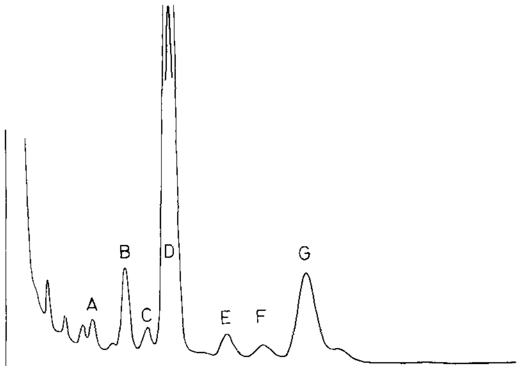

FIG. 3. Gas chromatogram of the methyl esters of dicarboxylic acids produced by KM_nO_4-oxidation of trienoic fatty acids from human adrenal cholesterol ester (column temperature, 184 C, 20% DEGS on celite). See text and Table V.

acids by repeating argentation TLC. The dimethylated acids produced by KM_nO_4-oxidation of the isolated docosatrienoate, at least most of them, had a chain of 10 carbon atoms (Fig. 4).

These results showed that the major portion of docosatrienoate from human adrenal cholesterol ester is that of the linoleate family, $22{:}3\Delta^{10,13,16}$.

Fatty Acid Composition of the Adrenal Lipids of Rats

Fatty acids of the adrenal lipids of fat deficient rats and of control rats were analyzed in the same manner as the human adrenal lipids (Table VI). Arachidonate and 22:4ω6 represented the major cholesterol ester fatty acids, and arachidonate represented more than 40% of phospholipid fatty acids in the control rats. In the cholesterol ester of fat deficient rats eicosa- and docosa-trienoate of the oleate family increased, and linoleate and arachidonate decreased, but the percentages of 22:4 and 22:5 of the linoleate family were unchanged, in comparison with those of the control rats.

In a separate examination, rats given a fat free diet for 15 weeks and rats given 40 mg of methyl linoleate per day for one more week after the fat free diet were compared. In the adrenal cholesterol esters, 20:4ω6 and 22:4ω6 represented 3.9% and 7.2%, respectively, in the fat free rats, and increased to 8.1% and 10.7%, respectively, after linoleate-feeding. Eicosatrienoate (ω9) and 22:3ω9 represented 15.6% and 24.3%, respectively, in the fat free rats and decreased to 11.6% and 16.5%, respectively, after linoleate-feeding.

These results suggested that the percentage of arachidonate fell faster than that of 22:4ω6 when rats were fed on a fat free diet and that the percentages of both 20:4ω6 and 22:4ω6 increased simultaneously when rats were given linoleate after the fat free diet.

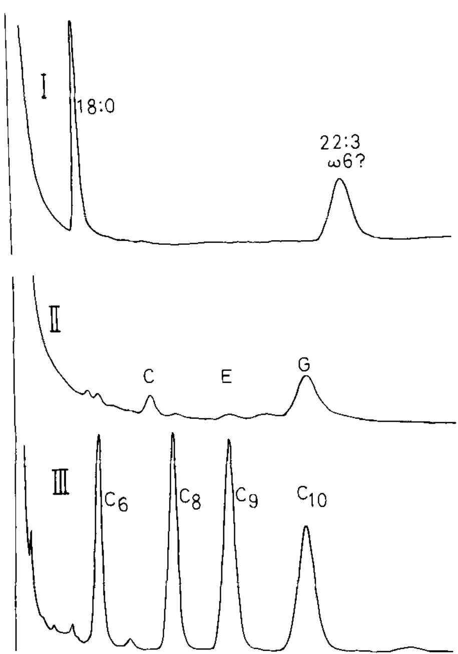

FIG. 4. (I) Gas chromatogram of ω6 (?) docosatrienoate isolated by repeating argentation TLC (column temperature, 206 C). Methyl stearate was added as a standard. (II and III) Gas chromatograms of dimethylated acid produced by KM_nO_4-oxidation of the docosatrienoate (II) and standard dimethylated acids having chains of 6,8,9 and 10 carbon atoms (III) (column temperature, 184 C). Peaks C, E and G correspond to those in Figure 3.

DISCUSSION

Since most of the fatty acids were sharply fractionated according to the degree of unsaturation, they were easily identified by their retention time on GLC. However fatty acids with short retention times, such as 20:2ω9 and 20:2ω6, might not be accurately identified. Autoxidation of unsaturated fatty acids is thought to be negligible even if it could occur, for the following reasons: firstly, the procedures and the storage were carried out under nitrogen gas as much as possible; secondly, the results in which BHT was used for all the solvents were not significantly different from those without BHT; and thirdly, no significant variation of the fatty acid pattern was shown after repeating any step of TLC. However it was not evident whether or not human adrenal lipids deteriorated during the period from death till autopsy.

TABLE V

Composition of Dimethylated Acids Produced by KM_nO_4 – Oxidation of Trienoates From Human Adrenal Cholesterol Ester and Comparison With the Composition of the Original Trienoates

	1	2	3	4	5
Retention time ratios on gas chromatograms[a]					
Standard dimethyl	1.00 (C6)[b]		1.82 (C8)	2.45 (C9)	3.29 (C10)
Peak in Figure 3	1.00 (A)	1.34 (B)	1.82 (D)	2.42 (E)	3.27 (G)
Peak area ratios[c]					
Ratio involved	C6/18:3ω6	C7/22:3ω9?	C8/20:3ω6		C10/22:3ω6?
Ratio expected	2.7	7.4	100.0		17.1
Ratio from Figure 3	2.4	9.0	100.0	4.1	22.6

[a]Ratios (C6 = 1.00). When the standard dimethyl ester of dicarboxylic acid having 6 carbon atoms was added into the sample shown in Figure 3, the retention time of the standard acid agreed with that of Peak A.

[b]Number of carbon atoms in the carbon chain of dimethylated acid.

[c]Ratios (area of C8 dimethyl and of 20:3ω6 = 100.0).

In human adrenal cholesterol ester, monoenoic acids (ω9) were predominant, and 20:3 and 20:4 of the linoleate family represented relatively high percentages, similar to the past report (8). The present report shows the existence of 22:1ω9 and 24:1ω9 and a high percentage of 22:4ω6 in cholesterol ester. In the phospholipid fraction, arachidonate represented a high percentage in four cases out of five. Human adrenal glands seemed to require the long chain polyunsaturated fatty acids of the linoleate family.

TABLE VI

Fatty Acid Composition of Adrenal Lipids From Rats Given Fat Free Diet for 10 Weeks and Rats Given Commercial Solid Diet (Control)

	Fat free diet[a]			Control[a]		
Fatty acids	CE (%)	PL (%)	TG (%)	CE (%)	PL (%)	TG (%)
14:0	Trace	Trace	1.8	b	Trace	b
16:0	10.5	8.7	25.7	10.5	6.9	25.6
16:1	4.5	0.5	9.3	2.0	0.1	5.2
18:0	2.9	28.9	6.2	2.6	31.9	7.4
18:1	19.7	17.2	54.9	11.9	8.3	37.8
18:2	1.2	0.5	0.4	4.5	4.7	19.1
18:3ω6	Trace	---	---	0.2	Trace	Trace
18:3ω3	0.1	---	---	0.2	Trace	0.3
20:0	2.2	0.3	Trace	0.9	0.3	0.1
20:1	5.9	0.8	0.5	2.5	0.5	1.2
20:2ω9	1.2	---	0.1	---	---	---
20:2ω6	Trace	---	---	0.7	0.3	0.1
20:3ω9	6.0	3.0	0.3	Trace	0.1	Trace
20:3ω6	0.7	0.2	---	2.2	0.4	0.1
20:4ω6	6.3	36.7	0.4	16.2	42.6	1.4
20:5ω3	Trace	---	----	1.6	Trace	0.1
22:0	0.4	0.3	---	0.8	0.2	---
22:1ω9	2.4	---	---	1.4	---	---
22:3ω9	8.8	---	---	---	---	---
22:4ω6	20.0	2.1	0.3	21.5	1.8	0.6
22:5ω6	3.3	---	---	1.7	Trace	Trace
22:5ω3	0.6	---	---	5.0	0.3	0.3
22:6ω3	1.8	---	---	12.3	0.8	0.7
24:0	Trace	0.4	---	Trace	0.5	---
24:1ω9	1.5	0.4	---	1.3	0.3	---

[a]CE, cholesterol ester; PL, phospholipid, TG, triglyceride.

[b]Not calculated because BHT was used.

Docosatrienoic acid was found in the adrenal cholesterol esters of all five cases. Since fatty acids could not be singly isolated except 24:1 by argentation chromatography, the KM_nO_4-oxidation products of them contained various components. However repeated argentation chromatography could separate a major portion of the docosatrienoate from the other trienoates of human adrenal cholesterol ester. The dimethylated acid with a chain of 10 carbon atoms produced by KM_nO_4-oxidation showed that the docosatrienoate belongs to the linoleate family. This fatty acid could not be found in any lipid fraction of human plasma by similar methods in our laboratory and also in the precise analysis of Phillips (33). It was not found in the liver, plasma and adrenal gland of rat. In the past reports on rat liver (34,35), human plasma (36) and human red cell (27), docosatrienoic acid was stated to be of the oleate family.

In the chain elongation reaction of fatty acid in vitro with rat liver microsomes, a small amount of 22:3ω6 was synthesized from 18:3ω6 (37) and 20:3ω6 (38). Because of the high percentages of 20:3ω6 in human adrenal glands, a portion of the acid may be converted into 22:3ω6, but it is not obvious whether the 22:3ω6 could be converted into 22:4ω6.

The fact that 20:4ω6 and 22:4ω6 were rich in rat adrenal cholesterol ester and that 20:3ω9 and 22:3ω9 increased in fat deficient rats agrees with the previous reports (16,23). The percentages of 22:4ω6 and 22:5ω6 in the 10-week fat free rats were retained on the same level as those of control rats, and when a small amount of linoleate was given to the 15-week fat free rats for one week, 20:4ω6 and 22:4ω6 increased in the similar degree. This suggests that the metabolic turnover rates of cholesterol esters with 22:4ω6 and 22:5ω6 were slower than those of other cholesterol esters in rat adrenal gland. It seems to agree with the experiment of Gidez and Feller (17), in which cholesteryl arachidonate decreased in concentration more than any other esters, followed by the esters with linoleate and oleate, in the adrenal gland of unilaterally adrenalectomized rats.

It should be considered that small amounts of periadrenal adipose tissues might be mixed in adrenal lipids. However the difference of fatty acid composition of triglyceride between adenoma and normal adrenal tissue was too large to be ascribed to the mixture of adipose triglyceride. It might be due to the histological difference between the adrenocortical adenoma and the whole adrenal gland, more than to differences in their lipid metabolism.

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Fatty Acid and Long Chain Base Composition of Adrenal Medulla Gangliosides

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ABSTRACT

Five ganglioside fractions from bovine adrenal medulla were analyzed with respect to their fatty acid and long chain base compositions. The five fractions included two hematosides and three hexosamine-containing species, the latter having chromatographic properties comparable to the major gangliosides of brain. The fatty acid compositions of all five were similar: 22:0 was the most abundant, but significant amounts of 16:0, 18:0, 24:0 and 24:1 were also present. No hydroxy fatty acids were detected. The principal long chain base in each fraction was 4-sphingenine (sphingosine), with lesser amounts of the C_{16} and C_{17} homologues. Minor quantities of the corresponding saturated bases were also detected. These were identified by two gas liquid chromatography methods: (a) trimethylsilyl ether derivatives, (b) aldehydes formed by periodate oxidation of the long chain bases. No 4-eicosasphingenine (C_{20}-sphingosine), characteristic of brain gangliosides, was found in any of the fractions. The results demonstrate that gangliosides of the adrenal medulla show tissue specificity in fatty acid and long chain base composition which is independent of carbohydrate structure.

INTRODUCTION

Gangliosides were recently shown to be present in one component of the autonomic nervous system, the adrenal medulla (1). The total level of ganglioside in this tissue was surprisingly high, amounting to slightly over 50% of the level in brain gray matter when expressed as μmoles of ganglioside per weight of fresh tissue. That study also demonstrated the presence of two categories of ganglioside in the same tissue: (a) hematosides, consisting of ceramide-lactoside attached to one sialic acid, and (b) hexosamine-containing gangliosides, which resembled the major gangliosides of brain in their slower migration on thin layer chromatography (TLC) and in their more complex oligosaccharide structures. However, the latter category comprised less than 10% of total ganglioside, which is contrary to the distribution observed in brain tissue (2).

Both of the major fractions were shown to be hematosides which differed from each other in the type of sialic acid; the slower migrating fraction (AG_5) contained N-glycolylneuraminic acid (NGNA) while the faster (AG_6) had N-acetylneuraminic acid (NANA). The three hexosamine-containing fractions possessed two or more sialic acid units, with both types of sialic acid appearing in the same structure in two of the fractions. The present study was undertaken to determine the nature of the fatty acids and long chain bases (LCBs) associated with each of the five ganglioside fractions from bovine adrenal medulla. It was of particular interest to ascertain whether the hexosamine-containing species, similar to brain gangliosides in several aspects of carbohydrate structure, also resembled the latter in regard to lipophilic constituents.

MATERIALS

Fatty acid standards and boron trifluoride-methanol (14% by weight) were obtained from Applied Science Laboratories, Inc., State College, Pa. *Rac*-4-sphingenine and *rac*-sphinganine were purchased from Miles Laboratories, Inc., Elkhart, Ind. Several other LCBs employed as standards included *rac-threo*-4-heptadecasphingenine, *rac-threo*-heptadecasphinganine, *rac-threo*-4-nonadecasphingenine, *rac-threo*-nonadecasphinganine, *rac-threo*-heneicosasphinganine and *rac-threo*-tricosasphinganine. Four LCBs were prepared (as a mixture) from brain gangliosides: 4-sphingenine, sphinganine, 4-eicosasphingenine and eicosasphinganine. Standard aldehydes containing two carbons less than the respective LCBs were prepared by periodate oxidation of the above bases. Myristic aldehyde was synthesized by controlled oxidation of myristic alcohol with C_rO_3 in pyridine. Hexane and dichloromethane were Matheson, Coleman and Bell spectroquality solvents and were used without further purification. Chloroform and methanol, Merck reagent grade, were distilled prior to use. Silica

Gel HR (without binder) from E. Merck, A.G., Darmstadt, Germany, was used for TLC purification of fatty acids, while Silica Gel G from the same source was employed for TLC of gangliosides.

METHODS

Gas Liquid Chromatography

Gas liquid chromatography (GLC) analyses were carried out using an F & M model 1609 gas chromatograph equipped with a flame ionization detector. The columns were stainless steel coils, 6 ft long and 1/4 in. i.d. Two types of packing were employed: 15% EGSS-X (polar) on Gas-Chrom P and 6% JXR (silicone) on Gas-Chrom Q. These materials were obtained from Applied Science Laboratories, Inc. Helium was used as the carrier gas at a flow rate of 55 ml/min. Peak areas were determined with a Disc integrator and also from the product of peak height by width at half height. Data are presented as weight percentages and each value is an average of at least three GLC determinations.

Sample Preparations

Gangliosides were isolated from bovine adrenal medulla and resolved into five fractions according to methods previously described (1). The conditions for methanolysis were very similar to those described by Gaver and Sweeley (3) in which a small amount of water is added to the methanolic-HCl in order to reduce the quantity of methylated sphingosine side products. Approximately 0.4 μmole ganglioside was dissolved in 2 ml methanol-HCl-water (82:8.6:9.4) in a small tube with teflon lined screw-cap and the mixture heated under nitrogen at 70 C for 23 hr. Four extractions with 2.5 ml hexane removed the fatty acids which were set aside for subsequent analysis. The remaining methanolic solution was evaporated to half its original volume with a stream of nitrogen and made basic with approximately 2 ml of 1 N (aq) sodium hydroxide. Two milliliters of chloroform were added, the mixture shaken well and the aqueous upper phase discarded. The chloroform phase was washed twice with dilute aqueous ammonia, once with water and finally evaporated to dryness. The residue, consisting of LCB mixture, was dissolved in dry chloroform for analysis.

Analysis of Long Chain Bases

The chloroform solution of LCBs was divided into two equal portions and analyzed by two GLC methods:

Trimethylsilyl (TMS) Derivatives. This procedure was patterned after that of Gaver and Sweeley (3), the TMS derivatives being prepared by the method of Carter and Gaver (4). Following reaction, the pyridine solution was evaporated to dryness with a stream of dry nitrogen and the TMS derivatives were then dissolved in hexane, filtered and evaporated to a small volume for GLC injection. The JXR column was employed at two isothermal temperatures: 195 C and 215 C. Peaks were identified by comparison with standards and from graphs of carbon number versus log retention time. For the latter method, portions of each sample were chromatographed together with a mixture of several TMS standards.

Periodate Oxidation of LCBs to Aldehydes. This procedure was patterned after that of Sweeley and Moscatelli (5). Following periodate oxidation of the LCB mixture in 2 ml methanol solution, 1 ml of water was added and the aldehydes removed by three extractions with 2 ml portions of hexane. The combined hexane extracts were carefully evaporated to a small volume with a stream of dry nitrogen, care being taken to maintain reduced temperature to avoid loss of the more volatile aldehydes. GLC analysis of the aldehyde mixture was accomplished with the JXR column, peaks being identified by comparison with standards and from graphs of carbon number versus log retention time.

A portion of each fraction was hydrogenated over platinum oxide catalyst and then subjected to GLC analysis by the above procedures. Comparison with the corresponding unhydrogenated samples assisted in establishing LCB identities.

Analysis of Fatty Acids

The hexane extracts obtained following methanolysis (vide supra) consisted of mixtures of methyl esters and free fatty acids due to the water present. To convert the acids to esters, the hexane solutions were evaporated to dryness and the residues heated briefly with boron trifluoride-methanol. Extraction with hexane removed the methyl esters which were washed once with water, evaporated to a small volume with nitrogen and purified by TLC on a plate coated with Silica Gel HR. Standards of methyl stearate and methyl-2-hydroxystearate were spotted adjacent to the samples and the plate was developed in heptane-ether (2:1). Water spray revealed a clear band parallel to methyl stearate which was scraped from the plate. The band parallel to methyl 2-hydroxystearate was also scraped, even though the spray revealed no visible material in this region. Both fractions were eluted from the silica gel with dichloromethane, and after evaporation of the

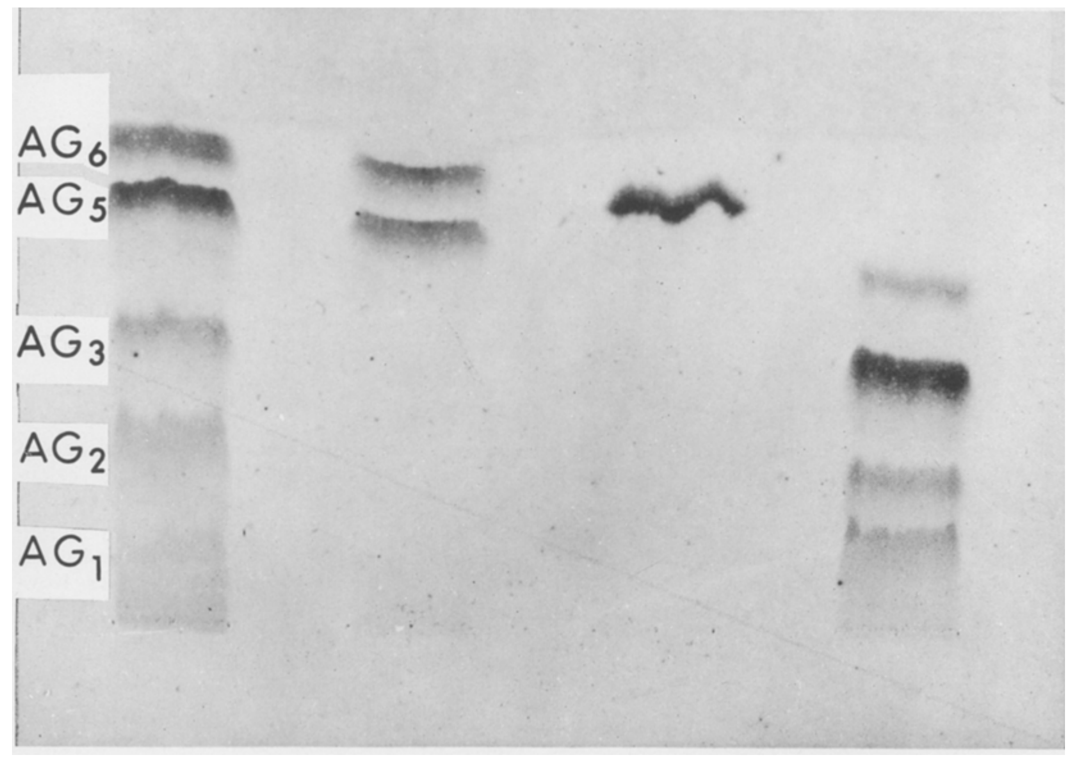

FIG. 1. Thin layer chromatogram of gangliosides from adrenal medulla and brain. Plate: 20 x 20 cm, coated with 250 μ of Silica Gel G. Solvent: chloroform-methanol-2.5 N ammonia (60:40:9), two ascending runs. Spray: resorcinol–all visible bands are purple. (a) Adrenal medulla, Folch upper phase; (b) adrenal medulla, Folch lower phase; (c) Tay-Sachs ganglioside; (d) beef brain ganglioside mixture.

solvent the normal fatty acid ester fraction was taken up in a small volume of hexane for GLC injection. The samples from the zone parallel to hydroxy fatty acid esters was first treated with trimethylchlorosilane and hexamethyldisilizane to form TMS derivatives of any hydroxy fatty acid esters which might be present. Both the JXR and EGSS-X columns were used for identification and the latter column for quantitative analysis.

RESULTS AND DISCUSSION

The TLC pattern of bovine adrenal medulla gangliosides is shown in Figure 1. As reported previously (1), the two major components are hematosides (AG_5 and AG_6), while the slower bands are hexosamine-containing gangliosides. A sixth fraction (AG_4) was faintly visible on the plate but could not be obtained in sufficient quantity for analysis. The five fractions were isolated by preparative TLC as previously described (1).

The results of LCB analysis are summarized in Table I. The major component of each ganglioside was 4-sphingenine (sphingosine), while the dihydro derivative (sphinganine = dihydrosphingosine) was present in varying amounts ranging from 2% to 8%. Also present in each fraction were small amounts of the C_{16} and C_{17} homologues, with the unsaturated forms predominating. The GLC peaks representing these six LCBs (as TMS derivatives), as well as the aldehydes derived by periodate oxidation, were identified in most instances by comparison with standards. Although no standard was available for 4-hexadecasphingenine (C_{16}-sphingosine), this LCB was identified by hydrogenation and oxidation with periodate to myristic aldehyde, for which a standard was available. The original base (as TMS derivative) and the unsaturated aldehyde from periodate oxidation both fit well on their respective semilog plots obtained from mixtures of samples and standards.

Identification of 4-heptadecasphingenine (C_{17}-sphingosine) and its dihydro derivative were also based on the semilog plot, as well as on comparison with standards. For the latter we were obliged to employ the *threo*

TABLE I

Long Chain Base Composition of Adrenal Medulla Gangliosides[a]

LCB	AG_1	AG_2	AG_3	AG_5	AG_6
16:0	Trace	5.9	2.1	1.4	0.8
16:1	3.3	2.6	3.5	6.4	3.9
17:0	2.3	Trace	Trace	Trace	0.9
17:1	9.5	2.8	2.4	6.7	4.0
18:0	7.5	2.6	7.7	2.4	3.1
18:1	77.4	86.1	84.3	83.0	87.4

[a]Bases were converted to aldehydes with periodate and analyzed by GLC on the JXR column. Quantification was achieved with a Disc integrator and by multiplying peak height by width at half-height. Data are presented as weight percentages and each value is an average of at least three determinations.

diastereomers because of their availability, although we recognized that the naturally occurring LCBs probably have the *erythro* configuration. [In all cases studied to date naturally occurring LCBs have been found to possess the D-*erythro* configuration. However, since the analytical methods employed here do not differentiate between *erythro* and *threo* isomers, the configuration of carbons 2 and 3 of these LCBs have not been rigorously established. It may be noted that the two stereoisomers can be distinguished by GLC analysis of the TMS derivatives of the N-acetylated bases (4).] This substitution appears legitimate in view of the observation (3) that the TMS derivatives of *erythro* and *threo*-4-sphingenine do not separate on an SE-30 column. We found the same two substances to be inseparable on the JXR column employed in this study, and we have made the reasonable assumption that the same relationship applies to the stereoisomers of lower and higher homologues. Aldehydes derived from these LCBs by periodate oxidation present no stereochemical problem since the asymmetric centers of the original bases are destroyed.

Some differences were noted in the results obtained by the two GLC procedures. The mixture of TMS derivatives gave a number of additional small peaks which were subsequently shown to be artifacts, whereas the periodate procedure gave very few false peaks. A small peak which appeared to represent O-methylated sphingenine was detected with both methods, despite addition of water to the methanolysis medium (vide supra); this was absent when hydrogenated samples were analyzed. Blank runs starting at the methanolysis stage gave very few false peaks with the periodate method but a considerable number with the TMS procedure. The former method was therefore used for quantification, while qualitative identifications were established with both procedures. The small peak representing O-methyl derivative was disregarded in quantitative calculations.

To our knowledge, C_{16} and C_{17} homologues of sphingosine or sphinganine have not previously been reported in gangliosides. However, these bases have been observed as minor constituents of other sphingolipids, such as sphingomyelin from human plasma (3,6) and bovine heart (7). 4-Hexadecasphingenine was found in trace amounts in rat brain but in much higher levels in brains of lower species (8). 4-Eicosasphingenine (C_{20}-sphingosine), a major LCB of gangliosides from mature brain (9), was not found in any of the gangliosides of bovine adrenal medulla. The three hexosamine-containing species in the present study had essentially the same LCB composition as the two hematosides.

The results of fatty acid analysis are shown in Table II. Behenic acid (22:0) was the most abundant in each fraction but significant amounts of 16:0, 18:0, 24:0 and 24:1 were also present. The constituents listed were identified on both the polar and nonpolar columns with the aid of standards and semilog plots, while quantification was carried out with the EGSS-X column alone (vide infra). All the gangliosides had generally similar compositions with the possible exception of AG_1 which had somewhat more 22:0 and slightly less 18:0, 24:0 and 24:1 than the others.

Hydroxy fatty acids were not detected in any of the fractions, which is consistent with the findings for most gangliosides to date. An exception appears to be a minor fraction from brain containing both hydroxy and unsubstituted fatty acids, and possessing galactose and NANA as the only carbohydrates (10).

An important consideration in GLC analysis, particularly of small samples, is the possibility of artifacts arising from solvent impurities. In some instances false peaks of this type were

TABLE II

Fatty Acid Composition of Adrenal Medulla Gangliosides[a]

Fatty acid	AG_1	AG_2	AG_3	AG_5	AG_6
14:0	2.5	Trace	2.6	0.4	---
14:1	0.3	---	0.3	---	---
15:0	1.8	Trace	1.8	0.2	0.7
16:0	15.8	10.4	14.3	16.1	14.4
16:1	5.3	Trace	1.9	1.1	2.3
17:0	---	---	---	0.5	---
18:0	8.4	16.8	15.9	12.3	15.9
18:1	6.7	0.9	4.1	2.3	2.4
19:0	---	---	---	0.4	Trace
20:0	0.7	3.0	2.7	3.3	4.4
20:1	Trace	---	---	0.5	---
21:0	---	---	---	0.4	---
22:0	44.3	24.9	21.6	22.6	28.9
22:1	Trace	Trace	---	Trace	---
23:0	2.2	6.9	5.2	9.5	5.6
23:1	---	Trace	Trace	---	---
24:0	7.9	23.9	18.6	14.6	12.8
24:1	4.1	13.3	11.1	15.6	12.0

[a]Acids were analyzed as the methyl esters on EGSS-X column. Quantification was achieved with a Disc integrator and by multiplying peak height by width at half-height. Data are presented as weight percentages and each value is an average of at least three determinations.

observed which had the same retention times as fatty acid methyl esters (18,19).

We encountered the same problem in the present study but found that the appearance of such artifacts depended greatly on the type of column. Thus, when quantities of hexane and dichloromethane corresponding to the amounts employed for fatty acid isolation were evaporated down and the residues (in a small volume of hexane) were injected onto the JXR column, a number of medium sized peaks were observed. Three of these had retention times which corresponded closely to those of 19:0, 20:0 and 22:0 (methyl esters). However, when the same sample was chromatographed on the polar EGSS-X column no peaks were detected.

In another set of experiments to test for artifacts, blank runs were started at the methanolysis stage and carried through all subsequent steps employed for isolation of fatty acid esters. The final residue, dissolved in a small volume of hexane, showed a number of small and medium-sized peaks when injected onto the JXR column, and many of these had retention times similar to some of the fatty acids present in the gangliosides. The EGSS-X column, on the other hand, showed only a few such false peaks and these were generally too small to offer serious interference. Accordingly, we employed the latter column only for quantification of fatty acids.

As with the LCBs, fatty acids of the hexosamine-containing gangliosides (AG_1, AG_2, AG_3) were generally similar to those of the hematosides. This common pattern, however, was quite different from the major gangliosides of brain which contain 75% or more of 18:0 and only minor amounts of 16:0, 24:0 and 24:1 (11-13). Hematoside from brain partially resembled the two hematosides of this study in containing more 16:0 and 18:1 along with less 18:0 in comparison to the other brain gangliosides (13,14). On the other hand, its content of 22:0 was negligible. Hematoside from horse erythrocytes was reported to contain predominantly 24:0 (15), while that from human erythrocytes contained the same major fatty acid along with substantial amounts of 16:0, 18:0 and 22:0 (16). Hematosides from canine erythrocytes was unique in containing both 24:0 and 24:1 as major fatty acids with the unsaturated predominating (17). It is evident that fatty acid composition is a highly variable factor, determined at least in part by the species and tissue from which the hematoside is isolated.

In summary, the five ganglioside fractions from bovine adrenal medulla all lack the characteristic features of brain gangliosides, i.e., a preponderance of stearate and the presence of 4-eicosasphingenine (C_{20}-sphingosine). The hexosamine-containing fractions, while resembling the major gangliosides of brain in several aspects of carbohydrate structure, bear little resemblance to these in lipophilic composition. The fact that all five fractions show

similar fatty acid and LCB patterns indicates tissue specificity for these units which is independent of carbohydrate structure.

ACKNOWLEDGMENT

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Blood α-Tocopherol: Erythrocyte and Plasma Relationships in Vitro and in Vivo

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ABSTRACT

When rat plasma labeled with ^{3}H-α-tocopherol was incubated with tocopherol-deficient red cells, uptake of radioactivity by cells was rapid with a maximum being reached in 6-8 hr. The equilibrium ratio of red cell-plasma α-tocopherol, from 0.3-0.6, was independent of the initial plasma concentration. When labeled red cells were incubated with tocopherol-deficient plasma, radioactivity in plasma became maximal in 11.5 hr. When both plasma and cells had normal α-tocopherol concentrations initially, the rate of exchange was similar in either direction with equilibrium of specific activity being attained in 6 hr. After a single oral dose of ^{3}H-α-tocopherol the activity in both plasma and cells reached a maximum in 8 hr and then fell rapidly during the next four days. The ratio of red cell-plasma radioactivity at all intervals after 8 hr was 0.5.

INTRODUCTION

Evaluation of nutritional status with respect to vitamin E is often determined by the susceptibility of red cells to peroxidative hemolysis in vitro. Although plasma α-tocopherol concentrations have been related to in vitro hemolysis, only recently has an attempt been made to relate hemolysis to the red cell content of α-tocopherol. Very little information exists on the red cell content of α-tocopherol or on the relationship between α-tocopherol in the red cell and plasma. In previous studies in which we analyzed rat erythrocytes as well as plasma for α-tocopherol (1,2), it was found that red cells always contained lower concentrations than plasma. The ratio of red cell-plasma α-tocopherol was generally 0.4-0.5. The present experiments were intended to provide more detailed information on the uptake and release of α-tocopherol by erythrocytes both in vitro and in vivo.

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EXPERIMENTAL PROCEDURES

Animals

Male weanling rats of the Sprague-Dawley strain were caged individually and fed a purified diet deficient in tocopherols (3). After five weeks, red cell and plasma α-tocopherol levels were below 70 μg/100 ml and the animals were considered to be sufficiently depleted. Supplements of α-tocopherol were either fed daily in the diet or were administered by stomach tube. Rats were fasted overnight and blood was drawn with a heparinized syringe from the abdominal aorta while the animal was under ether anesthesia. Red cells were separated at 1000 x *g* at 4 C for 15 min and washed three times with 2 vol of 0.85% saline. In one experiment (Fig. 3 and 4) the cells were washed only once. Analyses made on red cells from normal stock rats revealed that the α-tocopherol content of unwashed cells was not significantly different from that of cells washed once or three times.

α-Tocopherol

Tritiated 5-methyl-*d*,α-tocopherol was synthesized (1) by a minor modification of the procedure of Green et al. (4). The specific activity was 2.28 x 10^8 disintegrations/min per mg. Unlabeled *d*,α-tocopherol was obtained from Eastman Organic Chemicals, Rochester, N.Y. When fed, the compounds in 0.5 ml ethanol were mixed daily with 14 g of diet, an amount slightly less than the animals were normally consuming. For stomach tubing, the compounds were prepared in an aqueous dispersion containing 10% ethanol and 5% Tween 80.

Incubation and Analysis of Blood

Equal volumes of plasma and red cells were incubated in 15 ml beakers covered with paraffin film in a Dubnoff shaking incubator at 37 C. Aliquots were removed at intervals, using siliconized pipets, and centrifuged as described above. The washed cells were hemolyzed with an equal volume of water for 5 min.

A lipid extract of plasma or hemolyzed red cells was made by adding 3 vol of methanol cooled to -20 C slowly while mixing, followed by three extractions with hexane. The extracts

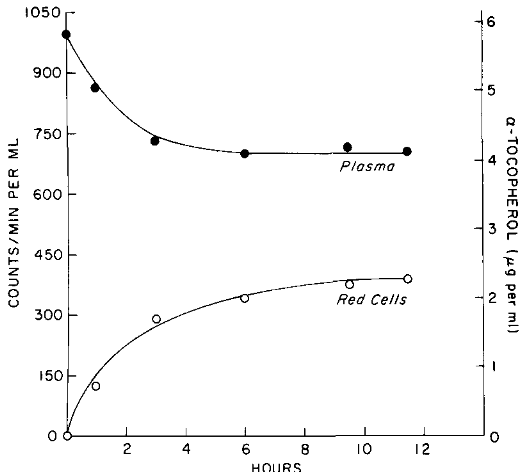

FIG. 1. Uptake by deficient red cells of α-tocopherol from labeled plasma. A tocopherol deficient rat was fed daily for four days 5 mg *d*,α-tocopherol containing 855,000 counts/min. Plasma from this rat was incubated in air at 37 C with an equal volume of washed cells from a tocopherol-depleted rat. Glucose, 2 mg/ml, was added to the mixture.

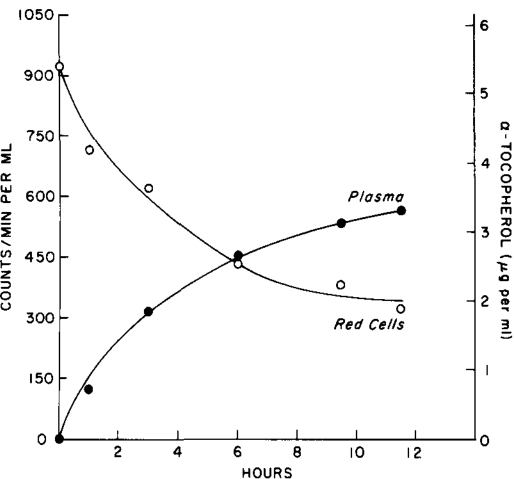

FIG. 2. Release of radioactive α-tocopherol from red cells to tocopherol deficient plasma. Source of red cells and plasma and incubation conditions were the same as in Figure 1.

were evaporated, Liquifluor (New England Nuclear, Boston, Mass.) was added, and the samples were counted in a Packard Model 3003 scintillation counter with an efficiency of 35% for tritium. Determinations of unlabeled α-tocopherol were made by a combined thin layer and gas chromatographic procedure previously described (1).

RESULTS

In Vitro Studies

Figure 1 shows the uptake by tocopherol deficient red cells of radioactive α-tocopherol from plasma. The uptake was rapid and essentially linear for the first 2-3 hr, then began to plateau with equilibration being reached in about 6-8 hr. The ratio of red cell-plasma α-tocopherol at equilibrium was 0.54. The reverse experiment, in which red cells containing radioactive α-tocopherol were incubated with tocopherol-deficient plasma, is shown in Figure 2. Release of α-tocopherol by the cells was rapid and essentially linear for 4-6 hr, then fell off between 6-9.5 hr. Equilibrium between cells and plasma was approached at about 11.5 hr, the last sampling period. At this time, the ratio of red cell-plasma α-tocopherol was 0.57.

The effect of varying plasma concentration of α-tocopherol on the red cell uptake of ^{3}H-α-tocopherol at equilibrium is given in Table I. The initial plasma concentrations varied four fold with the highest level being about two to three times that normally found in rats with adequate dietary α-tocopherol. As can be seen from the last column, the equilibrium ratio of red cell-plasma α-tocopherol was essentially the same regardless of initial plasma concentration. The average ratio of 0.3 is lower than that in Figures 1 and 2 and suggests that equilibrium may not have been reached by 6 hr. In analyses of a large number of rats, however, occasional ratios as low as 0.3 were found (1).

The exchange of ^{3}H-α-tocopherol between erythrocytes and plasma when both had equilibrium concentrations of α-tocopherol at the beginning of incubation is shown in Figures 3 and 4. For these experiments, two vitamin E depleted rats with the same weight were fed 10 mg *d*,α-tocopherol daily for four days. The tocopherol fed to one rat was unlabeled while the other rat received ^{3}H-α-tocopherol. Plasma and red cells from the two rats had relatively high concentrations of α-tocopherol as determined by gas chromatography (1) for the unlabeled blood or by counting of the labeled blood. It was not possible to analyze either plasma or cells for their total α-tocopherol content after incubation, since the procedure for the gas chromatographic determination of α-tocopherol utilized the isotope dilution technique. Consequently, it was assumed that the concentration in each compartment remained constant throughout the incubation.

When labeled plasma was incubated with unlabeled cells (Fig. 3), constant specific activity in both compartments was reached in 6 hr. When labeled red cells were incubated with un-

TABLE I

Effect of Plasma α-Tocopherol Concentration on Red Cell Uptake of ^{3}H-α-Tocopherol

Initial plasma α-tocopherol[a]		After 6 hr incubation				Total radioactivity recovered		Red cell-plasma radioactivity
		Red cells		Plasma				
(counts/min per ml)	(μg/ml)	(counts/min per ml)	(μg/ml)	(counts/min per ml)	(μg/ml)	(counts/min per ml)	(%)	
641	4.08	126	0.80	442	2.82	568	88.6	0.29
1283	8.17	265	1.69	940	6.00	1205	93.9	0.28
1708	10.9	403	2.57	1369	8.72	1772	104.0	0.29
2562	16.3	583	3.71	1756	11.2	2339	91.3	0.33

[a]The pooled plasma from two rats previously fed 10 mg ^{3}H-α-tocopherol (157,000 counts/min per mg) for four days was diluted with plasma from α-tocopherol deficient rats to give the plasma concentrations shown. Equal volumes of plasma and α-tocopherol deficient red cells were incubated as described in Figure 1.

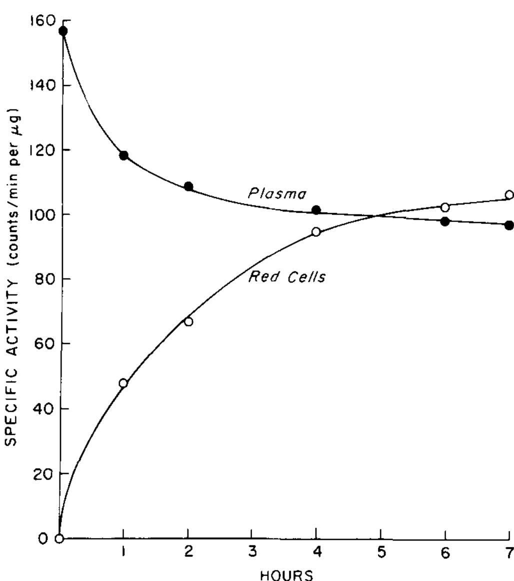

FIG. 3. Exchange in vitro of ^{3}H-α-tocopherol (156 counts/min per μg) between plasma and red cells containing equilibrium concentrations of α-tocopherol. Plasma containing 16.5 μg of labeled α-tocopherol/ml was incubated with an equal volume of unlabeled red cells containing 5.9 μg α-tocopherol/ml and the radioactivity determined at intervals as indicated. Cells were washed once before extracting. Incubation conditions as in Figure 1.

labeled plasma (Fig. 4), constant specific activity in both compartments was again attained in 6 hr. The release of α-tocopherol by erythrocytes under these conditions was slow during the first 2 hr so that the rate was almost linear for 4 hr. This may have been due to the relatively high initial concentration of α-tocopherol in these cells (ratio of red cells-plasma was 0.7). There would be a net loss of α-tocopherol to the plasma during the first few hours and the red cell specific activity would not change rapidly.

In Vivo Studies

The equilibration between plasma and erythrocytes in vivo and the rate of loss of α-tocopherol from the two blood compartments is shown in Figure 5. Four hours after dosing, a marked increase in blood level was found which peaked at 8 hr and fell sharply over the next four days. Between 11 and 18 days there was essentially no change in the very low activity retained by both cells and plasma which was about 8% of the maximum present at the 8 hr interval. The ratio of radioactivity between the compartments at 4 hr was low, 0.21, but by 8 hr an equilibrium value of about 0.5 was reached which was maintained at all subsequent periods.

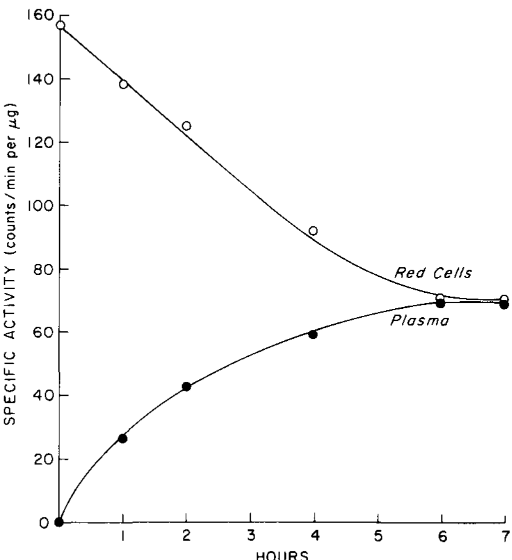

FIG. 4. Exchange in vitro of ^{3}H-α-tocopherol (156 counts/min per µg) between red cells and plasma containing equilibrium concentrations of α-tocopherol. Red cells containing 8.5 µg of labeled α-tocopherol/ml were incubated with an equal volume of unlabeled plasma containing 12.0 µg α-tocopherol/ml and the radioactivity determined at intervals as indicated. Cells were washed once before extracting. Incubation conditions as in Figure 1.

DISCUSSION

These experiments show that the exchange of α-tocopherol between erythrocytes and plasma is rapid with equilibration being reached in about 6-8 hr. Since the completion of these studies, a report by Silber et al. (5) of similar in vitro and in vivo experiments has appeared. These workers did not determine the actual red cell concentrations of α-tocopherol, however, and thus did not show that constant specific activity was reached in both plasma and red cells at equilibrium. One other difference in the two studies was that Silber et al. used dl-α-tocopherol whereas we used *d*-α-tocopherol. It has been shown that the absorption and elimination of the 1-isomer is faster than that of the naturally occurring *d*-isomer (6).

In spite of these differences, results from the two studies are remarkably similar. Both found that when labeled α-tocopherol was transferred from plasma or serum to cells containing unlabeled α-tocopherol, equilibrium was reached in about 6 hr. Calculation of the time required for 50% equilibration ($T_{1/2}$) of the data in Figure 1, as described by Silber et al. (5) gave a value of 1.6 hr. They reported a $T_{1/2}$ of 1.8 hr for the transfer of α-tocopherol from red cells to serum, whereas we found a $T_{1/2}$ of 2.5 hr in a comparable experiment. These differ-

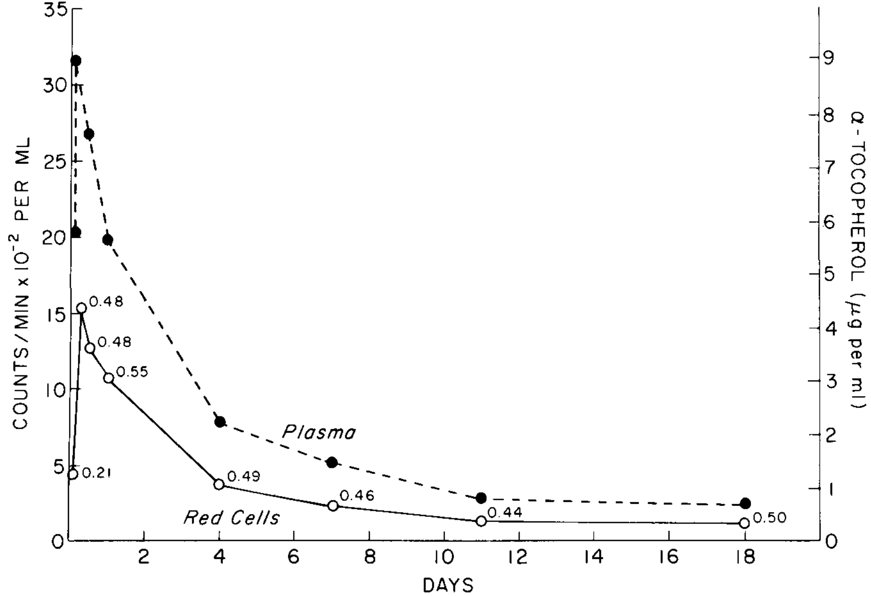

FIG. 5. Decay of α-tocopherol from plasma and erythrocytes of rats given a single oral dose of ^{3}H-α-tocopherol. A group of rats depleted of α-tocopherol for five weeks were given by stomach tube 1.4 mg *d*,α-tocopherol (358,000 counts/min per mg) in aqueous dispersion. Three rats were sacrificed at each time interval and the analyses averaged. Numbers at each time period are the ratios of red cell-plasma radioactivity.

ences are probably not significant.

The finding that constant specific activity in both cells and plasma was obtained after 6 hr indicates that all of the α-tocopherol in both compartments is exchangeable. Thus, there does not appear to be any fixed α-tocopherol which does not enter into the general blood pool.

There is a slight discrepancy in the time required for equilibration when α-tocopherol was transferred from red cells to plasma, depending on the initial plasma content of α-tocopherol (Fig. 2 and 4). As mentioned above, this may have been due to a net transfer of labeled α-tocopherol from the cells to plasma (Fig. 4), so that specific activity would have been reached quicker than if the amount of α-tocopherol in each compartment had remained constant.

The time of 1.4 hr for 50% equilibration of α-tocopherol, calculated for the data in Figure 3, is similar to the time of 1 hr found by Hagerman and Gould (7) for 50% equilibration of cholesterol between dog plasma and erythrocytes. Since Reed (8) found that the exchange of phospholipids between red cells and plasma was considerably slower, it would appear that similar factors may be operative in the exchange of both α-tocopherol and cholesterol.

As noted in Table I, the recovery of radioactivity was frequently less than 100%, usually about 85-90%. Losses of this nature were reported earlier in the development of an analytical method for α-tocopherol in red cells (1), and is considered to be due to oxidative destruction of tocopherol, probably coupled to heme-catalyzed oxidation of the polyunsaturated fatty acids of the cell membrane. The α-tocopherol oxidation products are primarily α-tocopheryl quinone but a significant fraction is so extensively oxidized that it is no longer soluble in hexane.

In previous analyses of rat blood for α-tocopherol (1,2) we found that over a wide range of plasma concentrations the ratio of red cell-plasma was 0.4-0.5. This in vivo ratio was also obtained in the present in vitro studies. When the data shown in Figures 1 and 2 were plotted as the reciprocal of the concentration vs. the reciprocal of time, the intercept on the ordinate gave the red cell or plasma concentration at infinite time. For the experiment in Figure 1, the values for red cell and plasma were 256 and 409 μg α-tocopherol/100 ml, respectively (ratio, 0.63), while for Figure 2 the values were 185 and 400 μg/100 ml, respectively (ratio, 0.46). This provides further evidence that a ratio of about 0.5 represents equilibrium conditions.

The rate of appearance of α-tocopherol in the blood after an oral dose, and its relatively rapid in vivo decay is similar to that found by Silber et al. (5). Almost identical uptake and decay curves have been reported for chickens (9,10), and peak absorption of labeled α-tocopherol in 4-6 hr in man was found by Blomstrand and Forsgren (11). It thus appears that in several species the absorption of α-tocopherol is relatively rapid but the compound is removed from the blood quickly if tissues stores are not adequate.

In contrast to the ratio of red cell-plasma α-tocopherol of about 0.5 found in rat blood, this ratio in human blood is considerably lower, about 0.27 (1). An interesting exception to this ratio, however, was found in several patients with *a*-betalipoproteinemia who had been treated with α-tocopherol (12). The ratio in these patients was approximately 1.0, with the red cells having a normal concentration while the plasma level was about one fourth of normal. These observations suggest that the affinity of the human red cell for α-tocopherol is different from that of the rat red cell. Also the equilibrium relationships demonstrated for rat blood may not be valid for human blood when low plasma concentrations are maintained over a long period. Further studies with human blood and other tissues will be required to clarify how blood tocopherol concentration reflects the general body status of the vitamin.

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Biosynthesis of Triglycerides From Triose Phosphates by Microsomes of Intestinal Mucosa

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ABSTRACT

Microsomes of hamster intestinal mucosa synthesize triglycerides from dihydroxyacetone phosphate or DL-glyceraldehyde 3-phosphate in the presence of palmitate, ATP, CoASH and NADPH or NADH but in the absence of soluble enzymes. An inhibitor of triose phosphate isomerase, 1-hydroxy-3-chloro-2-propanone phosphate, completely inhibits glyceride synthesis from glyceraldehyde 3-phosphate. This compound does not inhibit glyceride synthesis from dihydroxyacetone phosphate. These reactions confirm the conversion of glyceraldehyde 3-phosphate to dihydroxyacetone phosphate prior to glyceride synthesis.

INTRODUCTION

Results of recent studies have shown that phosphatidate may be synthesized from dihydroxyacetone phosphate (DHAP) by the combined action of the mitochondria and microsomes of guinea pig liver (1) and from DHAP or DL-glyceraldehyde 3-phosphate (GAP) by rat liver microsomes (2). Also, GAP has been observed to be the glyceride-glycerol precursor for alkyl glyceryl ether synthesis by the microsomes from preputial gland tumors (3), digestive glands and gonads (4). Subsequent studies with microsomes from mouse brain, preputial gland tumors or rat liver as well as with mitochondria from guinea pig liver have demonstrated that DHAP produced from GAP by the action of the particulate triose phosphate isomerase is the glyceryl precursor in the biosynthesis of alkyl glyceryl ether and phosphatidate (5-7).

These studies with DHAP as an acyl acceptor led us to investigate the possible utilization of this intermediate in the intestinal mucosa as an alternative to monoglyceride (MG) or glycerol 3-phosphate (GP). The results herein presented demonstrate that the microsomes of the intestinal mucosa are indeed capable of triglyceride (TG) synthesis from triose phosphates without their prior conversion to GP by the soluble enzymes.

MATERIALS AND METHODS

Diethyl acetal of GAP and dimethyl ketal of DHAP cyclohexylamine salt were purchased from Sigma Chemical Company and before use were regenerated as the K^+ salts according to the instructions supplied by Sigma. Glyceraldehyde 3-phosphate dehydrogenase and triose phosphate isomerase were obtained from Calbiochem. 1-^{14}C-palmitate from Volk Radiochemical Company was used after purification (>99%) by thin layer chromatography (TLC). The isolation of microsomes from hamster intestinal mucosa was accomplished as previously described (8). They were washed by resuspension in 0.01 M Tris-maleate, pH 7.0, containing 0.278 M mannitol with the volume corresponding to that of the original homogenate and recentrifugation at 104,000 x *g* for 1 hr. The conditions of incubation are described in Table I. The reaction was terminated by the addition of 5 ml chloroform-methanol (2:1, v/v). After acidification with 0.2 ml of 2N HCl the lipids were extracted and the neutral glycerides and phosphoglycerides were separated and quantitatively determined by previously published methods (9). Protein was estimated by the method of Lowry et al. (10). Glycerol 3-phosphate dehydrogenase and triose phosphate isomerase were assayed as described by Biesenherz et al. (11) and Biesenherz (12), respectively.

RESULTS AND DISCUSSION

The capacity of microsomes from the intestinal mucosa to convert DHAP to TG in the presence of palmitate, ATP, CoASH and NADPH is shown in Table I (Flask 1). In this conversion NADH is only half as active as NADPH (Flask 2) while in the rat liver microsomal system NADPH is half as active as NADH (2). Phosphatidate synthesis by the action of mitochondria and microsomes from guinea pig liver was observed earlier in the presence of NADPH but the substitution of NADH for NADPH in this process was not reported (1). Phosphatidate and higher glycerides are also synthesized from GAP by microsomes of the intestinal mucosa (Flasks 3-4). As with DHAP, NADPH is more effective than NADH. GP is

TABLE I

Conversion of Triose Phosphate to Triglycerides[1]

Flask	Additions	Total esters	Phosphatidate	1,2-DG	TG
			mμmoles		
1	DHAP + NADPH	205	13	8	33
2	DHAP + NADH	120	7	5	15
3	GAP + NADPH	175	17	8	21
4	GAP + NADH	110	6	3	10
5	GP	220	12	8	39
6	DHAP	95	1	3	2
7	GAP	80	2	2	2
8	NADPH	80	1	3	2
9	NADH	80	1	3	2
10	None	55	---	3	2

[1]Incubation mixture contained ATP (2 μmoles), CoASH (0.1 μmole), GSH (5 μmoles), KF (12.5 μmoles), $MgCl_2$ (5 μmoles), potassium 1-^{14}C-palmitate (0.5 μmole), potassium phosphate buffer pH 7.4 (200 μmoles), crystalline bovine serum albumin (3 mg) and washed microsomes (800 μg protein) from hamster intestinal mucosa. NADPH (2 μmoles), NADH (2 μmoles), DHAP (4 μmoles), DL-GAP (6 μmoles), DL-GP (5 μmoles) were included as indicated in the Table. Incubations were carried out for 30 min at 37 C in a Dubnoff shaker. Phosphatidate, 1,2-DG and TG were determined from the mμmoles of 1-^{14}C-palmitate incorporated into these fractions. Recovery of 1-^{14}C-palmitate in the total lipids was 97-100%.

converted to TG even in the absence of reduced pyridine nucleotides (Flask 5). The dependency of glyceride synthesis on the presence of triose phosphates and reduced pyridine nucleotides or on GP is shown by the results in the control flasks (Flasks 6-10). When triose phosphates are included in the absence of NADH or NADPH, (Flasks 6-7), palmitate incorporation and glyceride synthesis is minimal. However, appreciable synthesis of palmitoyl dihydroxyacetone (30-35 mμmoles compared to 10 mμmoles in the control flasks) is observed in these flasks. This ester migrates between MG and DG on thin layer chromatographs, co-chromatographing with authentic palmitoyl dihydroxyacetone, and yields MG upon $NaBH_4$ reduction. This lipid may have been produced by dephosphorylation of palmitoyl DHAP. No appreciable synthesis of palmitoyl-DHAP is observed in the absence of reduced pyridine nucleotides and since the incorporation of palmitate into phosphatidate is stimulated by the presence of NADPH or NADH, it would appear that in the intestinal mucosa as in rat liver (15), esterification of DHAP is stimulated only under conditions which favor the reduction of acyl-DHAP to lysophosphatidate and subsequent acylation.

When the reaction mixture contains neither triose phosphates (Flasks 8-9) nor reduced pyridine nucleotides (Flask 10), the incorporation of palmitate into phosphatidate and higher glycerides is meager. The labeled palmitate incorporated is primarily associated with the phosphatidylcholine, phosphatidylethanolamine and cholesterol ester fractions. The synthesis of these lipids is also observed in all the flasks and may be due to the acylation of endogenous precursors.

Earlier observations on glyceride synthesis by microsomes from intestinal mucosa by the GP pathway showed phosphatidate to di- and triglyceride ratios of 1:0.06 to 1:0.15. These ratios increased to 1:0.5 to 1:1.5 only in the presence of appreciable amounts of particle-free supernatant which contained a phosphatidate phosphohydrolase (9,13). However, in our studies of the conversion of triose phosphates or GP to TG, these ratios varied from 1:2 to 1:4 demonstrating the hydrolysis of the major portion of the phosphatidate to 1,2-DG which in turn is acylated to TG. This may be explained by the fact that when small amounts of microsomes are used for glyceride synthesis, high phosphate buffer (pH 7.4) concentration (133×10^{-3}M) gives optimal microsomal phosphatidate phosphohydrolase activity (14). Furthermore, under similar conditions the addition of bovine serum albumin stimulates DG-acyltransferase activity (unpublished).

Since washed microsomes are used in these studies, the participation of the soluble enzymes for the conversion of triose phosphates to L-GP need not be considered. As observed in preputial gland tumors (6) and rat liver (2), the microsomes of the intestinal mucosa are devoid of glycerol 3-phosphate dehydrogenase but do contain triose phosphate isomerase activity which is not dissociated by the washing procedures. In the presence of GAP

and GP dehydrogenase 238 mμmoles of NADH are oxidized per minute per milligram of microsomal protein from the intestinal mucosa. The requirement for this particulate triose phosphate isomerase in the conversion of GAP to glycerides is confirmed by experiments analogous to those reported earlier in the synthesis of glyceryl alkyl ether by preputial gland tumor microsomes (6) and in the synthesis of phosphatidate by rat liver microsomes (7). When an irreversible inhibitor of this enzyme (1-hydroxy-3-chloro-2-propanone phosphate, 3×10^{-4}M) is used in trials similar to those reported in Table I, the synthesis of phosphatidate and higher glycerides from DHAP or GP is not affected, whereas that from DL-GAP is completely inhibited.

The presented data demonstrate that microsomes of intestinal mucosa can synthesize triglycerides from triose phosphates without the aid of the soluble enzymes. In the mucosal cell, however, glyceride synthesis also occurs by the MG pathway and, following the conversion of triose phosphates to GP, by the GP pathway. Hence, this tissue is unique in that it possesses all the three routes for TG synthesis and it is likely that the various enzymes involved are associated as a multi-enzyme complex. A partially purified enzyme preparation capable of converting MG to TG has been reported (8). It remains to be determined whether this enzyme can also convert GP or DHAP to glycerides.

It is known that TG synthesis by the 2-MG pathway does not exhibit acyl specificity (16); furthermore, the intermediate 1,2-DG produced does not equilibrate with that of the GP pathway even when the two pathways proceed together in an in vitro milieu. Only the DG of the GP pathway forms phosphatidylcholine although DG from both pathways form TG (17,18). Enzyme preparations from liver have been shown to possess a degree of specificity for the acylation of the 2-C of GP (19,20) and the 1-C of DHAP (1) by unsaturated and saturated fatty acids, respectively. Similar phenomena in the intestinal mucosa could explain the observed differences in the fatty acid distribution of the TG and phosphatidylcholine fractions of thoracic lymph during fat absorption.

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Effect of Bound Gossypol in Cottonseed Meal on Enzymic Degradation

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ABSTRACT

The nutritive value of cottonseed protein is lowered by the presence of bound gossypol. Samples of cottonseed protein containing from 0.003% to 1.03% bound gossypol were hydrolyzed enzymically and the amounts of free amino acids released were determined. The amounts of amino acids contained in peptides not precipitated by 1% picric acid were also determined. The proportionate reduction in the release of free and peptide amino acids in the presence of bound gossypol was noted. Although the amount of lysine released is reduced in the presence of bound gossypol as expected, there is a markedly larger reduction in the release of a number of other amino acids.

INTRODUCTION

Under the processing conditions normally used in the extraction of oil from cottonseed, the pigment glands of the seed are ruptured by a combination of heat and moisture and the major pigment, gossypol, is bound to the surrounding protein (1). The principal binding site is thought to be the free epsilon-amino group of lysine (2-4). The detrimental effect of bound gossypol on the digestibility of cottonseed protein (approximately 83% digested) has been demonstrated by in vitro studies of the action of pepsin and trypsin on complexes of gossypol with cottonseed globulin (5) and purified cottonseed protein (4). In vivo studies with sheep fed soybean protein which had been heated with gossypol (6) also demonstrated a reduction in digestibility. This lowered digestibility presumably results from blocking of the action of proteolytic enzymes by the presence of gossypol in combination with the epsilon-amino group of lysine.

Lyman et al. (4), found that addition of 1.38% bound gossypol to purified cottonseed protein caused a 37% reduction in peptic digestion and a 58% reduction in tryptic digestion. This amount of reduction is far greater than the amount of lysine (3%) which is present in the protein and, therefore, must reflect the influence of bound gossypol on enzymic hydrolysis of bonds involving many amino acids other than lysine itself. A series of experiments was designed to evaluate the extent of the influence of bound gossypol on enzymic hydrolysis of cottonseed protein and the identity of the amino acids whose availability was decreased as a result of this influence.

[1]Deceased, March 9, 1969.

EXPERIMENTAL PROCEDURES

Methods and Materials

Free gossypol was determined by the method of Pons and Guthrie (7) with the exception that 80% 2-propanol (2-PrOH:H_2O, 80:20, v/v) was used as the diluting solvent instead of 95% ethanol. Total gossypol determinations were made by the method of Pons et al. (8).

Quantitative determination of amino acid composition was performed by the method of Spackman, et al. (9), using Beckman amino acid analyzers. The results were not adjusted for destruction of amino acids during acid hydrolysis.

Crude trypsin (1-100-Matheson, Coleman and Bell, Lot No. 1, and crystalline trypsin (lx crystallized from 50% $MgSO_4$) from bovine pancreas, B Grade, Calbiochem. Lot No. 32054, were used in tryptic digests. Pepsin (3x crystallized), Pentex, Inc., Lot No. C3708, provided the material for peptic digestion.

Preparation of Cottonseed Protein Samples

Low-gossypol cottonseed meal was prepared from glanded cottonseed flakes by successive extractions with hexane, 2-butanone, acetone and ether at room temperature. Gossypol was added back to this product and converted to the bound form by suspending 10 g of the meal in 100 ml of anhydrous methanol containing 100 mg of pure gossypol and stirring in the dark at room temperature for 15 hr. The methanol was removed by filtration and the product was washed three times with 25 vol of anhydrous ether to remove any free gossypol. A

TABLE I

Gossypol Content of Gossypol-Protein Complexes

Sample description	Free gossypol, %	Total gossypol, %
Low-gossypol cottonseed meal	0.01	0.32
Low-gossypol cottonseed meal with gossypol added	0.04	0.86
Glandless cottonseed meal	0.01 (0.005)[a]	0.01 (0.008)[a]
High-gossypol cottonseed meal extracted with 2-propanol	0.05	1.08

[a](Actual calculated values).

quantity of expeller-processed cottonseed meal, known to contain a high level of bound gossypol, was extracted with 10 vol (10 ml/g) of 2-propanol during four successive 9 hr periods at room temperature to remove free gossypol. A quantity of glandless cottonseed meal was prepared by hexane extraction at room temperature of glandless cottonseed which had been decorticated and flaked. These meals were all air-desolventized at room temperature and ground in a Wiley mill to pass through a 40 mesh sieve. Nitrogen and free and total gossypol determinations were made on all four samples.

Enzymic Hydrolysis of Samples

One gram samples of low gossypol cottonseed meal and low gossypol cottonseed meal with gossypol added were incubated with 250 mg of crude trypsin in 100 ml of 0.067 M K_2HPO_4 buffer, pH 7.6, containing 0.005 M calcium chloride and 0.002% merthiolate at 37 C for 0, 2, 4, 8, 16 and 24 hr. At the end of the hydrolysis periods, 10 ml aliquots from each sample were placed in 250 ml centrifuge bottles containing 50 ml of 1% picric acid for de-proteinization by the method of Hamilton and Van Slyke (10). These solutions were centrifuged at about 60 x *g* for 10 min and the excess picric acid removed by passing 50 ml aliquots through a 2 x 8 cm column of Dowex 2-X10 ion-exchange resin (chloride form) in the manner of Moore and Stein (11). The samples were reduced in volume on a rotary evaporator, diluted to a known volume with deionized water, and analyzed for amino acid content.

One half gram samples of high-gossypol cottonseed meal and glandless cottonseed meal were weighed into 250 ml erlenmeyer flasks containing 15 glass beads. Twenty-five milliliters of 0.03 M phosphate-0.015 M citrate buffer, pH 3.0, was added to each flask along with 0.05 ml of phenol and 2.5 ml of pepsin (1 mg/ml). The stoppered flasks were incubated at 37 C for 12 hr, at which time a second 2.5 ml of pepsin was added and the incubation continued. After 24 hr of peptic digestion, 1.05 ml of 1 N NaOH and 25 ml of 0.03 M Na_2HPO_4 buffer, pH 7.4, was added along with 2.5 ml of trypsin (1 mg/ml) and incubation was continued. A second increment of 2.5 ml of trypsin was added 12 hr later and digestion continued for another 12 hr. At the end of 48 hr of combined peptic and tryptic hydrolysis, the digestion mixtures were removed from the incubator and 20 ml aliquots were transferred to centrifuge bottles for de-proteinization as previously described. The effluent from the Dowex column was reduced to dryness with a rotary evaporator and re-dissolved in 10 ml of 0.1 N HCl. A 5 ml aliquot was taken for the determination of free amino acids and the remaining 5 ml was subjected to hydrolysis with 6 N HCl for the determination of total amino acid content of the de-proteinized sample.

Estimation of Number of Amino Acid Residues in Peptides not Precipitated by 1% Picric Acid

Solutions of insulin (51 residues), insulin A-chain (21 residues), insulin B-chain (30 residues) and glutathione (3 residues) were made with the concentration approximating that of the supernatant solutions. A proportionate amount of 1% picric acid was added and the results observed.

RESULTS AND DISCUSSION

The content of free and total gossypol in the four samples of cottonseed meal prepared is listed in Table I, showing a range of total gossypol from 0.01% in the glandless meal to 1.08% in the high-gossypol 2-propanol-extracted cottonseed meal. The free gossypol content was sufficiently low as to have a negligible effect on the experiments. The effect of a 0.51% increase in bound gossypol on enzymic digestion by crude trypsin is tabulated in Table II. When low-gossypol cottonseed meal and low-gossypol cottonseed meal with gossypol added were incubated with crude trypsin, a progressive release of amino acids was noted with increase in time in each instance. However, in the case of the sample to which gossypol was added, there was a reduction in the amount of amino acids released. This was expected in view

TABLE II

Effect of Bound Gossypol on the Liberation of Amino Acids From Cottonseed Meal by a Crude Preparation of Trypsin[a]

Amino acids	A Cottonseed meal containing 0.32% bound gossypol, %	B Cottonseed meal containing 0.86% bound gossypol[b], %	Reduction in amino acid liberation, %
Lysine	4.41	3.70	16.1
Histidine	2.46	1.99	19.1
Arginine	11.02	10.74	2.5
Aspartic acid	2.05	0.79	61.5
Threonine	2.84	1.73	39.1
Serine	7.98	5.35	33.0
Glutamic acid	5.69	2.89	49.2
Proline	1.80	1.01	44.0
Glycine	1.99	1.16	41.7
Alanine	3.89	2.61	32.9
Valine	4.88	3.23	33.8
Methionine	1.75	1.28	26.9
Isoleucine	3.53	2.57	27.2
Leucine	6.48	5.60	13.6
Tyrosine	4.00	3.32	17.0
Phenylalanine	5.41	4.06	13.9
Total	70.18	52.04	

[a]Expressed as percent of protein, duplicate analyses.
[b]Meal A after reaction with gossypol.

of the known reaction of gossypol with the epsilon-amino group of lysine and the consequent blocking effect on the action of trypsin whose specificity requires either lysine or arginine, with unsubstituted side chains, as substrates.

Since crude trypsin contains chymotrypsin, which cleaves bonds involving the carboxyl groups of tyrosine and phenylalanine, and carboxypeptidase, which removes the carboxyl-terminal residues from peptides, the total amino acids released by its action were greater than would have been expected if a pure preparation of trypsin had been used. However, even in the presence of these additional enzymes, there was a proportionally greater reduction in the release of a number of other amino acids as compared with lysine. Two factors may be involved here: (a) When the epsilon-amino group of lysine is blocked, with gossypol in this instance, the peptide chain is not cleaved at that point by trypsin and longer peptides result with fewer amino acids being released. (b) When gossypol is bound to a protein molecule, due to the coiling of the peptide chain and the size of the gossypol molecule, proteolytic enzymes may be sterically hindered from attacking certain segments of the protein molecule. It would be expected that those amino acids which showed the greatest reduction were contained within these longer peptides in association with the lysine-gossypol or possibly other amino acid-gossypol complexes.

The action of pepsin, although relatively nonspecific, is favored by the presence of an aromatic ring in the side chain of the amino acid on either side of the peptide bond. Pepsin is specific in that it hydrolyzes only peptide bonds and not amides or esters. Trypsin, on the other hand, hydrolyzes peptide, amide and ester bonds involving the carboxyl groups of lysine and arginine. In the absence of exopeptidases or dipeptidases, the action of crystalline pepsin and trypsin on protein would be expected to produce a small amount of free amino acids and a large quantity of peptides of various lengths. The number and length of the peptides produced would be affected by gossypol bound to the protein molecule.

The results of the sequential digestion of glandless cottonseed meal and high-gossypol 2-propanol-extracted cottonseed meal with crystalline pepsin and trypsin are shown in Table III. Amino acid analysis of the supernatant solution after picric acid treatment produced a number of peaks on the chromatogram in addition to the normal peaks for the individual amino acids. It was assumed that peptides which had not been precipitated by picric acid were responsible for these peaks and aliquots of the supernatant solutions were hydrolyzed with 6 N HCl and analyzed for amino acid content. The increased amounts of

TABLE III

Effect of Bound Gossypol on Liberation of Amino Acids and Peptides From Cottonseed Meal by Crystalline Pepsin and Trypsin[a]

Amino acids	Total amino acids liberated by hydrolysis with 6 N HCl		Free amino acids liberated by enzymatic hydrolysis			Free AA + Peptide AA liberated by enzymatic hydrolysis		
	Glandless CSM, %	2-Propanol Extr. CSM, %	Glandless CSM, %	2-Propanol Extr. CSM, %	Reduction, % %	Glandless CSM, %	2-Propanol Extr. CSM, %	Reduction, %
Lysine	3.97	3.22	0.13	0.06	43	2.68	1.25	42
Histidine	2.50	2.02	0.06	0.02	59	1.45	0.57	51
Arginine	10.18	8.17	1.08	0.57	34	6.75	3.35	38
Aspartic acid	7.36	9.55	0.18	0.08	66	5.87	3.44	55
Threonine	2.53	3.53	0.01	0.02	+43	2.06	1.22	58
Serine	3.50	4.79	0.02	0.03	+10	2.07	1.87	34
Glutamic acid	16.88	22.94	0.20	---	100	11.33	8.28	46
Proline	2.91	3.95	---	0.12	---	2.30	1.22	61
Glycine	3.46	4.96	0.03	0.03	30	2.75	1.48	62
Alanine	3.23	4.13	0.07	0.08	11	2.26	1.52	47
Valine	3.64	5.10	0.06	---	100	2.99	1.64	61
Methionine	0.96	1.09	0.05	0.04	30	0.60	0.07	90
Isoleucine	2.57	3.45	0.06	0.05	38	1.96	1.09	59
Leucine	4.36	6.39	0.08	0.05	57	3.37	1.83	63
Tyrosine	2.32	2.76	0.07	0.07	16	1.64	0.79	60
Phenylalanine	4.22	5.63	0.47	0.31	51	3.32	1.85	58
Total	75.76	92.79	2.70	1.63	51	53.89	31.80	52

[a]Expressed as per cent of protein, duplicate analyses.

amino acids present in the acid-hydrolyzed supernatant solutions confirmed the assumed presence of peptides.

In the procedure used to estimate the size of peptides which are precipitated by 1% picric acid, it was found that insulin and the B chain of insulin precipitated immediately upon the addition of picric acid. However, the insulin A chain precipitated very slowly, with the total precipitate appearing to be less than the original solute, and the glutathione remained in solution. From this evidence, it is proposed that under the conditions used in this procedure, and depending on their amino acid composition, peptides containing fewer than 15 to 20 residues are not precipitated by 1% picric acid.

Comparison of the total amino acids liberated from glandless cottonseed meal and high-gossypol 2-propanol-extracted cottonseed meal revealed that amino acids make up a larger percentage of the protein (Kjeldahl nitrogen x 6.25) in the 2-propanol-extracted material than in the glandless meal. Extraction of non-protein nitrogen by 2-propanol probably accounts for this in view of the findings of Harris and Hayward (12), who showed that 2-propanol extracts pigments, phospholipids, carbohydrates and smaller amounts of sterols from cottonseed meats.

The use of crystalline pepsin and trypsin produced the anticipated results, in that a small amount of free amino acids were released by their action, with much larger amounts of amino acids being contained in small peptides which were not precipitated by 1% picric acid. In the digestive tract of an animal, the presence of other endopeptidases, exopeptidases and dipeptidases would undoubtedly degrade many of these small peptides to their component amino acids. However the blocking effect of bound gossypol on the action of these two enzymes is indicative of its action in the digestive tract. Further studies, using the complete spectrum of digestive enzymes should help to more clearly identify those amino acids rendered unavailable by the presence of bound gossypol in cottonseed protein and aid in proper supplementation of rations containing this material.

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Triose Phosphates as Precursors of Glyceride Biosynthesis by Rat Liver Microsomes[1]

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ABSTRACT

Washed rat liver microsomes synthesize phosphatidate and di- and triglycerides from dihydroxyacetone phosphate (DHAP) or DL-glyceraldehyde 3-phosphate (DL-GAP) and fatty acids. The cofactors required in either case are ATP, CoASH, Mg^{++}, and either NADH or NADPH. NADH is twice as active as NADPH. In the presence of γ-^{32}P-ATP, 1-^{14}C-palmitate, CoASH and NADH, the combined actions of glycerol kinase (ATP-glycerol phosphotransferase EC 2.7.1.30), and microsomes convert dihydroxyacetone to phosphatidate with the molar ratio of ^{14}C:^{32}P approximately 2:1. Since glycerol 3-phosphate dehydrogenase (L-glycerol 3-phosphate: NAD oxidoreductase EC 1.1.1.8) is not present in microsomes, the synthesis of glycerides from either DHAP or GAP need not occur via their conversion to glycerol 3-phosphate (GP). However, the presence of triose phosphate isomerase (D-glyceraldehyde 3-phosphate:ketol-isomerase EC 5.3.1.1) in microsomes, suggests the conversion of GAP to DHAP during glyceride synthesis. The requirement for this conversion was confirmed by the use of 1-hydroxy-3-chloro-2-propanone phosphate (HCPP), an irreversible inhibitor of triose phosphate isomerase. The synthesis of glycerides from DL-GAP was completely inhibited by HCPP, whereas that from DHAP was not.

INTRODUCTION

The biosynthesis of glycerides has been known to occur by the glycerol 3-phosphate pathway in several mammalian tissues. The reduction of DHAP by the action of cytoplasmic glycerol 3-phosphate dehydrogenase and NADH, or the phosphorylation of glycerol by glycerol kinase and ATP, yielded GP for glyceride synthesis. Recently Hajra has reported the conversion of DHAP to phosphatidate by the combined action of the mitochondria and microsomes of guinea pig liver (1). Snyder and co-workers (2,3) and Hajra (4) have also shown triose phosphates to be precursors of alkyl ethers. Preliminary studies in this laboratory have suggested that DHAP and GAP may act as acyl acceptors and that their conversion to GP may not be necessary (5,6). However, a demonstration of this process required several detailed studies, the results of which are presented in this paper. These results demonstrate that in the absence of either the particle free supernatant or an appreciable amount of mitochondrial protein, rat liver microsomes can utilize either DHAP or GAP as precursors for glyceride synthesis.

MATERIALS AND METHODS

The dimethyl ketal of dihydroxyacetone phosphate, the dimethyl acetal of DL-glyceraldehyde 3-phosphate and dihydroxyacetone (DHA) were purchased from Sigma Chemical Co. The free carbonyl compounds were regenerated by hydrolysis with Dowex 50-X4,H^+, and converted to their potassium salts using $KHCO_3$. Glycerol 3-phosphate dehydrogenase and triose phosphate isomerase were obtained from Calbiochem. Glycerol kinase was obtained from Boehringer Mannheim Corp. Glycerol dehydrogenase (glycerol:NAD oxidoreductase, EC 1.1.1.6) was purchased from Worthington Biochemical Corp. 1-^{14}C-Palmitate from Volk Radiochemical Co. was used after purification (>99%) by thin layer chromatography (TLC). γ-^{32}P-ATP was purchased from Amersham Searle Corp. Monopalmitoyl dihydroxyacetone was prepared by the method of Hajra and Agranoff (7). Purified palmitic acid was purchased from the Hormel Institute. Phosphatidic acid used as standard was obtained from Pierce Chemical Co.

ENZYME ASSAYS

Succinate dehydrogenase (succinate: phenazine methosulfate oxidoreductase EC 1.3.99.1) activity was measured as described by Arrigoni and Singer (8). The method of Cooperstein and Lazarow (9) was used to assay cyto-

[1]Presented in part at the AOCS Meeting, Minneapolis, October 1969.

TABLE I

Conversion of Triose Phosphates Into Phosphatidate and Neutral Glycerides[a]

Flask	Additions	Phosphatidate mμmoles	1,2-DG mμmoles	TG mμmoles
1	None	28	2	7
2	NADH	19	3	6
3	NADPH	34	6	6
4	DHAP	32	3	6
5	DL-GAP	38	3	12
6	DHAP + NADH	256	27	19
7	DL-GAP + NADH	192	18	26
8	DHAP + NADPH	148	22	20
9	DL-GAP + NADPH	82	11	20

[a]Incubation mixture contained ATP (2.5 μmoles), CoASH (0.1 μmole), GSH (12.5 μmoles), KF (12.5 μmoles), $MgCl_2$ (5 μmoles), potassium 1-^{14}C-palmitate (1 μmole), potassium phosphate buffer pH 7.4 (200 μmoles) and washed rat liver microsomes (4 mg protein) in a total volume of 1.5 ml. DHAP (5 μmoles), DL-GAP (10 μmoles) and NADH or NADPH (2 μmoles) were added as shown in the Table. Incubations were carried out at 37 C in a Dubnoff shaker for 30 min. The amount of glyceride synthesis was calculated from the mμmoles of 1-^{14}C-palmitate incorporated in each lipid class. The phosphatidate synthesized was calculated from the 1-^{14}C-palmitate incorporated in that lipid fraction as well as that in the 1,2 di- and triglycerides (1,2-DG, TG) formed from phosphatidate.

chrome oxidase (cytochrome C:O_2 oxidoreductase EC 1.9.3.1). The procedure described by Beizenherz et al. (10) and Beisenherz (11) was employed to determine glycerol 3-phosphate dehydrogenase and triose phosphate isomerase activities, respectively. Glucose 6-phosphatase (D-glucose 6-phosphate:phosphohydrolase EC 3.1.3.9) was measured as described by Swanson (12). Glycerol dehydrogenase was assayed according to Burton (13). Protein determinations were carried out by the method of Lowry et al. (14).

PREPARATION OF MICROSOMES

Rat livers were removed immediately after killing the animals, and washed in ice cold 0.25 M sucrose. All subsequent operations were carried out at 0-5 C. Liver homogenates were prepared in 2 vol of 0.25 M sucrose using a loose fitting Potter Elvehjem homogenizer. The homogenate was centrifuged at 1,000 x *g* for 15 min and the pellet of whole cells, nuclei and cell debris was discarded. The supernatant was centrifuged at 8,000 x *g* for 10 min to isolate the mitochondria when this organelle was required. The mitochondria were washed by resuspension in 0.25 M sucrose and centrifugation before use as an enzyme source.

To prepare the microsomes the 1,000 x *g* supernatant was centrifuged at 15,000 x *g* for 15 min to remove the mitochondria and lysosomes. The supernatant was siphoned carefully so as not to disturb the pellet, leaving 2-3 ml supernatant above it. The supernatant was then centrifuged at 104,000 x *g* for an hour. The microsomal pellet was suspended in 0.25 M sucrose in a volume corresponding to that of the original homogenate and centrifuged at 104,000 x *g* for one hour. In some experiments this washing procedure was repeated once more. Washed microsomes were suspended in a volume of 0.25 M sucrose corresponding to one-half the volume of the original homogenate and aliquots containing 3-4 mg protein were used in the experiments.

Incubations were carried out as described in the tables and terminated by adding 5 ml chloroform-methanol (2:1, v/v). After acidification with 0.2 ml 2N HCl, glycerides and phosphoglycerides were isolated and analyzed by procedures described by Johnston et al. (15).

RESULTS AND DISCUSSION

Conversion of Triose Phosphates to Glycerides. In order to determine the ability of microsomal enzymes to convert DHAP and GAP to glycerides without the participation of the soluble enzymes, the experiments reported in Table I were performed. When the reaction mixture was devoid of triose phosphates (Flask 1), or contained NADH (Flask 2) or NADPH (Flask 3), a minimal synthesis of phosphatidate and higher glycerides occurred. Neither the reactions nor the endogenous substrates responsible for this synthesis are known. When DHAP or DL-GAP was included and reduced pyridine nucleotides were excluded, glyceride synthesis was also minimal (Flasks 4,5). However, when the incubation mixture contained DHAP or GAP and NADH, phosphatidate synthesis was stimulated severalfold (Flasks 6,7). NADPH was also effective in this process

TABLE II

^{32}P-Phosphatidate Synthesis From Dihydroxyacetone[a]

Flask	Glycerol Glycerol kinase	NADH	NADPH	DHA	Phosphatidate ^{32}P mμmoles	^{14}C mμmoles	$^{14}C/^{32}P$
1	+	+	-	+	58		
2	+	-	+	+	25		
3	+	-	-	+	3		
4	-	+	-	+	3		
5	+	+	-	-	3		
6	+	+	-	+	47.5	85	1.8

[a]Incubations were carried out for 2 hr at 37 C in the presence of glycerol kinase (40 μg), γ-^{32}P-ATP (2 μmoles, 0.5 μc), $MgCl_2$ (0.4 μmole), triethanolamine-HCl buffer pH 7.4 (4 μmoles) and DHA (3 μmoles) in a total volume of 0.5 ml. Incubation was continued for 30 min after the addition of CoASH (0.1 μmole), GSH (12.5 μmoles), KF (12.5 μmoles), $MgCl_2$ (5 μmoles), ATP (2.5 μmoles), potassium palmitate (0.5 μmole), NADH or NADPH (2 μmoles) and microsomes (4 mg protein)in a total volume of 1.5 ml. In Flask 6, preincubation with glycerol kinase was avoided. Instead, a single incubation in the presence of DHA, γ-^{32}P-ATP, CoASH, GSH, NADH, glycerol kinase and microsomes was carried out for 30 min.The unlabeled palmitate was replaced by 1-^{14}C-palmitate (0.5 μmole).

although not as active as NADH (Flasks 8,9). Previous studies had demonstrated the activation of the microsomal phosphatidate phosphohydrolase (EC 3.1.3.4) by a high concentration of buffer in the incubation medium (16). This can explain the increase in di- and triglyceride synthesis that occurred with increased phosphatidate synthesis (Flasks 6-9). Maximal synthesis of higher glycerides requires the participation of the soluble fraction containing a phosphatidate phosphohydrolase which acts specifically on membrane bound biosynthesized phosphatidate (15,17). However, this fraction was not used since it contains enzymes that convert DHAP and GAP to GP under these experimental conditions. ATP and CoASH are needed for the activation of palmitate and hence when either of these were not included in Flasks 6-9, phosphatidate synthesis was found to be meager. Furthermore, Mg^{++} was included in the reaction mixture since this cation stimulated the 1-^{14}C-palmitate incorporation into phosphatidate two- to threefold. The appreciable synthesis of phosphatidate that occurred in the absence of Mg^{++} may be due to the presence of this cation in the microsomal protein.

Recovery of ^{14}C activity of palmitate obtained in the lipids isolated subsequent to incubation was 97-100% demonstrating no loss of any specific lipid during the isolation procedures. Unlike the direct measure of labeled glycerides, the oxidation of reduced pyridine nucleotide was an inconclusive assay for the conversion of triose phosphates to glycerides. In the absence of triose phosphates, 133 mμmole NADH/min (Flask 2) and 71 mμmoles NADPH/min (Flask 3) were oxidized. Significantly higher rates of oxidation, 171 mμmoles NADH/min (Flask 6) and 95 mμmoles NADPH/min (Flask 8) were obtained in the presence of DHAP. Microsomal desaturase as well as enzymes not related to glyceride synthesis may be responsible for the oxidation of pyridine nucleotides in the absence of DHAP. The oxidation observed in the presence of DHAP may be due to the synthesis of phosphatidate along with oxidation from DHAP independent reactions.

Appreciable incorporation of 1-^{14}C-palmitate (125-175 mμmoles) into phosphatidylcholine and phosphatidylethanolamine fractions was also observed in these studies. However, the ^{14}C incorporation was of the same order of magnitude in all the flasks and hence did not depend on the presence of either triose phosphates or reduced pyridine nucleotides. These phosphoglycerides may have been produced either by the acylation of endogenous lysophosphoglycerides (18,19) or by the transesterification reactions catalyzed by phospholipases (20).

Acylation of DHAP by either the mitochondria or microsomes from guinea pig liver to yield acyl-DHAP has been described by Hajra (21). In our studies, in the absence of NADH or NADPH, significant conversion of DHAP to 1-^{14}C-palmitoyl-DHAP was not observed, although a small amount of palmitoyl-DHA was synthesized. It appears that the palmitoyl-DHAP is hydrolyzed to palmitoyl-DHA by a phosphohydrolase present in rat liver microsomes. Chemically synthesized palmitoyl-DHA and 1-^{14}C-palmitoyl-DHA from the lipid mixture isolated following incubation had R_f values of 0.3 on TLC using silica gel G plates with a

solvent system consisting of *n*-hexane-diethyl ether, methanol-acetic acid (90:22:3:2, v/v) and 0.2 on Adsorbosil-5 plates with a solvent system consisting of petroleum ether (b.p. 30-60 C)-diethyl ether-acetic acid (70:30:1.5, v/v). The identity of this ^{14}C-palmitoyl-DHA was further confirmed by the association of the radioactivity with monoglycerides following reduction with $NaBH_4$. The presence of NADH or NADPH enhances the fatty acid incorporation severalfold indicating that in rat liver microsomes, acylation of DHAP is coupled to and dependent on the reduction of acyl-DHAP to lysophosphatidate and further conversion to phosphatidate.

Employing approximately equal amounts of microsomal and mitochondrial proteins from guinea pig liver, and utilizing NADPH as the cofactor for reduction, conversion of DHAP to phosphatidate has been reported recently (1). Although either of these organelles catalyzed the synthesis of acyl-DHAP (21), only mitochondria was shown to be capable of reducing this derivative to lysophosphatidate and only NADPH was active in the reaction (22).

Our attempts to study the enzymic reduction of acyl-DHAP and to compare the effectiveness of NADH or NADPH in this process have not been successful. Oxidation of NADH or NADPH by rat liver microsomes dependent on the presence of palmitoyl-DHAP was not observed. It is likely that the microsomal dehydrogenase acts on the biosynthesized acyl derivative rather than on the added lipid substrate. The lipids isolated after incubating palmitoyl-DHAP in the presence of reduced pyridine nucleotides and microsomes contained palmitoyl-DHA and palmitate but no lysophosphatidate. Furthermore, addition of glycerol 3-phosphate dehydrogenase to the protein free supernatant of such incubation resulted in the oxidation of NADH. The lack of activity of this enzyme on acyl-DHAP suggested the presence of DHAP in such incubations. These observations demonstrate that the microsomal deacylases and phosphohydrolases are able to cleave palmitoyl-DHAP to palmitate, palmitoyl-DHA and DHAP. Our studies of glyceride synthesis by rat liver microsomes show that this organelle has all the enzymes necessary for the conversion of DHAP to phosphatidate and that NADH is more active as a hydrogen donor than NADPH. The contamination of the microsomes by mitochondrial fragments was found to be not more than 12% based on succinic dehydrogenase and cytochrome oxidase activities. Furthermore, when the conditions for the conversion of triose phosphates to phosphatidate given in Table I were extended to mitochondria, phosphatidate synthesis was found to be 20-30% of that observed with microsomes. The mitochondrial protein may have contained 20% microsomes as indicated by the glucose-6-phosphatase activity. Thus the observed phosphatadiate synthesis by mitochondria may have been due to the microsomal enzymes present.

TABLE III

Effect of HCPP[a] on Phosphatidate Synthesis From Triose Phosphates

Flask	Triose phosphate	HCPP	Phosphatidate (mμmoles)
1	DHAP	-	136
2	DHAP	+	118
3	DL-GAP	-	95
4	DL-GAP	+	2

[a]1-hydroxy-3-chloro-2-propanone phosphate. Incubation conditions are the same as those given in Table I except that HCPP (0.4 μmole) was also included in Flasks 2 and 4. The phosphatidate synthesized was calculated as indicated in Table I.

^{32}P-Phosphatidate Synthesis From Dihydroxyacetone. In order to confirm the conversion of DHAP to phosphatidate by rat liver microsomes, experiments reported in Table II were carried out. Since glycerol kinase catalyzes the phosphorylation of DHA (23-25), its incubation with γ-^{32}P-ATP should produce ^{32}P-labeled DHAP. This was confirmed by the observation of the oxidation of NADH spectrophotometrically in the presence of crystalline glycerol 3-phosphate dehydrogenase and by the association of ^{32}P with DHAP subsequent to paper chromatography or high voltage electrophoresis separations (7). ^{32}P-labeled DHAP is then converted to ^{32}P-phosphatidate by the microsomal enzymes in the presence of NADH, fatty acids and the cofactors necessary for fatty acid activation. As was observed in Table I, either NADH or NADPH are active although the former is twice as active as the latter (Flasks 1,2). When reduced pyridine nucleotides were excluded from the second incubation, phosphatidate synthesis was meager (Flask 3) which confirms the absence of ^{32}P-GP in the reaction mixture. Furthermore, the dependency on the presence of glycerol kinase or on DHA is shown by the minimal synthesis observed in Flasks 4 and 5, respectively. Although preincubation with glycerol kinase, DHA and ATP was carried out to assure the synthesis of DHAP, several experiments showed that the simultaneous action of glycerol kinase and microsomes on DHA, fatty acids, ATP, CoASH and NADH also produces phosphatidate. Thus in Flasks 6, γ-^{32}P-ATP and 1-^{14}C-palmitate were included

and as expected, phosphatidate had a molar ratio of ^{14}C:^{32}P of approximately 2:1. Since neither oxidation of NADH in the presence of DHA nor the reduction of NAD in the presence of glycerol was observed, it can be concluded that glycerol dehydrogenase is not present in the microsomes. Hence, phosphatidate synthesis by the combined action of glycerol kinase and microsomes occurred via the conversion of DHA to DAHP and not the reduction of DHA to glycerol and the subsequent phosphorylation to GP.

Absence of Glycerol 3-Phosphate Dehydrogenase and Presence of Triose Phosphate Isomerase in Microsomes. The participation of triose phosphates as glyceride-glycerol precursors has been generally considered to occur via their conversion to GP by the action of soluble triose phosphate isomerase and glycerol 3-phosphate dehydrogenase. In the presented studies, washed microsomes were used for the conversion of triose phosphates to glycerides and hence the involvement of the soluble enzymes was avoided. However, if these enzymes are also present in the microsomes, a direct conversion of triose phosphates to glycerides without the intermediate production of GP would be doubtful.

Several experiments were performed to test the presence of glycerol 3-phosphate dehydrogenase in washed microsomes. Oxidation of NADH or NADPH by microsomes (10-100 μg protein) dependent on the presence of DHAP does not occur. Also, NAD is not reduced by microsomes in the presence of GP. The absence of glycerol 3-phosphate dehydrogenase as suggested by these assay procedures is not due to an inhibition by the microsomes since the enzymatic activity of crystalline glycerol 3-phosphate dehydrogenase is not hampered by the presence of this organelle. We also carried out incubations using 10 times the microsomal protein NADH and DHAP of Flask 6 in Table I, in the absence of palmitate and cofactors for its activation. Following incubation, the procedure for isolation of ^{14}C-GP described by Hajra (21) showed no GP as measured by the reduction of NAD in the presence of added glycerol 3-phosphate dehydrogenase. From these studies it can be concluded that microsomes do not contain glycerol 3-phosphate dehydrogenase and that the conversion of DHAP to glycerides occurs without its prior conversion to GP. The absence of this dehydrogenase in the microsomes isolated from preputial gland tumors has also been observed by Wykle and Snyder during their investigations of the biosynthesis of alkyl glyceryl ethers from DHAP (3).

When washed rat liver microsomes were incubated in the presence of DL-GAP, glycerol 3-phosphate dehydrogenase and NADH, appreciable oxidation of NADH was observed (350 mμmoles NADH oxidized/min/mg microsomal protein). In spite of washing the microsomes twice, the specific activity of this enzyme was not decreased appreciably (320 mμmoles NADH oxidized/min/mg protein). Hence it can be concluded that triose phosphate isomerase is closely associated with the microsomes. Recent studies on alkyl ether synthesis have resulted in similar conclusions regarding the presence of this isomerase in microsomes from preputial gland tumors (3). The presence of this enzyme in rat liver microsomes raises the question as to whether GAP must be converted to DHAP for glyceride synthesis. Recent investigations by Wykle and Snyder (3) and Hajra (4) demonstrate that indeed this conversion occurs in the synthesis of alkyl glyceryl ethers from GAP. Wykle and Snyder used 1-hydroxy-3-chloro-2-propanone phosphate (HCPP), an irreversible inhibitor of triose phosphate isomerase, to demonstrate that the conversion of GAP to DHAP is necessary (3). We have also used this inhibitor to determine whether similar triose phosphate conversion is needed for phosphatidate synthesis from GAP by rat liver microsomes. The results of these studies are given in Table III. While the phosphatidate synthesis from DHAP was not affected appreciably, that from DL-GAP was completely inhibited by HCPP. It can be concluded from these observations that GAP has to be converted to DHAP by the microsomal triose phosphate isomerase prior to its utilization for glyceride synthesis.

Investigations reported in this paper and those that have been reported recently (1-4) demonstrate that triose phosphates may be converted to glycerides without the participation of the enzymes in the cytosol. The extent of reactions as related to their conversion to GP by the soluble enzymes in the overall process of glyceride synthesis awaits further investigation. Although these reactions have been demonstrated using enzyme preparations from rat, guinea pig livers or preputial gland tumors, it is likely that they are of a general nature and may occur in all species and in several tissues. It has been reported that tumor cells are either devoid of or deficient in glycerol 3-phosphate dehydrogenase (26). In these cells, the production of GP from DHAP or GAP will not occur or will occur to a limited extent. An extension of the presented studies to cancer cells will be necessary to determine whether glyceride synthesis occurs in them preferentially by the utilization of triose phosphates rather than of GP.

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Tumor Lipids: Carbon Number Distribution of Triglycerides and Glyceryl Ether Diesters

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ABSTRACT

Triglycerides and glyceryl ether diesters were isolated from a number of transplantable rat and mouse tumors and analyzed intact by gas liquid chromatography (GLC). The carbon number distributions of the triglycerides were distinctly different from those of the glyceryl ether diesters. Both glyceride classes contained abnormally high molecular weight species. The data support earlier evidence that indicated diglycerides used for the biosynthesis of phosphatides are also precursors of triglycerides in neoplasms. Glycerides isolated from three centrifuged fractions of Ehrlich ascites cells exhibited similar carbon number distributions. The results indicate a random distribution of triglyceride and glyceryl ether diester molecular species among cellular organelles. Triglycerides, glyceryl ether diesters and neutral plasmalogens of the same carbon number were partially resolved by GLC.

INTRODUCTION

The occurrence of an unidentified neutral lipid in tumor tissue was first reported by Bollinger (1) and Cheng et al. (2) and later confirmed (3,4). The lipid, isolated from Ehrlich ascites cells, was subsequently identified as a glyceryl ether diester (GEDE) (5). Reduction of total neutral lipids from several transplantable rat and mouse tumors with lithium aluminum hydride (6) has revealed relatively high levels of alkyl ethers of glycerol in these neoplasms (7). However, free alkyl glyceryl ethers and alkyl acyl glycerides (8), in addition to GEDE, contribute to the total percentage of glyceryl ethers obtained from the neutral lipids by hydrogenolysis. Thin layer chromatography (TLC) of the neutral lipids of the rat and mouse tumors indicated the presence of GEDE (7), but class identity and purity of GEDE, and even triglycerides, are difficult to establish by adsorption TLC. Baumann et al. (9) have demonstrated the similarities in the TLC R_f values of diacyl and alkyl acyl glycerides of diols (ethane and propane) and that of GEDE and triglycerides. The occurrence of diol lipids is covered in a review by Bergelson (10).

The use of high temperature gas liquid chromatography (GLC) to identify and quantify intact GEDE, triglycerides, alkyl acyl, diacyl and dialkyl glycerides has been well established (5,8,11). This report describes the GLC analysis and comparison of carbon numbers of triglycerides and GEDE isolated from several transplantable rat and mouse tumors.

EXPERIMENTAL PROCEDURES

Lipid Material

Transplantable rat and mouse tumors were grown and harvested as described previously (7). A non-transplantable fibroadenoma that occurs in rats approximately one year after exposure to 800 R total body irradiation was also examined. Ehrlich ascites cells were disrupted in a French pressure cell and separated into three fractions [15,000 *g*(10 min), 34,000 *g*(3 hr), and supernate] by centrifugation. The term fraction is used instead of names of specific organelles usually sedimented at these centrifugation forces because of considerable contamination from other cell organelles and cell fragments. However, the impure nature of these fractions does not affect the validity of the data. The total lipids were extracted according to the Folch et al. procedure (12). Neutral lipids were separated from the phospholipids by silicic acid chromatography (13), and the triglycerides and GEDE were isolated by preparative TLC (5,8). Both lipid classes were purified by rechromatography in a hexane-diethyl ether 90:10 v/v solvent system. Quantification was carried out on hydrogenated samples (14), which prevented peak broadening caused by highly unsaturated glyceride species.

Gas Liquid Chromatography

The satisfactory and reliable high temperature chromatographic system used for the quantitative GLC analysis of intact triglycerides and related high molecular weight compounds was constructed from an Aerograph Model 204

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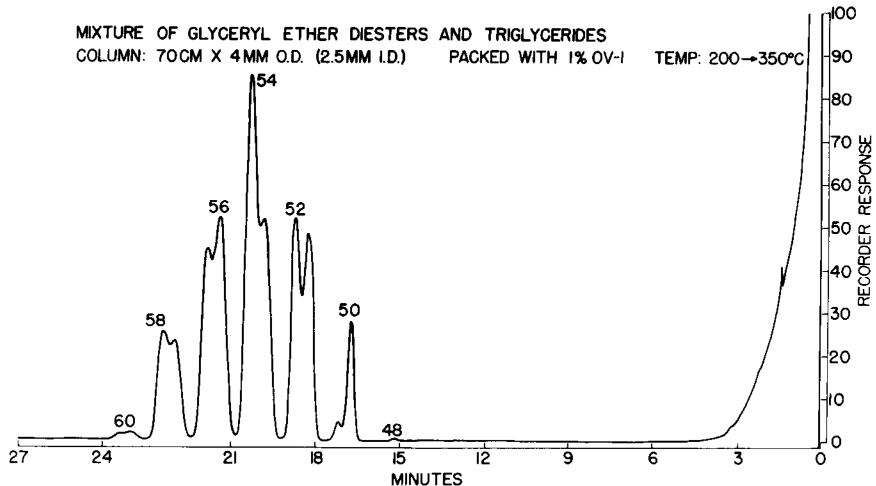

FIG. 1. A chromatogram tracing depicting the partial resolution of triglycerides from glyceryl ether diesters of the same carbon number. Analysis was carried out on a 70 cm x 4 mm O.D. (2.5 mm I.D.) Pyrex column packed with 1% OV-1 and manually temperature-programmed from 200-350 C. The large solvent peak was due to a large amount of benzene solvent.

chromatograph. The modified chromatograph has been used to analyze a number of high molecular weight lipids (8,11,14-16), but the modifications necessary for quantitative analysis have not been described.

The column inlet, flash heater and column exit were modified to accept a 4 mm O.D. (2.5 mm I.D.) glass column (70 cm), fitted with 3/16 in. Kovar Pyrex to metal seals described by Radin (17) and Litchfield et al. (18). Extension of the column through the flash heater to the injection septum allows on-column sample injection without exposure to metal surfaces. Unpacked columns were installed while being heated to the softening point to allow tightening of the Swagelock fittings without breaking the column. Gas pressure was used to pack and unpack the installed column. The column was packed with either 1% OV-1 or 1% OV-17 coated on 100-120 mesh Gas Chrom Q. The temperature was manually programmed from approximately 200-335 C at approximately 5 C/min.

Detector sensitivity and stability was improved by the following modifications: (a) installation of a ceramic flame tip (0.03 in. I.D.); (b) the use of oxygen instead of air; (c) installation of oxygen and hydrogen gas preheaters (20 ft x 1/16 in., 0.04 in. I.D., stainless steel tubing) and pressure regulators (Brook Model 8743); and (d) grounding the positive electrode to the flame tip. Spurious peaks and excessive baseline drift were eliminated by preconditioning septums at 300 C under vacuum for 4 hr. Temperatures of the flash heater and detector were maintained at 325 C and 350 C. Helium carrier gas, hydrogen and oxygen flow rates were 100, 50 and 300 ml/min, respectively.

Peak areas were measured by the triangulation method (mean of three measurements) or with a Datex Model DIR-1 digital integrator. A standard triglyceride mixture (trilaurin, trimyristin, tripalmitin, tristearin, triarachidin and tribehenin) was used to calibrate the instrument daily and the mole percentage given has been corrected for slight losses of the high molecular glycerides unless noted otherwise.

Materials

Purified triglycerides were purchased from The Hormel Institute, Austin, Minn. Glass-distilled solvents were purchased from Burdick and Jackson Laboratories, Inc., Muskegon, Mich. Other chemicals were reagent grade or better and were used without further purification.

RESULTS AND DISCUSSION

Resolution of Triglycerides, GEDE and Neutral Plasmalogens by GLC

The large quantity of triglycerides relative to the quantity of GEDE and the marginal TLC resolution of GEDE and the neutral plasmalogens enhanced the possibility of cross contamination between these lipid classes. Therefore, before quantitative analyses of the triglycerides and GEDE could be carried out, we had to demonstrate that such class contamination

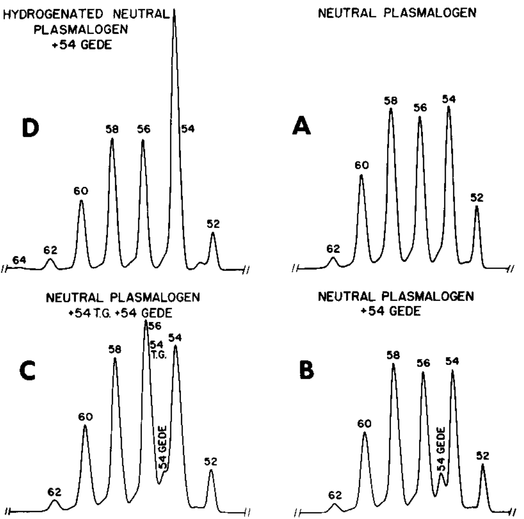

FIG. 2. Chromatogram tracings that illustrate the elution order of neutral plasmalogens relative to the elution order of a triglyceride and glyceryl ether diester of the same carbon number. The chromatograms represent A, neutral plasmalogens isolated from ratfish liver oil (18); B, neutral plasmalogens spiked with a standard carbon number 54 glyceryl ether diester; C, neutral plasmalogens co-chromatographed with carbon number 54 glyceryl ether diester and tristearin; and D, hydrogenated neutral plasmalogen sample spiked with standard glyceryl ether diester. Chromatographic conditions were the same as noted in Figure 1.

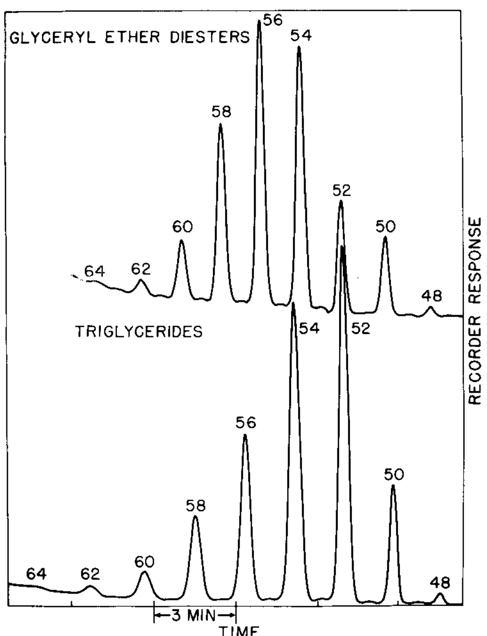

FIG. 3. Representative chromatogram showing the carbon number distribution of triglycerides and glyceryl ether diesters isolated from several transplantable rat and mouse tumors. These chromatograms are of glycerides isolated from Friend virus leukemia mouse tumors. Chromatographic conditions were the same as indicated in Figure 1 except that the liquid phase was OV-17, instead of OV-1.

could be detected. Figure 1 shows the partial resolution of a mixture of triglycerides and GEDE. The GEDE were eluted immediately ahead of the corresponding carbon number, instead of directly between triglyceride carbon numbers as might have been expected, since they differed in molecular weight by only 14. The OV-17 liquid phase gave slightly improved resolution of the mixture of triglycerides and GEDE, but the slight retarded elution of the GEDE relative to the corresponding triglyceride was still observed. Complete resolution of triglycerides and GEDE was not obtained on either liquid phase but was sufficient to enable detection of triglycerides contaminating GEDE and vice versa.

The chromatograms shown in Figure 2 illustrate the elution order of neutral plasmalogens relative to standard GEDE and triglycerides. Although the alk-1-enyl ether bond of the neutral plasmalogen is generally considered very labile, the neutral plasmalogens were able to withstand the high GLC temperatures without any apparent degradation (Fig. 2A). The neutral plasmalogens were eluted ahead of GEDE of the same carbon number (Fig. 2B). Triglycerides and neutral plasmalogens of two higher carbon numbers were eluted as a single peak (Fig. 2C). Figure 2D shows that upon hydrogenation neutral plasmalogens give rise to GEDE. The early elution of the neutral plasmalogens relative to the GEDE of the same carbon number enable contamination from this class to be detected by GLC analysis of the GEDE fraction before hydrogenation. Triglyceride and GEDE samples that showed any contamination with the analogues were repurified by TLC.

Despite the slight difference between the triglyceride, GEDE and neutral plasmalogen structures, they can be resolved by adsorption chromatography (19). The present data demonstrate that the effect of the alkyl and alk-1-enyl ether bonds permit GLC to partially resolve triglycerides, GEDE and neutral plasmalogens of the same carbon number.

Triglycerides and GEDE Isolated From Various Tumors

The lipids of each neoplasm examined contained glyceryl ether diesters. Only triglycerides and GEDE were found in the respective fractions, as determined by the absence of other

TABLE I

Rat and Mouse Tumor Triglycerides and Glyceryl Ether Diesters

Tumor[a] tissue	Lipid[b] class	Per cent carbon number (mole)[c]									
		46	48	50	52	54	56	58	60	62	64
Walker 256	TG	---	1.1	7.0	20.6	29.1	20.2	4.2	5.3	1.6	0.7
	GEDE	---	0.6	4.3	15.2	25.9	29.9	6.4	4.7	2.1	0.9
R-3259	TG	---	2.5	18.2	42.6	31.8	3.3	0.9	0.5	---	---
	GEDE	---	0.7	10.7	24.9	24.8	24.5	11.9	2.3	0.4	Trace
TBI	TG	1.6	4.6	16.5	30.6	24.6	13.0	5.0	1.5	0.7	---
	GEDE	0.4	4.4	23.0	40.4	14.3	11.1	4.9	1.3	0.2	---
EAC	TG	0.4	1.3	6.9	21.7	32.4	19.1	3.2	3.8	1.1	---
	GEDE	0.8	6.3	18.2	24.4	20.1	17.6	9.2	2.5	1.1	---
TLT	TG	---	0.9	12.6	42.4	41.0	2.0	0.8	0.4	0.1	---
	GEDE	0.7	4.1	16.3	31.5	23.5	13.4	7.4	2.3	0.7	0.2
FVL	TG	---	0.8	8.5	30.6	28.8	15.3	8.9	3.3	1.7	1.3
	GEDE	---	0.8	6.4	17.6	21.8	25.2	17.4	6.6	3.4	0.7
S-180	TG	---	1.1	11.1	37.9	45.0	3.0	1.3	0.6	---	---
	GEDE	---	1.8	12.2	32.4	20.9	17.5	11.4	2.6	0.8	0.4
T-241	TG	---	0.3	5.7	38.1	53.1	2.0	0.5	0.2	---	---
	GEDE	---	2.0	11.0	26.1	22.1	21.3	13.5	3.0	0.7	0.2
B-16	TG	---	0.7	13.1	43.6	39.5	2.6	0.4	0.1	---	---
	GEDE	1.9	8.3	22.0	29.5	18.1	13.3	5.5	1.1	0.2	---
E-0771	TG	---	1.8	19.8	42.1	34.5	1.4	0.4	---	---	---
	GEDE	---	2.0	14.0	26.0	22.5	23.1	10.1	1.7	0.4	---

[a]The tumor abbreviations are Walker 256, Walker carcinosarcoma 256 (rat); R-3259, R3259/96A sarcoma (rat); TBI, non-transplantable fibroadenoma occurring in rats approximately one year after 800R total body irradiation; EAC, Ehrlich ascites carcinoma cells (mouse); TLT, taper liver tumor (mouse); FVL, Friend virus leukemia (mouse); S-180, sarcoma 180 (mouse); T-241, sarcoma T241 (mouse); B-16, melanoma B16 (mouse); and E-0771, adenocarcinoma E0771 (mouse).

[b]TG, triglyceride; GEDE, glyceryl ether diester.

[c]Percentages represent the mean of duplicate analyses of two samples. Agreement between percentage of major components was ± 5% and for minor components ± 10%.

peaks from the chromatograms, indicating the absence of diol lipids (20) in these fractions. The carbon number distribution of both triglycerides and GEDE showed very high molecular weight species for each lipid class of each neoplasm (Table I). Typical chromatograms shown in Figure 3 illustrate the resolution and wide carbon number distribution of the tumor triglycerides and GEDE. Carbon number percentages of triglycerides and GEDE obtained for ten tumors are given in Table I. Carbon number percentage (mole) of triglycerides differed greatly from the GEDE percentages, but usually the range of carbon numbers was similar for both lipid classes derived from the same neoplasm. Carbon number 52 and 54 were the major triglyceride species, whereas a major percentage of the GEDE species was distributed more evenly from carbon number 50 to 58 (Table I). The dissimilarity of carbon numbers between these two lipid classes supports the pathways proposed earlier (8) which suggested independent routes of biosynthesis. Generally the carbon number percentages of the GEDE from the various tumors showed a closer agreement than the agreement between triglyceride percentages. The carbon number percentages of triglycerides from some of the neoplasms agreed rather closely. Adenocarcinoma E0771, R3259/96A sarcoma, melanoma B16, sarcoma 180 and taper liver tumor triglyceride percentages were reasonably close. The triglyceride percentages of Walker carcinosarcoma 256 and Ehrlich ascites carcinoma were similar. The GEDE percentages of R3259/96A sarcoma, adenocarcinoma E0771, sarcoma 180 and sarcoma T241 agreed, as did Ehrlich ascites carcinoma, melanoma B16 and taper liver

TABLE II

Comparison of Triglyceride and Glyceryl Ether Diester Distribution According to Carbon Number in Various Ehrlich Ascites Cell Fractions

Fraction	Glyceride type	Per cent carbon number (weight)[a]							
		48	50	52	54	56	58	60	62
Homogenate	TG	Trace	1.2	19.9	41.8	21.4	14.1	1.3	0.2
	GEDE	0.8	11.4	21.4	24.4	26.3	13.5	2.0	0.2
15,000 x *g* (10 min)	TG	Trace	1.0	21.9	42.9	19.5	12.5	1.0	0.1
	GEDE	1.0	11.9	22.8	24.1	23.9	12.5	1.7	0.2
34,000 x *g* (3 hr)	TG	Trace	1.2	23.1	45.0	19.5	10.4	0.8	0.1
	GEDE	1.4	14.8	24.3	24.1	23.8	10.4	0.9	Trace
Supernate	TG	Trace	1.9	24.5	42.1	19.0	11.7	0.8	Trace
	GEDE	0.4	8.0	23.7	25.9	26.2	11.6	1.3	Trace

[a]The percentages represent the mean of duplicate analyses on three separate samples and have not been corrected for slight losses of the higher molecular weights.

tumor percentages of GEDE.

Detailed analyses of triglycerides and glyceryl ether diesters of Ehrlich ascites cells earlier revealed that both lipid classes exhibited a 1-random-2-random-3-random distribution, and that 1,2-diacyl and 1,2-alkyl acyl glycerides derived from phosphatidylcholine were similar to the corresponding glycerides derived from triglycerides and GEDE (8). These observations suggested that the selectivity of diglycerides for the biosynthesis of triglycerides and phosphatidylcholine that occurs in normal tissue was absent in Ehrlich ascites cells, moreover the absence of diglyceride selectivity might be common to other neoplasms. The high molecular weight species of triglycerides found in the neoplasms of the present study indicated that the lack of diglyceride selectivity in triglyceride biosynthesis may represent a significant difference between the lipid metabolism of normal and neoplastic tissue. Carbon numbers of triglycerides of most mammalian tissues analyzed thus far have not contained significant quantities of triglyceride species longer than carbon number 54 (21-23). However, high molecular weight triglycerides are not restricted to neoplasms. Marine oil triglycerides have been shown to exhibit a wide carbon number distribution (18,23).

Glyceryl ether diesters of neoplasms were first identified in Ehrlich ascites cells (5). The present data demonstrates the occurrence of GEDE in eight additional transplantable rat and mouse tumors, implicated earlier by indirect evidence (7). The significance of glyceryl ether diesters in neoplastic tissue is not known, but they may occur as a result of increased concentrations of alkyl glyceryl ether precursors in the neoplastic cell. Glyceryl ether diesters have been isolated from human perinephric fat (24), beef heart lipids (25), and indirect evidence suggests that they occur in most mammalian tissues (6,26).

Carbon Number Distribution of Triglycerides and GEDE From Ehrlich Ascites Cell Fractions

The carbon number percentages (weight) of the homogenate triglycerides and GEDE showed small deviations from the corresponding Ehrlich ascites cells values given in Table I. The differences are probably due to different cell harvesting times after tumor transplantation. The values in Table I were obtained from seven day cells, whereas the percentages in Table II were obtained from cells harvested five days after transplantation.

The fractions obtained from the disrupted Ehrlich ascites cells by centrifugation contained different quantities of GEDE as estimated from the quantities isolated by TLC. The 15,000 *g* fraction contained approximately three times the quantity of GEDE as the 34,000 *g* and supernate fractions. Table II gives the carbon number percentages of triglycerides and GEDE derived from the various fractions. As expected, based upon prior analyses, the carbon number distribution of the GEDE of each fraction was distinctly different from the triglyceride distribution. More importantly, the carbon number percentages of the triglycerides and GEDE of one fraction were not significantly different from the corresponding percentages of the other fractions. This similarity between the carbon number percentages of the fractions for both lipid classes suggests a random distribution of triglycerides and GEDE molecular species among cellular components, although the cellular components may differ significantly in the percentage of each lipid class present. Similar carbon number distributions in the

various cell fractions could have resulted from random distribution of preformed glycerides or biosynthesis of the glycerides in the components of the cell fractions from a common source of precursors. Bartley (27) has reported that lipids of various cell organelles obtained from the same tissue have approximately the same fatty acid composition. Our results, which go a step further, indicate a random distribution of triglyceride and GEDE molecular species among the cellular components. Such a distribution of phosphoglyceride molecular species may also occur in normal and neoplastic tissue.

ACKNOWLEDGMENTS

E.A. Cress prepared the Ehrlich ascites cell fractions, and Arlene Wood typed and reviewed this manuscript. Synthetic GEDE (glyceryl 1,2-dipalmitoyl-3-octadecyl and glyceryl 1,2-distearoyl-3-hexadecyl ethers) and the neutral plasmalogens (alk-1-enyl 2,3-diacyl glyceryl ethers) were gifts from H.K. Mangold, W.J. Baumann and H.H.O. Schmid of the Hormel Institute.

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A Simple Preparative Procedure for the Rapid Isolation of Phytosphingosine From the Yeast *Hansenula ciferrii*

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ABSTRACT

Phytosphingosine and dihydrosphingosine were isolated as a mixture of their N-acetyl derivatives from the cells of the yeast *Hansenula ciferrii*. After alkaline hydrolysis of the N-acetyl compounds, the free base mixture was reacted with either acetone or *p*-nitrobenzaldehyde. The reaction products consisting of the carbonyl derivative of phytosphingosine and free dihydrosphingosine were separated on an acetyl cellulose column. The acetone or *p*-nitrobenzaldehyde derivative of phytosphingosine recovered from the benzene eluate contained no dihydrosphingosine as determined by thin layer and gas liquid chromatography. The operating time from preparation of the derivative through column purification was less than 3 hrs. Mass spectrometry of the acetone and *p*-nitrobenzaldehyde derivatives disclosed in each case that ring closure with the carbonyl reagent involved the amino and primary hydroxyl groups to give *D-ribo*-2-dimethyl-4-(2,3-dihydroxyhexadecyl) oxazolidine and *D-ribo*-2-p-nitrophenyl-4-(2,3-dihydroxyhexadecyl) oxazolidene, respectively.

INTRODUCTION AND DISCUSSION

The yeast *Hansenula ciferrii* produces partially (1) and fully acetylated derivatives of phytosphingosine and dihydrosphingosine (2,3). The fully acetylated bases, after isolation from the culture medium, were separated by counter-current distribution (4). Recently, the free bases were resolved on a Silica Gel S column by treatment with chloroform-methanol (9:1) followed by two successive continuous gradients containing varying proportions of chloroform-methanol-2M NH_4OH (5). Since these methods involve considerable time, manipulations and analyses, we sought an easy chemical procedure for the resolution of these compounds. Accordingly, a procedure for the rapid separation of phytosphingosine, via its acetone or *p*-nitrobenzaldehyde derivative, from dihydrosphingosine obtained from the cells of *H. ciferrii* is now described. In addition, proof of the structure of the acetone and *p*-nitrobenzaldehyde derivatives of phytosphingosine is presented. In this study cells were employed as the source material because they contained about nine times more base than that present in the extracellular medium.

It was found that phytosphingosine forms a complex with acetone (6,7). Use of this reaction, it was reasoned, could be made in the separation of phytosphingosine from dihydrosphingosine. We observed, however, that the complex was of limited stability under the initial conditions studied. A more stable derivative of phytosphingosine was formed with *p*-nitrobenzaldehyde; dihydrosphingosine was unreactive under the same circumstances. The *p*-nitrobenzaldehyde derivative of phytosphingosine was separated quantitatively within 2 hr from dihydrosphingosine on an acetyl cellulose column by development with benzene; no dihydrosphingosine was present in the product obtained from the benzene eluate as determined by thin layer chromatography (TLC) and gas liquid chromatography (GLC). Similarly, the acetone derivative of phytosphingosine was separated from dihydrosphingosine. The phytosphingosine obtained from the column could be stored until needed as the carbonyl derivative or as the sulfate salt which was formed by regeneration of the base from the derivative by treatment with dilute sulfuric acid.

Although analytical values were presented for the product formed from the reaction of acetone with phytosphingosine, no structural determinations were reported (6,7). We observed that the acetone derivative showed no UV absorption at 247 mμ for an azomethine linkage. The acetone and *p*-nitrobenzaldehyde derivatives gave negative ninhydrin reactions, had similar IR spectra with no absorption near 1600 cm^{-1} for a free amino group, and could be crystallized from various nonaqueous solvents. These observations, along with data from elementary and active hydrogen analyses, indicated that amino and hydroxyl groups were involved in ring formation with the carbonyl reagent. Attempts to ascertain which of the three hydroxyl groups participated in ring

closure by permethylation of the *p*-nitrobenzaldehyde derivative followed by ring cleavage and periodate oxidation were unsuccessful. The *p*-nitrobenzaldehyde derivative was recovered unchanged after treatment with sodium periodate in 90% aqueous methanol. Mass spectrometry of the acetone and *p*-nitrobenzaldehyde derivatives disclosed in each case that ring closure with the carbonyl reagent involved the amino and primary hydroxyl groups to give *D-ribo*-2-dimethyl-4-(2,3-dihydroxyhexadecyl) oxazolidine and *D-ribo*-2-p-nitrophenyl-4-(2,3-dihydroxyhexadecyl) oxazolidine, respectively.

EXPERIMENTAL PROCEDURES

Materials and Methods. Hansenula ciferrii, mating type F-60-10, strain NRRL-Y-1031 was provided by L.J. Wickerham. Silicic acid, acetyl cellulose and Adsorbosil-1 were products of Mallinkrodt, Woelm and Applied Science Lab, respectively. The silicic acid was washed with chloroform:methanol (2:1) and activated by heating 24 hr at 120 C. All solvents were distilled. TLC was conducted as previously described (8) on plates coated with Adsorbosil-1 and developed with chloroform-methanol (95:5) or chloroform-methanol-2M NH_4OH (40:10:1) (9). Bands were detected by exposure to iodine vapor. The trimethylsilyl derivatives of the bases were analyzed by GLC (10) on 2.5% SE-30 on Gas Chrom Q, mesh 100/200. The column, injector and detector temperatures were maintained at 210 C, 270 C and 240 C, respectively. IR spectra were obtained on KBr discs with a Perkin Elmer IR Spectrophotometer. High resolution mass spectrometry was performed by Gollob Analytical Service.

Isolation of N-Acetyl Bases. Each of ten 1 liter Erlenmeyer flasks containing 500 ml of medium (2) was inoculated with the growth from a single slant and grown 72 hr at room temperature under vigorous aeration. The cells were collected by centrifugation and washed once with 300 ml of cold isotonic saline. To the cells suspended in methanol, 25 g/100 ml, was added 1 ml of 10 N KOH per 100 ml of suspension which was stirred magnetically 4 hr at room temperature. After standing overnight, the suspension was centrifuged and the precipitate was washed by centrifugation three times with 200 ml portions of methanol. The combined supernatant solutions, after addition of several drops of Dow antifoam A, were concentrated below 55 C, on a water pump to approximately 150 ml. An equal volume of water was added and the solution was treated three times with 200 ml portions of ether. The combined ether extracts were washed with water until neutral and the residue, after ether removal, was dried over P_2O_5 and crystallized from 35 ml of acetonitrile; yield of crude N-acetyl bases from 52 g of dry cells, 780 mg (range 10 to 22 mg/g of dry cells). TLC showed N-acetylphytosphingosine and N-acetyldihydrosphingosine with trace amounts of phytosphingosine and dihydrosphingosine; less polar impurities appeared at the front.

Purification of N-Acetyl Bases. A 30 g silicic acid column, 2.5 x 50 cm, packed from chloroform, was loaded with 500 mg of crude N-acetyl bases in 30 ml of warm chloroform. The column was developed with 125 ml each of chloroform and methanol. The chloroform eluate was discarded and the residue obtained from concentration of the methanol eluate was crystallized from acetonitrile; yield 360 mg (range 67% to 74%).

Preparation of Free Bases. Purified N-acetyl bases, 720 mg, were refluxed 7 hr in 18 ml of ethanol and 2 ml of 2N aqueous KOH. An equal volume of water was added and the reaction mixture was treated three times with 30 ml portions of ether. After washing the combined ether extracts with water, the residue, obtained after removal of the solvent, was dried and washed by suspension in 35 ml of hot petroleum ether. Although further purification was unnecessary, they could be purified by application in 2% methanol in chloroform to a 20 g silicic acid column and developed with 125 ml each of 5% methanol in chloroform and methanol. The former eluate was discarded and the product was obtained by concentration of the methanol eluate; yield 544 mg (range 85% to 90%). The base mixture consisted of about 95% phytosphingosine and 5% dihydrosphingosine as determined by GLC.

P-Nitrobenzaldehyde Derivative of Phytosphingosine. To 318 mg of free bases in 35 ml of warm petroleum ether, bp 68-74 C (Skellysolve B), was added 164 mg of *p*-nitrobenzaldehyde; the reaction mixture was heated until a clear solution resulted. After cooling to room temperature, the precipitate was collected by centrifugation and resuspended in hot petroleum ether. The product obtained from the cool reaction mixture was dried, dissolved in 20 ml of benzene and applied to an 8.0 g acetyl cellulose column, 1.5 x 50 cm, which was packed in benzene and washed successively with 3 column volumes each of ethanol and benzene. After development with 125 ml of benzene the residue, obtained from concentration of the eluate, was dried and resuspended in hot petroleum ether. The cool suspension was centrifuged and the precipitate dried; yield

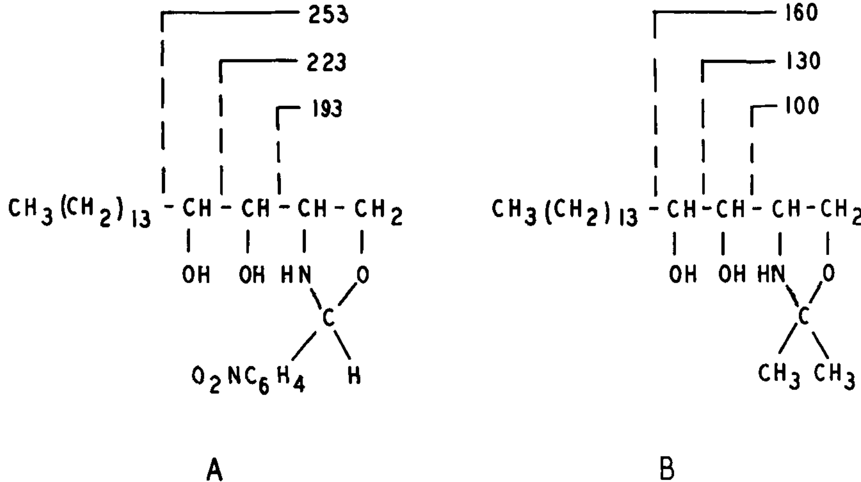

FIG. 1. The mass spectra of the *p*-nitrobenzaldehyde (A) and acetone (B) derivatives of phytosphingosine showed major peaks at m/e 193 and 100, respectively, which indicated participation of the amino and primary hydroxyl groups with the carbonyl reagent to give a substituted oxazolidine. See text for details.

356 mg (range 82% to 86%); mp 99-101 C. TLC revealed only one component, R_F 0.9; GLC showed no dihydrosphingosine.

Analysis. Calculated for $C_{25}H_{42}O_5N_2$ (450.3): C, 66.61; H, 9.40; O, 17.76; N, 6.22; Active H, 0.67. Found: C, 66.70; H, 9.50: O, 18.01; N, 6.26; Active H, 0.64.

Acetone Derivative of Phytosphingosine. Free bases, 318 mg, in 10 ml of acetone was heated to reflux, after cooling to room temperature, the acetone was removed under a stream of N_2 below 30 C. The dried residue was applied in 20 ml of hot benzene to an 8.0 g acetyl cellulose column. After washing the product into the column with several hot 15 ml portions of benzene, the column was developed with another 100 ml of benzene. The eluate was concentrated to dryness and the dried residue was crystallized from petroleum ether; yield 242 mg (range 70% to 74%); mp 102-104 C. TLC and GLC disclosed only one component.

Analysis. Calculated for $C_{21}H_{43}NO_3$ (357.3); C, 70.53; H, 12.12; O, 13.43; Active H, 0.84. Found: C, 70.08; H, 12.00; O, 13.66; Active H, 0.79. The acetone derivative of phytosphingosine obtained from a chilled acetone solution of the mixed bases melted at 105-107 C in agreement with that reported previously (7).

Regeneration of Phytosphingosine as Sulfate Salt. The *p*-nitrobenzaldehyde derivative, 180 mg, in 9.0 ml of 90% methanol and 1.0 ml of 0.5N aqueous sulfuric acid was heated to reflux. After cooling to room temperature, 10 ml of acetonitrile were added and the reaction mixture was centrifuged. The precipitate was washed successively by centrifugation with 3 ml portions of acetonitrile, two times, and once with ethanol. After drying, the precipitate was washed with 10 ml of hot petroleum ether; yield 127 mg (range 85% to 89%). GLC of the trimethylsilyl derivative formed directly from the sulfate salt showed only phytosphingosine, retention time 39 min.

Analysis. Calculated for $C_{36}H_{80}N_2O_{10}S$ (732.6); C, 58.96; H, 11.00; N, 3.82; O, 21.83; S, 4.37. Found: C, 58.52; H, 10.83; N, 4.02; O, 21.68; S, 4.34. The acetone derivative was treated in the same manner to yield the base sulfate.

Structure Proof of Derivatives of Phytosphingosine. Mass spectrometric analysis of the *p*-nitrobenzaldehyde derivative showed a major peak at m/e 193 which is the mass of a 5-membered ring (Fig. 1,A); this eliminated the hydroxyl on carbon atom 4 as a participant which would yield a 6-membered ring. If the hydroxyl on carbon atom 3 were involved, the ring mass would be 192 and the expected major peak would be at m/e 223 with smaller peaks at m/e 192 and 253. Since the fragment ion at m/e 193 was the largest with 223 and 253 progressively smaller, it was concluded that the terminal hydroxyl participated in ring formation. Similarly, the largest peak at m/e 100 corresponded to a 5-membered ring which involved the primary hydroxyl group of the acetone derivative (Fig. 1,B) with smaller peaks at m/e 130 and 160.

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Identification and Characterization of Fully Deuterated Fatty Acids Isolated from *Scenedesmus obliquus* Cultured in Deuterium Oxide[1]

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ABSTRACT

The alga *Scenedesmus obliquus* cultured in deuterated water, synthesized fully deuterated saturated and unsaturated long chain fatty acids. Their methyl esters were purified and their equivalent chain lengths were determined by gas liquid chromatography (GLC). They were characterized by mass spectroscopy, IR and near-IR spectroscopy, and NMR spectroscopy. Hexadeca-3,6-dienoic acid was identified. The fatty acid compositions of the total lipid and of individual lipid classes were measured. The melting point of methyl perdeuteriohexadecanoate was lower than that of its hydrogen counterpart. Methyl esters of perdeuterio fatty acids had shorter retention times in GLC chromatography on polar and nonpolar phases.

INTRODUCTION

For biochemical and analytical studies of saturated and unsaturated fatty acids, it was necessary to prepare fully deuterated fatty acids. Perdeuterio saturated fatty acids have been synthesized by Dinh-Nguyen (1), but chemical methods for preparation of perdeuterio unsaturated fatty acids have not been developed. Algae have been cultured in D_2O of high purity for preparation of perdeuterio proteins and other metabolites (2). The lipids from such cultures should be a good source of a variety of fatty acids in perdeuterio form. Therefore, a preparation of lipids from *Scenedesmus obliquus*, cultured in D_2O, was converted to methyl esters, which were separated and more thoroughly characterized individually. The IR spectrum of the methyl esters of mixed fatty acids isolated from this species has been reported previously (3).

[1]Presented before ISF-AOCS Congress, Chicago, September 1970.

EXPERIMENTAL PROCEDURES

Culture Conditions

Cultures of *S. obliquus* adapted to 99.7% D_2O were grown in a medium of 99.7% D_2O containing the following inorganic salts (gm/1): NH_4NO_3 2.00, $KHCO_3$ 0.20, $Ca(NO_3)_2 \cdot 4H_2O$ 0.03, NaCl 0.02, $MgSO_4$ 0.50, $FeSO_4 \cdot 7\ H_2O$ 0.045, Na_2HPO_4 0.75. The cultures were maintained in 2 liter Erlenmeyer flasks under an atmosphere of 5% CO_2 in air, illuminated by circular fluorescent lights below and around each flask, and stirred magnetically. Cultures were harvested at intervals of 10-14 days by centrifugation of the medium at 2000 rpm. The cell walls were ruptured by boiling in D_2O, and the insoluble material was collected by centrifugation and extracted with chloroform-methanol (2:1).

Analysis of Fatty Acid Composition

A portion of the total lipid was interesterified with 3 ml of boron trifluoride in methanol in a closed tube at 80 C for 30 min. After methanolysis was complete, 3 ml of distilled water was added, and the esters were extracted with petroleum ether. The extract was dried over sodium sulfate, filtered, concentrated and subjected to analytical gas liquid chromatography (GLC). The chromatograms for fatty acid composition and measurement of equivalent chain lengths (ECL) (4) were obtained with an F&M Model 810 gas chromatograph with a hydrogen flame detector. The flow rate was 75 ml helium per minute, and the injection port was kept at 250 C. An aluminum column 180 x 0.6 cm packed with 20% ethylene glycol succinate (EGS) plus 2% phosphoric acid on Gas Chrom P, 60-80 mesh was used, and the oven temperature was kept at 180 C. The aluminum column 180 x 0.6 cm packed with 60-80 mesh Gas Chrom P containing 3% OV-17 was operated at 200 C. When 1% OV-1 was the liquid phase the oven temperature was 180 C.

The extract was separated into several lipid classes by thin layer chromatography (TLC) on

Silica Gel H with petroleum ether (30-60 C)-diethyl ether-acetic acid (90:10:1, v/v/v) (5). The fractions were interesterified with boron trifluoride in methanol, and analytical GLC was performed on the EGS column. Retention times were measured on EGS, OV-1 and OV-17 phases and compared with authentic saturated methyl esters.

Isolation and Purification

The chloroform-methanol extract from the algae was interesterified using 50 ml of 5% HCl in methanol. Chlorophyll, carotenoids and unmethylated products were removed by chromatography on Florisil with petroleum ether (30-60 C): diethyl ether (80:20, v/v). The methyl ester fraction was checked for purity by TLC. The saturated and unsaturated methyl esters were fractionated by argentation TLC on 0.5 mm Silica Gel H plates impregnated with 3% silver nitrate, with petroleum ether-diethyl ether-acetic acid, 85:15:1 (6). The methyl ester fractions having 0, 1, 2 and 3 double bonds were scraped off separately, extracted with petroleum ether, and the π-complexes were broken by Cl-ions. Each fraction was fractionated according to chain length by preparative GLC (7).

Physical Characterization

IR spectra of liquid films were obtained with a Perkin Elmer Model 21 spectrometer. Near IR spectra were measured on carbon tetrachloride solutions with a Beckman DK-2 spectrometer. Spectra of the acids were measured on 140 mM solutions and of the esters on 150 mM solutions. The NMR spectrum of methyl perdeuteriohexadecanoate in $CDCl_3$ (29 mg/ml) was measured with a Varian 220 MHz instrument with trimethyl silane as internal standard. Mass spectra were measured in a Hitachi RMU6D mass spectrometer with the liquid sample insertion system, reservoir and molecular leak. Spectra were taken at ionization potentials of 70 and 15 eV at pressures near 1 to 2 x 10^{-7} Torr.

Methyl perdeuteriohexadecanoate was saponified in CH_4OD with a slight excess of KOD, and the acid was liberated with DCl in D_2O. The acid was crystallized four times in petroleum ether and the melting point was measured with a Kofler hot stage microscope.

Chemical Characterization

Ozonolysis of the unsaturated esters was performed according to the method of Nickell and Privett (8). The ald-esters and aldehydes were analyzed by GLC on a 180 x 0.6 cm aluminum column packed with 20% EGS plus 2% phos-

TABLE I

Fatty Acid Composition of Total Lipids and Individual Lipid Classes

Fatty acid	Total lipid	Sterol esters	Methyl esters	Triglycerides	Free fatty acids	Diglycerides	Phospholipids
a	1.7	--	--	--	--	--	--
b	0.6	--	--	--	--	--	--
13:0	3.5	10.6	--	--	--	12.5	3.4
c	1.8	--	--	--	--	2.8	1.0
d	1.1	3.1	--	--	--	4.4	1.8
14:0	1.8	3.1	1.2	--	0.1	5.3	2.7
e	1.5	--	--	--	--	0.2	1.3
15:0	3.2	2.5	0.8	0.9	0.2	4.3	1.6
16:0	22.3	24.5	29.9	26.1	27.4	17.6	22.6
16:1	4.5	0.8	4.0	1.6	5.7	1.8	4.5
16:2	12.2	8.9	5.0	8.5	18.2	19.2	13.8
18:0	1.1	--	0.8	2.7	1.0	2.0	0.9
18:1/16:3	10.4	6.1	10.8	25.8	16.3	11.8	10.4
18:2	30.7	38.3	44.1	32.3	29.8	18.3	30.2
18:3	1.8	1.5	3.2	2.1	1.4	0.9	1.7

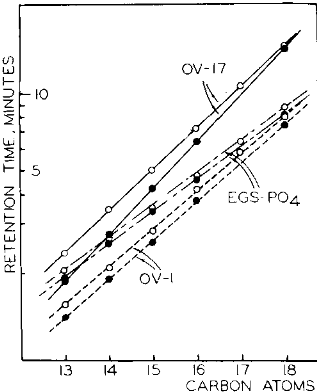

FIG. 1. The relationship of logarithm of retention time to the number of carbon atoms in methyl esters of protonated fatty acids, ○——○, and perdeuterio fatty acids, ●——● on three liquid phases in gas chromatography.

phoric acid, kept at 160 C or 180 C depending upon the chain length of the ozonized products. The ECL values of the ald-esters and aldehydes were compared with those produced from authentic methyl octadecenoates (9) of several structures.

Methyl 9,10,12,13-tetrahydroperdeuterio-octadecanoate was prepared from methyl perdeuterio-9,12-octadecadienoate by treatment with hydrazine hydrate (10). The mass spectrum of this compound was measured as described above.

RESULTS

Composition

The fatty acid compositions of the total perdeuterio lipids and of the several lipid classes are given in Table I. The composition of the total fatty acids of *S. obliquus* cultivated in D_2O differs somewhat from that of a wild type or that of a mutant analyzed by Erwin and Bloch (10), who reported only acids with ECL value greater than 15.0

Gas Chromatographic Behavior

A comparison between the retention times of methyl esters of the perdeuterio acids and the hydrogen analogs was made on the polar phase EGS, a medium polarity phase OV-17, and a low polarity phase OV-1. The plots of logarithm of retention times vs. chain length are shown in Figure 1. On the EGS column, and on the OV-1 column, the methyl esters of the perdeuterio acids differed from the hydrogen analogs only slightly. The plots were parallel and the perdeuterio compounds appeared earlier than the hydrogen compounds (3). On the medium polarity phase the plots were not parallel, and the divergence between retention of the deutero- and hydrogen analogs increased as chain length decreased. The values for saturated esters converged to the same value near 18 carbon atoms. The ECL values of methyl esters of identified deuterated and protonated acids determined on an EGS column are shown in Table II.

TABLE II

Equivalent Chain Lengths of Identified Methyl Esters of Fatty Acids as Measured on EGS-PO_4

Methyl ester of fatty acid	Deuterated	Protonated
16:0	15.93	16.00
16:1	16.50	16.65
16:2	17.31	17.42
16:3	18.30	18.43
18:0	17.76	18.00
18:1	18.30	18.48
18:2	19.20	19.36
18:3	20.22	20.33

Melting Points

The melting points of hexadecanoic acid, methyl hexadecanoate, perdeuteriohexadecanoic acid and methyl perdeuteriohexadecanoate were found to be 64.0, 30.0, 65.0 and 25.5-26.0 C, respectively. A mixed melting point of methyl hexadecanoate-methyl perdeuteriohexadecanoate (50/50) was 26.5 C. These results agree in general with those of Dinh-Nguyen (11).

Infrared Spectra

The infrared spectra of the methyl esters of the 16-carbon perdeuterio acids are shown in Figure 2 with a spectrum of methyl hexadecanoate for comparison. The asymmetric and symmetric C-D stretching vibrations of the polymethylene chain occur at 2206 and 2107 cm^{-1}, and a shoulder at 2240 cm^{-1} due to the double bond C-D stretching vibrations appears in the unsaturated compounds. The residual absorption in the 2800-3000 cm^{-1} region is accounted for largely by the C-H stretching vibration of the OCH_3 group. Based on the ratio of the intensities at 2206 cm^{-1} (C-D) and 2925 cm^{-1} (C-H), methyl perdeuteriohexadecanoate (16:1, Fig. 2) is the least contaminated with CH_2 groups, and the vibrations of the methoxyl group are best detected in this compound. Definite peaks of 2980 and 2940 cm^{-1} are attributed to asymmetric vibrations of the CH_3 group, and a strong shoulder at 2890 cm^{-1} to the corresponding symmetric mode.

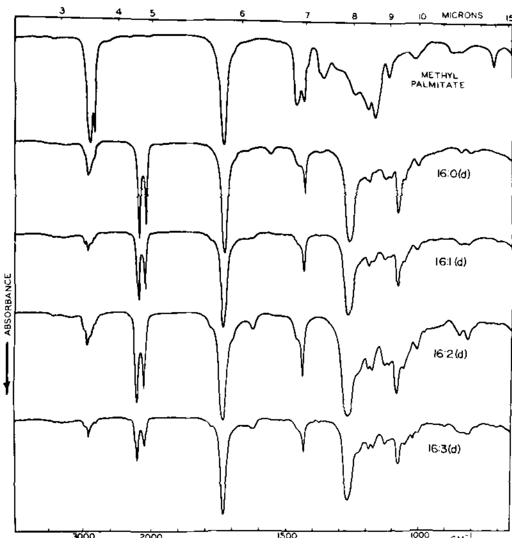

FIG. 2. IR spectra of thin films of methyl hexadecanoate and methyl esters of perdeuteriohexadecanoic acid 16:0 (d); perdeuteriohexadecenoic acid 16:1 (d); perdeuteriohexadecadienoic acid 16:2 (d); and perdeuteriohexadecatrienoic acid 16:3 (d).

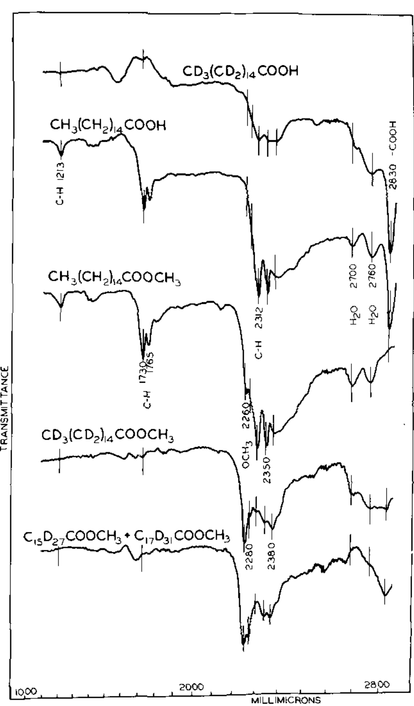

FIG. 3. Near IR spectra of perdeuteriohexadecanoic acid, methyl perdeuteriohexadecanoate and a mixture of methyl esters of perdeuteriohexadecadienoic and octadecadienoic acids compared with spectra of hexadecanoic acid and methyl hexadecanoate.

These frequencies are lower than those assigned to the OCH_3 groups (3025, 3000 and 2957 cm^{-1}) of methyl acetate by Wilmshurst (12) and of methyl laurate by Jones (13). However, the assignments of Wilmshurst and Jones were based on bands which disappeared in the deuterio methyl esters. In the original compounds, however, the methoxy vibrations could have been influenced by the other CH groups in the molecules. In our deuterated compounds, the only CH group present in the molecules were those of the carbomethoxyl and our observed frequencies, therefore, are probably more characteristic of the vibrations of this isolated group than those reported earlier. In this region a small shoulder at 2840 cm^{-1} is probably due to contamination with small amounts of undeuterated compounds which would also contribute slightly to the intensity of the 2940 cm^{-1} OCH_3 peak.

The ester carbonyl peak remains unaffected at 1735 cm^{-1} but the C=C stretch vibration is lowered to 1625 cm^{-1} in the deuterated unsaturated compounds.

In the C-H deformation region, the peak resulting from the scissoring vibrations of methylene groups is absent and the small amount of absorption remaining at 1465 cm^{-1} may be attributed to the asymmetric C-H vibration of the carbomethoxy group, while the sharp peak remaining at 1436 cm^{-1} is due to the symmetric vibration of the same group.

In an extensive study of the IR spectrum of methyl laurate, Jones (13) assigned the three bands at 1170, 1195 and 1255 cm^{-1}, characteristic of methyl esters of long chain fatty acids, to C-O stretching of C-O-C perturbed by C-H twisting, or wagging, or C-C skeletal vibrations. By replacing the ωCH_3, αCH_2 and OCH_3 groups of methyl laurate with deutero analogs, Jones found that a sharp band of medium intensity was present at 1088 cm^{-1} only in those compounds containing the OCD_3 group. Deuteration of the α-methylene group only resulted in the disappearance of the 1170 and 1195 cm^{-1} peaks, whereas the intensity of the 1250 cm^{-1} peak was nearly doubled. All our methyl deuterio esters show a band of medium intensity near 1085 cm^{-1} which cannot be due to the OCD_3 group. Furthermore, this band remains in perdeuteriohexadecanoic acid and in perdeuteriohexadecanol and, in our compounds it must be assigned to the C-D scissoring vibrations of the deuterated polymethylene chain. Our spectra agree with the view that the 1170, 1195 and 1250 cm^{-1} bands of methyl esters are not due solely to C-O-C vibrations since all these peaks are absent from the spectra

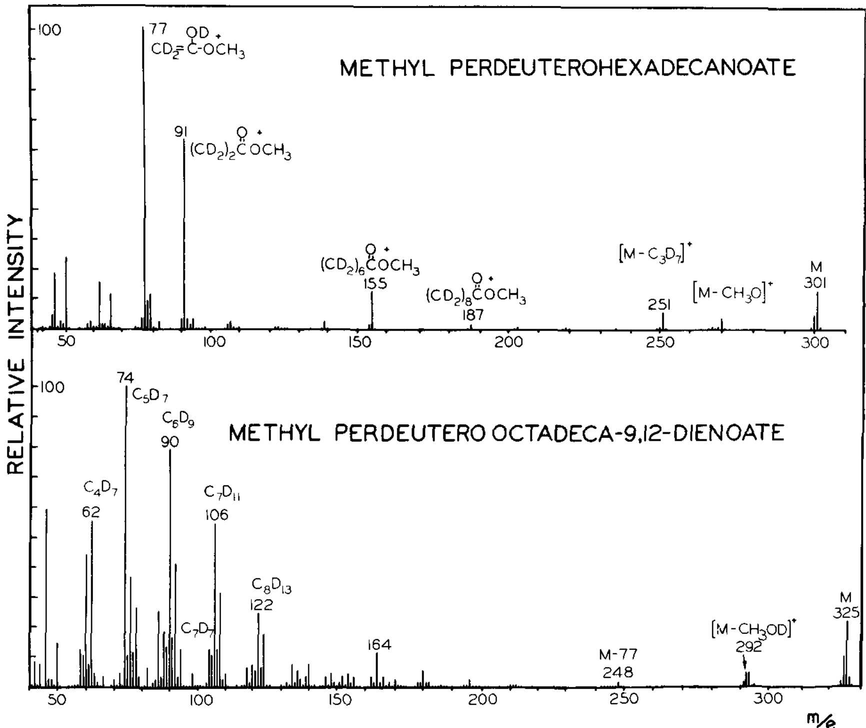

FIG. 4. Mass spectra of methyl perdeuteriohexadecanoate and methyl perdeuterio-octadeca-9,12-dienoate taken at 20 eV.

of the deuterated compounds. The only peak which can be associated with the C-O-C modes appears at 1270 cm^{-1} and probably corresponds to the single peak observed by Jones at 1252 cm^{-1} for αCD_2 methyl laurate. The higher frequency is undoubtedly due to the effect of the rest of the CD_2 chain on the 1250 cm^{-1} peak in agreement with the observations of Jones (14) who found this member of the methyl ester triplet to be more sensitive to the nature of the whole fatty acid chain. The intensity of the 1270 cm^{-1} peak is also greatly increased and approaches that of the carbonyl group. In perdeuteriohexadecanoic acid, this peak appears at 1307 cm^{-1} and it is absent from the spectrum of the alcohol. The free acid also shows a peak at 1435 cm^{-1} which, in this case, must be assigned to the OH deformation of the carboxyl group.

Absorption at 1170 and 1195 cm^{-1} may be due to normal methyl ester impurities. Peaks at 1137, 1007, 850 and 815 cm^{-1} have been associated with the structure CD_2COOCH_3 and peaks at 1115 and 1055 cm^{-1} to the asymmetric and symmetric C-D bending of ωCD_3 (13). A shoulder at 1235 cm^{-1} and a small peak at 904 cm^{-1} which increase in intensity with increasing unsaturation are possibly associated with unsaturation. In addition, a small but sharp peak appears at 1023 cm^{-1} in perdeuteriohexadecatrienoate (16:3, Fig. 2) but not in the spectra of the less unsaturated compounds. A similar peak was observed in the spectrum of methyl perdeuterio-9,12,15-octadecatrienoate but not in that of the corresponding diene or monoene. This small peak, therefore, may be associated with the *triene* group. The C-D deformation of -CD=CD- *trans* would be expected near 710 cm^{-1} and may account for the very small amount of absorption in this region by the three unsaturated compounds. The corresponding vibration for *cis* unsaturation would be outside of the range of these spectra.

Near Infrared Spectra

In palmitic acid and methyl palmitate, (Fig. 3) the two peaks at 1730 and 1765 mμ result from the first overtones and various combinations of the fundamental C-H stretching vibrations of the CH_3 and CH_2 groups at 2920 and 2850 cm^{-1}. The small peak at 1213 mμ is the second overtone of these vibrations. These peaks are absent in the spectra of the deuterated compounds.

In the 2200-2400 mμ regions the peaks

TABLE III

Structures and Isomeric Compositions of Unsaturated Perdeuterio Acids from *S. obliquus*

Fatty acid	Isomer	Per cent of fraction	Per cent of total fatty acids
16:1	Δ7	64.5	2.9
	Δ9	35.5	1.6
16:2	Δ3,6	50.8	6.2
	Δ7,10	49.2	6.0
16:3	Δ7,10,13	99.0	1.6
18:1	Δ9	85.8	7.6
	Δ11	14.2	1.2
18:2	Δ9,12	99.0	30.7
18:3	Δ9,12,15	99.0	1.8

observed for hexadecanoic acid and methyl hexadecanoate are due either to the second overtones of the C-H bending deformations at 1475-1420 cm^{-1} or to a combination of these with the fundamental C-H stretching vibrations. The relatively high intensity of these peaks favors the latter. Absorption at 2260 mμ which is present only in the methyl esters and persists with approximately the same intensity in the deuterated esters has been assigned to the $O\text{-}CH_3$ group. A similar peak has been reported previously in a number of other methyl esters (15). The other peaks in this region are much attenuated in the deuterated compounds. It should be considered that all absorption due to CH (other than $O\text{-}CH_3$) has disappeared and is replaced by the first overtones and combinations of the 2100-2200 cm^{-1} C-D stretching vibrations. The peak at 2280 mμ which appears in the deuterated methyl esters, however, is not present in the deuterated acid, and cannot be assigned solely to CD vibrations of the chain. Its origin is not clear.

Only second and third overtones of the C-D bending (1085 cm^{-1}) and $(CD_2)_nCO\text{-}O\text{-}C$ (1270 cm^{-1}) vibrations would absorb in the near IR regions and their intensity would be too low to be detected.

Mass Spectra

The mass spectra of all the methyl esters of perdeuterio acids isolated from *S. obliquus* indicate a high degree of isotopic purity, equal in all the compounds. The mass spectra of the esters indicate modes of fragmentation, the same as observed in the protonated analogs. Spectra of a saturated and an unsaturated ester are shown in Figure 4. In the perdeuterio compounds the rearrangement ion of McLafferty (16) has been shifted from m/e 74 to 77. The tropylium ion in the spectra of unsaturated esters (17) shifted from m/e 91 to 98 in the deuterated analogs. The series of hydrocarbon fragments had intervals of 16 rather than 14. In the methyl perdeuteriohexadecanoate, the ion $CH_3OCO(CD_2)_6^+$ appeared as reported by Dinh-Nguyen (18).

Nuclear Magnetic Resonance Spectroscopy

The NMR spectrum of methyl perdeuteriohexadecanoate showed the anticipated signal for the methyl ester CH_3 and several residual peaks in the vicinity of the methylene region. The total area of these peaks was 0.50 times that of the methyl ester peak, indicating a maximum proton content of 4.5%. At least part of this hydrogen contribution is due to residual hydrocarbon solvent used to purify the sample. This is evident in a sharp signal in one of the peaks, as opposed to the broad diffuse peak resulting from residual hydrogen in the fatty acid chain. The deuterium content estimated by this method is greater than 97%.

Ozonolysis

Ozonolysis of the purified fractions of unsaturated esters indicated the structures and compositions listed in Table III. The retention times for ald-esters and aldehydes from the esters of perdeuterio acids were the same as those from the protonated esters on the $EGS\text{-}PO_4$ phase used. The mass spectrum of the methyl ester of tetrahydroperdeuteriooctadecanoic acid revealed that the hydrogen atoms were in the 9,10,12,13 positions, thereby confirming that the structures deduced by ozonolysis were correct and that the parent acid was indeed perdeuteriolinoleic acid.

The Δ3,6-16:2 detected among the perdeuterio acids is unusual in having an ω10 structure. Monoenoic acids with Δ3 double bonds have been reported in plants but we are

unaware that a Δ3,6 dienoic acid has been found previously. Debuch (19) found Δ3-16:1 to be present in fatty acids from green algae and leaves. In the algae cultured in D_2O, the 16:1 was Δ7 and Δ9 as expected. It is possible that the D_2O caused a shift in the composition of the C_{16} fatty acids of *S. obliquus*. The normal structures for 18:1, 18:2 and 18:3 appeared in the fatty acids of *S. obliquus* cultured in D_2O, but the proportions of these acids were different from those reported by Erwin and Bloch (10).

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SHORT COMMUNICATION

Interaction of Detergents With Transfusion Gelatin

ABSTRACT

Binding of sodium dodecyl and octyl sulphate to transfusion gelatin has been studied at pH 7.7 by equilibrium dialysis. At lower detergent concentration the binding is statistical, whereas at higher concentrations the binding increases apparently without limit. This may be explained by assuming unfolding of the protein molecule with formation of detergent micelles around the binding sites.

Studies (1-4) have described the results of interaction of globular proteins with anionic detergents. However, investigation with fibrillar proteins have not been made. The present study uses a modified equilibrium dialysis method to determine binding of some typical anionic detergents (sodium dodecyl and octyl sulphate) to transfusion gelatin (5-6), a well characterized fibrillar protein (mol wt 75,000).

Sodium dodecyl sulphate (SDS) and sodium octyl sulphate (SOS) were obtained from Chemical De Universe, India. They were, respectively, 98.6% and 99.1% pure as evaluated by the Wickbold method (7). Their critical micelle concentration was found to be 0.0081 and 0.135 Moles/liter, respectively, at 25 C by conductance measurements. Transfusion gelatin was obtained from the National Chemical Laboratory of India, (N.C.L.) Poona, India. Phosphate-NaCl buffer (8) of pH 7.7 was prepared from reagent grade chemicals using doubly distilled water.

A modified equilibrium dialysis method (9) was employed in these studies. Aliquots (20.0 ml) of protein-detergent mixtures in buffer were first stored for at least two days at 25 C. They were then dialysed against equal volumes of buffer and the amount of free detergent in the dialysate determined spectrophotometrically (10). Visking Nojax sausage casing bags (23/32 in. diam) soaked overnight in the buffer were used.

The binding data are presented in Figure 1 where V_M the average number of moles of SDS or SOS bound to per mole of protein are plotted against log of unbound detergent concentration (log C_F). The binding data with only one soap (SDS) are given in Table I. Figure 1 shows that the mode of binding changes with increasing concentration of the detergent; the curves can be roughly divided into three regions A, B and C. In the lower concentration range (Region A) the plot is a straight line. It may be concluded, therefore, that in this region the

TABLE I

Binding of Sodium Dodecyl Sulphate to Transfusion Gelatin[a]

Concentration of detergent	Concentration of bound detergent	Concentration of free detergent	Moles of SDS bound per mole of transfusion gelatin
15	13.5	1.5	2.25
30	26.7	3.3	4.45
40	35	5	5.8
50	42	8	7
80	63	17	10.5
100	78	22	13
120	96	24	16
200	156	44	26
400	312	88	52
500	384	116	64
700	528	172	88
1000	780	220	130
1200	930	270	155
1400	1110	290	185
2000	1620	380	270

[a]Concentrations expressed in units of 1.0^{-5}M. Transfusion gelatin concentration is 6×10^{-5}M.

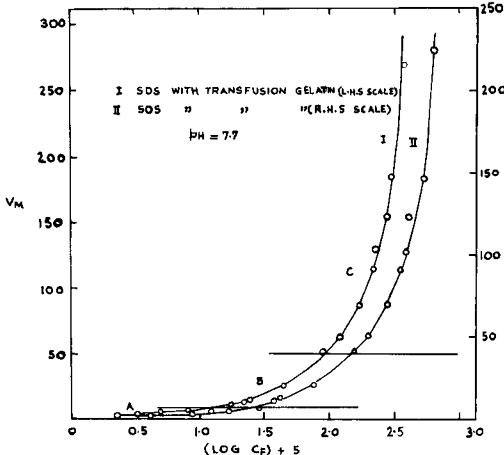

FIG. 1. Binding of detergents to transfusion gelatin.

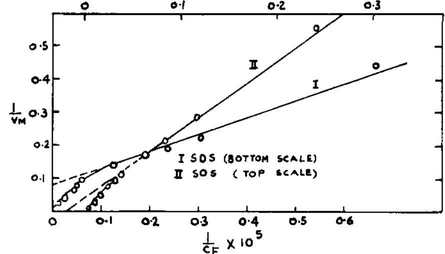

FIG. 2. Plot of $1/V_M$ vs. $1/C_F$ showing statistical binding of detergents to transfusion gelatin.

detergent is distributing itself over all the available protein molecule in a more or less statistical manner. This can be further tested by plotting $1/V_M$ against $1/C_F$ (11), in which case a straight line is obtained up to certain limit (Fig. 2). The reciprocal of the intercepts on the ordinate obtained from the extrapolation of the straight lines for SDS and SOS are 12 and 20 V_M, respectively. These values represent the maximum of V_M up to which statistical binding takes place.

Beyond the Region A, the mode of binding changes and the reaction does not appear to be statistical in nature. We speculate that after a certain number of sites have been occupied, detergent ions disrupt the tightly folded protein molecule and enter into combination with less accessible sites.

In Region C, the values of V_M far exceed the number of total cationic groups in the protein. Our results agree with Yang and Foster's studies (8) on the interaction of sodium dodecylbenzene sulphonate with serum albumin. Such extra bound detergent ions were also found by previous workers (3-4).

Excessive binding of detergents to protein can be explained by assuming that the binding of one detergent ion at a site on the protein favors the binding of additional detergents in its immediate vicinity through hydrophobic interactions of the paraffin chains. It may be concluded from this that detergent binding sites may act as a nuclei for the formation of micellar clusters on the protein (1).

Blei (11) reports that SDS well below critical micelle concentration forms a solubilizing complex with bovine serum albumin, a property which is shown by surfactants only above critical micelle concentration. This suggests that detergent on the protein forms a micelle or aggregate. A minimum concentration of the detergent is required to form these aggregates on the protein, which may correspond to the value of V_M at which extensive uncoiling and denaturation of the protein occurs. This is consistent with our results which indicate extensive unfolding in the upper range of Region B. Such micelles on protein may further be stabilized through the interaction of paraffin tails with hydrophobic residues of amino acids along the polypeptide chain.

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Presence of Antioxidant Materials in Bacteria

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ABSTRACT

Fourteen species of gram-positive and gram-negative bacteria from nine genera were investigated to determine if they produced compounds that have antioxidant activity. Washed bacterial cells were extracted with methanol for 24 hr. This extract was evaporated to dryness and extracted with benzene. The fractions were added to lard and incubated at 58 C. Antioxidant activity was determined by prolongation of the period required for the initiation of rancidity as measured by changes in peroxide value. The methanol soluble-benzene soluble fractions of *Bacillus cereus, Lactobacillus dextranicum, Micrococcus freudenreichii* and *Sarcina lutea* showed considerable antioxidant activity. Methanol fractions of three *Pseudomonas* species showed considerable activity that could not be extracted with benzene. The possibilities of using microbial growth on fats as well as extracts of microorganisms added to fats as antioxidants are discussed.

INTRODUCTION

The number of effective antioxidants that can be added to food products is limited and new compounds would be very useful. Lea (1), in 1938, suggested that because of the reducing action of microorganisms, the growth of a microbial flora on a fatty medium could lead to an inhibition of the oxidation of the fat by preventing the formation of peroxides and partially or completely destroying preformed peroxides.

In spite of this long-standing suggestion, few papers have appeared in which the antioxidant activity of microorganisms has been investigated. An oil soluble fraction of *Mycobacterium phlei* added to cottonseed oil protected it against oxidation (2) with the antioxidant activity apparently associated with the lipid component of the cell. Tatarenko and coworkers (3) were able to prevent rancidity in lard and butterfat by adding the nonsaponifiable portion of ether-extracted mycelia of *Naumoviella oleaginosa* to them. The antioxidant, 2-(hydroxy-2-methoxy-3,4-methylenedioxyphenyl)-benzofuran, isolated from baker's and brewer's yeast, was effective in protecting fats or food containing fats (4,5), and an oil soluble extract of *Aspergillus oryzae* contained a factor that prevented oxidative rancidity (6).

Recent investigations from our laboratory have shown that a large number of microorganisms have the ability to decompose the peroxides present in fresh and rancid lard (7,8). This peroxide-decomposing activity of microorganisms appears to be enzymatic in nature (9).

In attempting to extract the peroxide-decomposing material, it became apparent that a distinct antioxidant material also was present. The present investigation was undertaken to examine a wide range of bacteria as potential sources of nonenzymatic antioxidant materials. The preparation of these materials minimized the possibility that the activity was enzymatic. Crude extracts were prepared for comparison of the relative efficiency of the material from each source.

MATERIALS AND METHODS

Media for Growth

The microorganisms studied were representative strains from our culture collection and are listed in Table I. The media for production of cells were: Medium 1, Difco tryptose phosphate broth. Medium 2, Difco veal infusion broth. Medium 3, BBL APT broth. Medium 4, Case peptone, 1.0 g; 1 M phosphate buffer (pH 7, equimolar Na:K), 5 ml; distilled water, 94 ml. All media were sterilized by autoclaving at 121 C for 15 min.

Purification of Methanol and Benzene

Although reagent grade chemicals meeting American Chemical Society's requirements were used, the benzene and methanol contained substances that accelerated the oxidation of lard. To remove most of these prooxidants, benzene was passed through a celite-sulfuric acid column (10) and then distilled. Methanol was treated by the method of Lund and Bjerrum (11). The residue from 100 ml of benzene, or 400 ml of methanol, oxidized 15 g of lard to a peroxide value (PV) of 15 in 5.3 days and 2.3 days, respectively, as compared to

TABLE I

Bacterial Species Employed and Methods of Producing Cells for Isolation of Antioxidant Activity

Bacteria	Medium used[a]	Incubation conditions
Staphylococcus aureus No. 63 and No. 66	1	35 C; 24 hr, shaken[b]
Sarcina lutea No. 112	1	25 C; 24 hr, shaken[b]
Micrococcus cryophilus No. 90	1	20 C; 24 hr, shaken[b]
Micrococcus freudenreichii No. 115	1	20 C; 24 hr, static[c]
Bacillus cereus No. 283 and No. 284	2	25 C; 24 hr, shaken[b]
Escherichia coli No. 107	2	25 C; 24 hr, shaken[b]
Serratia marcescens No. 279	2	25 C; 24 hr, shaken[b]
Pediococcus cerevisiae No. 270	3	25 C; 48 hr, static[d]
Leuconostoc dextranicum No. 268	3	25 C; 48 hr, static[d]
Pseudomonas ovalis No. 36	4	20 C; 24 hr, shaken[b]
Pseudomonas fragi No. 43	4	20 C; 24 hr, shaken[b]
Pseudomonas species No. 92	4	20 C; 24 hr, shaken[b]

[a]The composition of each medium is given in the text.

[b]Two hundred milliliters of medium were dispensed into 1-liter Erlenmeyer flasks; after inlculation, the flasks were incubated on a rotary shaker at 200 rpm.

[c]One liter of medium was dispensed into 3 liter low form culture flasks.

[d]Two liters of medium were dispensed into 3 liter low form culture flasks.

10 days for the control. The purified solvents required 8.8 days and 6.7 days, respectively.

Preparation of Antioxidant Fractions and Assay of Antioxidant Activity

Appropriate media were inoculated with 18-24 hr cultures of the bacteria and incubated as indicated in Table I. Several solvents, including hexane, petroleum ether, methanol, acetone and benzene were examined for their ability to extract an antioxidant material. Sonication of the cells before extraction was of no value and the following procedure gave the best results. The cells were harvested by centrifugation and washed twice with 0.005 M phosphate buffer (pH 7, equimolar Na/K), and the cell pellets kept frozen until needed. Approximately 250 ml of methanol were added to 10-50 g wet weight of thawed cells and agitated on a magnetic stirrer for 24 hr at room temperature. The cellular debris was removed by centrifugation and the methanol removed by evaporation from a rotary evaporator at 50-60 C. Forty milliliters of benzene were added to the residue, mixed, and the material soluble in benzene removed. Both fractions were evaporated to dryness by rotary evaporation. Thus, a methanol soluble-benzene soluble fraction and a methanol soluble-benzene insoluble fraction were obtained. Fifteen grams of melted lard were added to a known weight of each fraction, the flasks rotated 5 min at 50-60 C, and the resulting mixtures placed in covered jars and incubated at 58 C in the dark. At intervals, approximately 200-250 mg of fat were removed and the PV determined by the iodometric method of Lea (12).

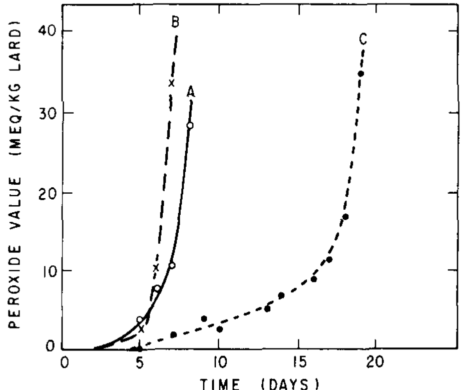


FIG. 1. Antioxidant activity of fractions from *Staphylococcus aureus* No. 63. A, methanol soluble-benzene insoluble, 253 mg; B, control; C, methanol soluble-benzene soluble, 275 mg.

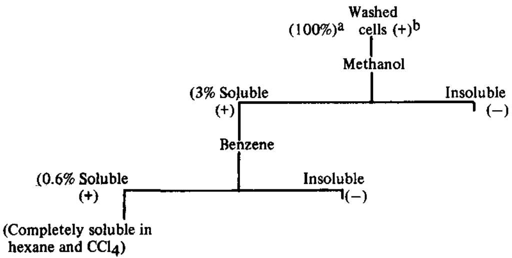
Mathematical Treatment of Data

The line of best fit was determined by the least squares analysis of regression (13).

RESULTS

The extractibility of antioxidant material from *Bacillus cereus* by different solvents is summarized in Scheme 1.

Initial extraction of cells with benzene yielded very little activity. When benzene was used to extract the methanol-soluble material from the *Pseudomonas* species, the extraction was incomplete and variable results were obtained.



Scheme I

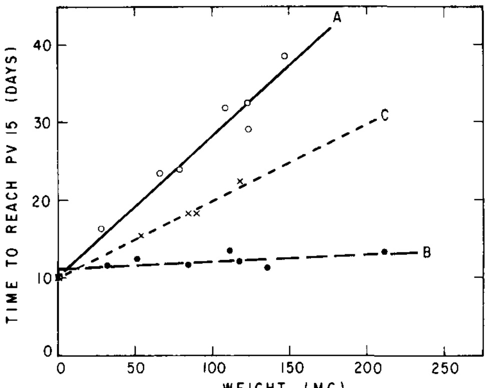


FIG. 2. Effect of varying concentrations of the methanol soluble-benzene soluble fraction from different bacteria on the stability of lard. A, *B. cereus* No. 284; B, *S. marcescens*; C, *M. cryophilus*.

Therefore, only the methanol fraction of these three cultures was tested in subsequent experiments. When cells were ashed, the antioxidant activity was destroyed. The supernatant fluid of the culture was examined for antioxidant activity, but none was detectable, even when an entire lot of growth medium was evaporated to dryness, extracted and tested.

In a typical pattern of fat oxidation there is an induction period, during which little or no change in peroxide value (PV) occurs. This is followed by a shorter period in which the rate of change in PV rapidly accelerates, reaching a high PV a short time after the oxidation begins. In Figure 1, a typical experiment with the methanol soluble-benzene soluble and methanol soluble-benzene insoluble fractions of *Staphylococcus aureus* No. 63 shows how these fractions affect the induction period. The primary effect was typical of known antioxidants in that it extended the induction period rather than decreasing the rate of oxidation once it had begun. All of the other microorganisms tested had a pattern similar to that of *S. aureus* although they varied in the time at which the upswing of the curve occurred.

There is no specific PV at which a fat can be considered rancid. However, by the time the lard in these experiments had reached a PV of 15, a rancid odor was detectable and the PV was increasing rapidly. Since different batches of fat will not oxidize at the same rate, the data from each experiment were normalized by arbitrarily adjusting the control to a PV of 15 in 10 days and then adjusting the other results in the same ratio. Utilizing this procedure, the effect of increasing concentrations of the methanol soluble-benzene soluble fraction of several microorganisms on the onset of rancidity was determined. Figure 2 indicates the linear responses obtained with three of the cultures. A linear response similar to that of *B. cereus* No. 284 was given by *B. cereus* No. 283, *Lactobacillus dextranicum, Micrococcus freudenreichii, Sarcina lutea* and *Escherichia coli*, and the methanol extract of *Pseudomonas fragi*. The two *S. aureus* strains were similar to *Micrococcus cryophilus*, and *Pediococcus cerevisiae* was similar to *Serratia marcescens*. The methanol extract of *Pseudomonas ovalis* and *Pseudomonas* sp. No. 92 gave a consistent curvilinear response as is shown in Figure 3.

The activity of the fractions from the various microorganisms is compared at the 100 mg level in Table II. With the exception of *E. coli*, the benzene insoluble fractions had little or no activity. Fractions from *M. freudenreichii, S. lutea, E. coli, B. cereus* No. 283 and No. 284, *L. dextranicum, P. fragi, P. ovalis* and *Pseudomonas* sp. No. 92, which protected lard against oxidation for more than 20 days, have strong antioxidant activity and warrant further investigation.

Lard reached a PV of 15 in 43.0, 33.5 and 22.2 days when butylated hydroxyanisole

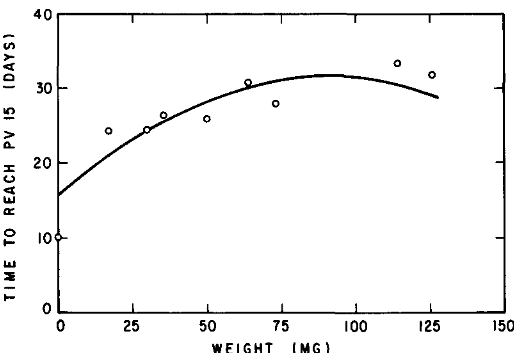


FIG. 3. Effect of varying concentrations of the methanol soluble material from *P. ovalis* on the stability of lard.

TABLE II

Comparison of the Ability of Fractions[a] From Microorganisms to Stabilize Lard

Microorganisms	Number of days for oxidation of lard[b]		
	Benzene insoluble	Benzene soluble	Methanol soluble
Micrococcus freudenreichii No. 115	10.8	23.4	---
Sarcina lutea No. 112	9.9	21.4	---
Micrococcus cryophilus No. 90	10.3	19.9	---
Staphylococcus aureus No. 66	10.5	18.6	---
Staphylococcus aureus No. 63	9.4	15.6	---
Escherichia coli No. 107	16.7	22.9	---
Serratia marcescens No. 279	10.8	12.0	---
Bacillus cereus No. 284	10.8	28.4	---
Bacillus cereus No. 283	10.7	26.4	---
Leuconostoc dextranicum No. 268	11.2	24.4	---
Pediococcus cerevisiae No. 270	11.1	12.8	---
Pseudomonas sp. No. 92	---	---	34.0
Pseudomonas ovalis No. 36	---	---	31.2
Pseudomonas fragi No. 43	---	---	29.7

[a]100 mg/15 g lard.

[b]Time for PV of lard to reach 15. Data are normalized to a value of 10 days for the controls.

(BHA), butylated hydroxytoluene (BHT) and α-tocopherol, respectively, were added at a concentration of 0.005 wt. %; the control value with no antioxidant was 10.0 days.

DISCUSSION

Although there are definite differences in concentration of antioxidant materials found in the crude extracts from the various bacteria, it remains to be determined whether these differences were a function of concentration, ease of extraction, presence of prooxidants which were simultaneously extracted, or to actual chemical differences in the compounds involved. On the basis of the results reported here, the extracts from *P. ovalis* were the most active since as little as 25 mg/15 g of lard extended the shelf life beyond 20 days. At higher concentrations, however, the curvilinear response of this extract became a factor and at 100 mg/15 g *Pseudomonas* sp. No. 92 gave better protection. The lack of any antioxidant activity in the supernatant fluid indicates that the material is intracellular and the cells must be harvested and extracted to obtain it. Since the active material is destroyed by ashing, it is not a simple inorganic substance; however, the harsh solvent extraction makes it unlikely that it is enzymatic.

The difficulties in extraction of antioxidant materials were greatest with the four gram-negative bacteria (*E. coli* and the three *Pseudomonas* species). Whether or not this problem was related to the known higher lipid content of the cell walls of gram-negative bacteria (14) awaits further study.

These data indicate that retardation of oxidation by microorganisms actively growing on the fatty medium, as suggested by Lea (1), would require extensive growth of the culture to have an appreciable effect. Such growth would likely be undesirable because of other changes such as lipolysis or proteolysis that accompany metabolism. However, on the surface of some meats, such as aged hams (15), dry sausages (16), and other foods normally accompanied by extensive microbial growth, a significant amount of antioxidant activity might develop.

On a weight basis, the bacterial fractions were not as active in protecting fats against oxidation as were BHT, BHA, and α-tocopherol. However, the microbial fractions were crude preparations and must be further purified before a suitable comparison can be made.

The work reported here establishes the potential of bacteria as sources of antioxidant materi s. The chemical nature of the compound or compounds responsible is being actively investigated, and from these studies more definite information on the economic aspects should be forthcoming.

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A Quantitative Determination of Phosphonate Phosphorus in Naturally Occurring Aminophosphonates

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ABSTRACT

A new method is described for the quantitative determination of naturally occurring aminophosphonates. Samples are treated sequentially with hydrochloric acid and alkaline phosphatase to convert esters to inorganic phosphate without destruction of phosphonates. The difference between inorganic phosphate and total phosphorus is then a measure of phosphonate phosphorus.

INTRODUCTION

The discovery of naturally occurring compounds containing the carbon-phosphorus bond (1) has necessitated the development of methods for the quantitative measurement of phosphonates. Methods previously reported can be considered in three categories. The first methods (2-4) rely upon the stability of the carbon-phosphorus bond to prolonged and vigorous acid hydrolysis followed by the separation of organic and inorganic phosphorus by ion exchange chromatography. The methods in the second category (5,6) again rely upon the acid stability of phosphonates, but they utilize differential analytical methods for the determination of total phosphorus and inorganic phosphate in the crude hydrolysate. All of these procedures are time-consuming except for the method of Aalbers and Bieber (6) which is rapid, but involves the use of very strong acid, resulting in some phosphonate destruction. Finally, ^{31}P NMR has been used to estimate the amount of phosphonate phosphorus in natural samples (7). This method is entirely nondestructive, but it has the disadvantage of low sensitivity.

MATERIALS

E. coli alkaline phosphatase was obtained from Worthington Biochemical Corporation. Ampules (2 ml, No. 12012-L) were purchased from Kimble and Silica Gel G analytical thin layer plates were a product of E. Merck (Brinkman). O-phosphoethanolamine, DL-O-phosphoserine, 2-amino-3-phosphopropionic acid, and 2-aminoethylphosphonic acid were obtained from Calbiochem. DL-serine was purchased from Nutritional Biochemicals Corporation, and phosphocholine chloride from Sigma Chemical Company. Azotobacter phosphatidylethanolamine was prepared according to Essén and Law (9).

METHODS

The lipid sample, containing approximately 1 μmole of P, was placed in a 2 ml ampule and the solvent was evaporated with a stream of N_2. After the addition of 0.1 ml of 6 N HCl the ampule was sealed and heated at 120 C for 2 hr. The ampule was cooled, opened, and the HCl was evaporated under N_2 in a warm water bath (65 C), after which the sample was dried in vacuo over NaOH for approximately 1 hr. To

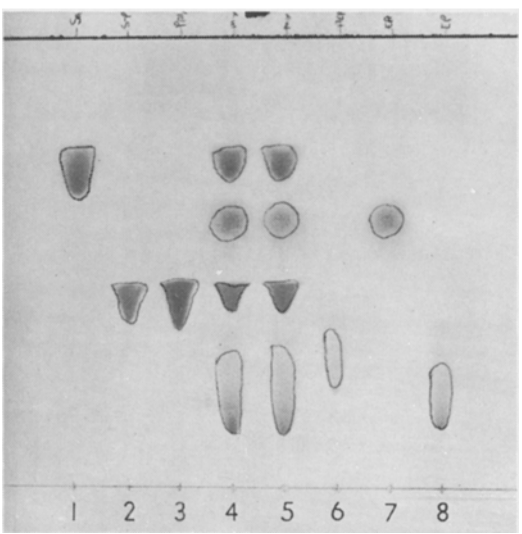

FIG. 1. Thin layer chromatogram of standard aminophosphates, aminophosphonates and phospholipid bases. (1) DL-serine; (2) DL-O-phosphoserine; (3) 2-amino-3-phosphopropionic acid; (4 and 5) mixture of the six standards; (6) 2-aminoethylphosphonic acid; (7) ethanolamine; (9) O-phosphoethanolamine. The chromatogram was developed in the solvent system i-propanol-acetic acid-58% ammonium hydroxide-water (5:2:4:3) and the spots were visualized with ninhydrin.

[1]Predoctoral trainee of the U.S. Public Health Service.

TABLE I

Enzymatic Cleavage of Various Phosphate Esters

Ester	Time, min	Per cent inorganic phos.
α-Glycerol phosphate	30	96.8
	60	99.4
	120	100
Phosphocholine	30	80.9
	60	87.8
	120	100
Phosphoserine	30	97.3
	60	98.1
	120	99.2
Phosphoethanolamine	30	89.4
	60	93.8
	120	97.2
2-Aminoethylphosphonate	30	0
	60	0
	120	0
2-Amino-3-phosphonopropionic acid	30	0
	60	0
	120	0
75% Aminoethylphosphonate 25% Phosphoethanolamine	120	23.7

the dry sample was added 0.3 ml of 0.1 M Tris-HCl buffer, pH 7.8 and 12 μg of alkaline phosphatase. The mixture was incubated at 37 C for 2 hr and aliquots were taken for determination of inorganic phosphate by the method of Chen et al. (10) and total phosphorus by the method of Ames (11).

Thin layer chromatographic (TLC) separation of aminophosphonates and their analogues phospholipid bases on Silica Gel G was carried out using the solvent system i-propanol-acetone-58% ammonium hydroxide-water 5:2:4:3 and the spots were developed with ninhydrin.

RESULTS

We first examined the action of alkaline phosphatase on various phosphates and phos-

TABLE II

Examples of Determination With Some Natural Products

Sample	Treatment	Per cent of total phosphorus: Organic[a]	Per cent of total phosphorus: Inorganic[b]
Phosphatidylethanolamine	Complete	0	100
	6N HCl, 4 hr, no enz.	41	59
Aminoethylphosphonate	Complete	100	0
Crude *Tetrahymena* lipids[c]	Complete	43.6	56.4
Phosphonolipid from *Tetrahymena* cilia[d]	Complete	98	2

[a] $\frac{\text{Total phosphorus-inorganic phosphate}}{\text{total phosphorus}} \times 100.$

[b] $\frac{\text{Inorganic phosphate}}{\text{total phosphorus}} \times 100.$

[c] This sample was obtained from Glonek et al. (7) who estimated the phosphonate content at 40% by phosphorus NMR.

[d] Reference 8.

phonates to determine appropriate conditions for quantitative cleavage of phosphomonoesters. Table I shows that 2 hr treatment with the enzyme results in complete cleavage of several phosphate esters without cleavage of the carbon-phosphorus bond. Only phosphoethanolamine was not quite completely hydrolyzed under these conditions.

We then applied the sequential treatment with 6 N HCl (to cleave phosphodiesters) followed by enzymatic digestion on some natural products to test the method. These results are reported in Table II. It can be seen that the treatment of a natural phosphatidylethanolamine for 4 hr with 6 N HCl at 120 C results in only 59% cleavage of phosphate ester bonds, while similar treatment for only 2 hr followed by enzymatic digestion results in complete cleavage. No destruction of phosphonate was observed by the complete treatment. Analysis of a crude phospholipid mixture gave results in good agreement with those obtained by a nondestructive physical method (NMR). This indicates that the presence of phosphonates did not seriously interfere with enzymatic hydrolysis of phosphate esters, which is also strengthened by the observation that phosphoethanolamine in the presence of a large amount of aminoethylphosphonate is hydrolyzed to the extent of 94.5% by alkaline phosphatase (Table I).

We have found it convenient to monitor hydrolyses occasionally by withdrawing samples for direct application to silica gel plates for TLC. The solvent system, i-propanol-acetone-58% ammonium hydroxide-water 5:2:4:3, does not separate phosphonates from the analogous phosphate esters (Fig. 1), but it is useful for judging completeness of hydrolysis with standard phosphate esters and for qualitative analysis of the components of lipid hydrolysates.

DISCUSSION

The procedure we have described is simple, accurate and reasonably rapid. A possible source of small error lies in the resistance of phosphoethanolamine to complete hydrolysis by alkaline phosphatase. However, since a natural phosphatidylethanolamine gave a quantitative yield of inorganic phosphate, this is probably not important in the analysis of natural phospholipid.

Certain natural products, such as the serine ethanolamine phosphodiester (12) would be resistant to both acid hydrolysis and alkaline phosphatase treatment, and would be measured as phosphonate. There is no evidence that this compound occurs in lipids and would cause no difficulty if only lipid extracts are examined.

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Structure Determinations and Synthesis of Pharmacologically Active Phospholipids From Kidney and Intestine

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ABSTRACT

The chemical structures of an acidic phospholipid originally isolated from equine intestine by Vogt and of a phospholipid isolated from rabbit kidney medulla by Walaszek are shown by total synthesis to be similar mixtures of 2-alkyl (and alkenyl)-4-hydroxymethyl-1,3-dioxolane-dihydrogen phosphate esters. In these materials, the alkyl residues derive primarily from oleyl, palmityl and linoleyl aldehydes. The smooth muscle contracting activity observed in the natural substances is shown to reside exclusively in the oleyl aldehyde derivative.

INTRODUCTION

Vogt reported (1a) in 1949 the isolation from horse intestine of an acidic phospholipid, which he named at that time Darmstoff, and which was capable of effecting smooth muscle contraction. It was suggested, mostly on the basis of chromatographic evidence, that the substance liberated α-glycerophosphate and a fatty aldehyde upon hydrolysis, and that the structure was of the form depicted as I below (1b).

CH_2 – O, C (H, R), CH – O, CH_2 – OPO_3H_2

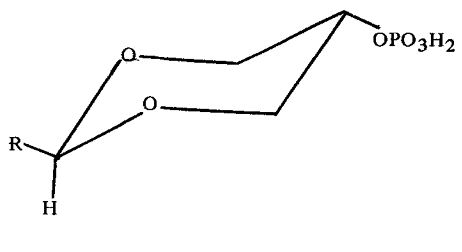
I

CH_2 – O–CH=CH–R
CH–O–C(=O)–R
CH_2 – O – P(=O)(OH) – $OCH_2CH_2NH_2$

II

The nature of the aldehyde was never elucidated, and we were struck by the fact that Vogt had isolated the phospholipid by boiling tissue in 0.2 N sodium hydroxide followed by acidification. A similar isolation procedure apparently resulted in the plasmalogens (example shown as II) being erroneously assigned a cyclic acetal-type structure I). It was shown (2) that exposure of plasmalogens to alkali caused hydrolysis of the fatty acid ester at position 2, and that subsequent acid treatment catalyzed cyclization of the enol ether-type moiety shown in II to the corresponding cyclic acetal.

Since Vogt's chromatographic evidence for the liberation of α-rather than β-glycerophosphate was conclusive, the alternative dioxane formulation III for the acetal was apparently excluded, but we desired to check this point also.



III

Dyer and Walaszek (3) have recently reported the isolation from rabbit kidney medulla of a phospholipid with pharmacological properties similar to those of Darmstoff. It has been suggested that the observed biological properties of Darmstoff and the kidney phospholipid are due to the presence of trace amounts of prostaglandins. This does not appear to be the case, since there are prominent qualitative differences in relative pharmacological properties. For example, highly purified phospholipid preparations fail to potentiate the action of acetylcholine on intestinal smooth muscle, a known effect of the prostaglandins. Moreover, Lee et al. (4) in a report concerning identification of prostaglandins from rabbit kidney, showed the presence of a lipid fraction chromatographically similar to the substances here under consideration. This material was easily differentiable from prostaglandins, but was not further characterized by Lee and co-workers.

It was of interest to ascertain the chemical structure of Darmstoff and of the kidney phospholipid, and to confirm the composition of these substances by synthesis. In this way, the possibility that the biological properties of the lipids resulted from the presence of contaminants could be decisively eliminated.

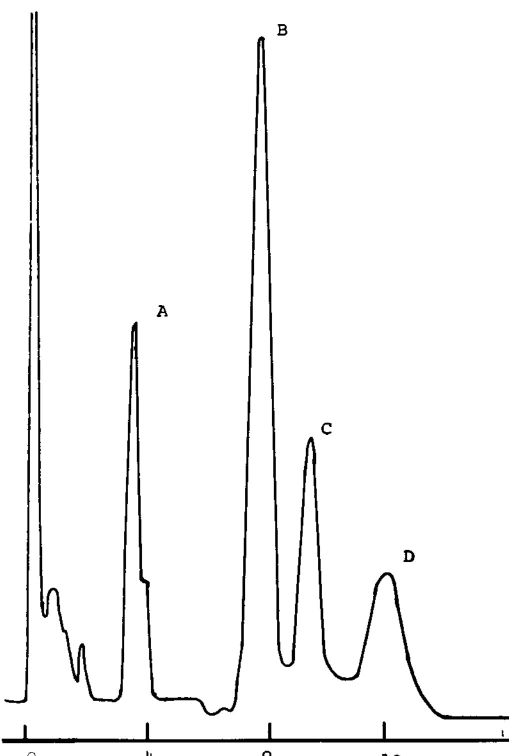


FIG. 1. Gas chromatogram of methyl esters from rabbit kidney extract. A, methyl palmitate; B, methyl oleate; C, methyl linoleate; D, methyl linolenate (tentative).

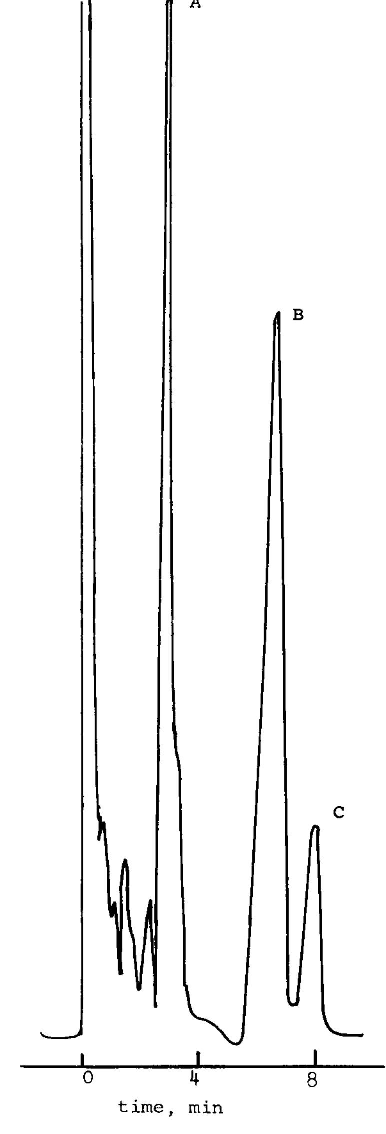


FIG. 2. Gas chromatogram of methyl esters from Darmstoff. A, methyl palmitate; B, methyl oleate; C, methyl linoleate.

CHARACTERIZATION METHODS

Extraction and Purification

The initial extraction of Darmstoff was accomplished by the procedure of Vogt (1b). The rabbit kidney lipid was extracted as follows. Frozen rabbit kidney medulla (Pel-freeze Biologicals, Rogers, Arkansas, 26 g) was homogenized in 100 ml water. The solution was acidified with 3 N HCl to pH 1, and twice extracted with 250 ml ether. The resulting emulsion was broken by centrifugation, and the combined ether extracts dried (Na_2SO_4) and evaporated. During the entire extraction process, the temperature was maintained below 20 C.

The initial extracts were purified by column chromatography on Brinkmann silica gel, 70-325 mesh. In a typical run, the ether extract from 25 g of tissue was placed on a column containing 25 g of silica gel. Following elution of nonpolar impurities with 100 ml of chloroform, the active materials was eluted with 200 ml of methanol. This was rechromatographed on Florisil as follows. Two grams of Florisil prepared according to Carroll (5) was added to the methanol eluate from the silica gel column, from which the methanol was then evaporated without the aid of heat. The resulting Florisil residue was then placed at the top of a 50 g Florisil column, and purified using the stepwise gradient elution procedure. Fractions of volume equal to that of the column were collected. Elution was begun with ethyl acetate, and methanol was added in 10% increments. The desired material began to be eluted with 40% methanol in ethyl acetate. The solvent was evaporated without heating, and the residue

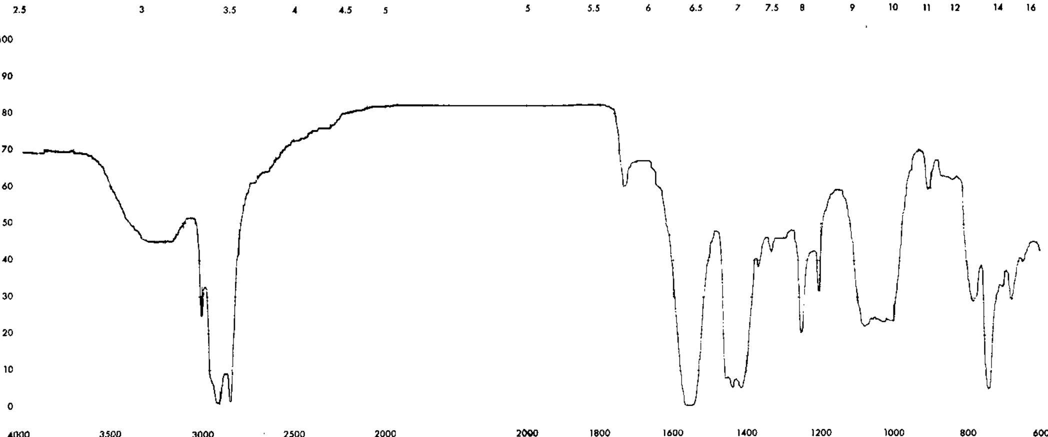


FIG. 3. Infrared spectrum of phospholipid extract from rabbit kidney.

suspended in 5 ml methanol and filtered to remove residual Florisil, which was then re-extracted with 5 ml methanol, the methanol solutions combined and evaporated to yield about 3 mg of pure lipid. Purity was established by determination of biological activity and by analytical thin layer chromatography (TLC). When chromatographed on thin layer plates prepared by dipping microscope slides into a suspension of Merck Silica Gel G in chloroform-methanol 9:1, using as developing solvent $CHCl_3$-methanol-concentrated NH_4OH (60:35:5) a reproducible R_f of 0.52 ± 0.02 was obtained for both extracts. The spot was visualized by I_2 vapor, the fuchsin aldehyde reagent of Skidmore and Enterman (6), the phosphate reagent of Hanes and Isherwood (7), and phenol red spray in weakly basic solution. The material was also homogenous on silica gel using several other solvent systems, and on Whatman No. 1 paper, using as developing solvent *n*-butanol saturated with water and *n*-propanol-NH_4OH-H_2O (70:10:20). Both Darmstoff and the kidney phospholipid were shown to exhibit pKa values near 2.5 and 6.8, thus affording additional indication that both are phosphatidic acids. For storage, it was desirable to convert the extracts to the corresponding sodium salts. This was done by suspending a large excess of Dowex 50 (Na^+ form) in water, adding the lipid, and stirring for 1 hr. The solution was then filtered and lyophilized.

Identification of Aldehydes

One milligram of purified extract, 25 ml of methanol, and a trace of HCl were heated at reflux for 12 hr. The solvent was evaporated to a volume of about 0.5 ml, and the fatty acid methyl esters produced by oxidation of the aldehyde mixture with O_2 and esterification of the resulting fatty acids with methanol in the presence of acid were identified by comparison of their gas chromatographic retention times with authentic standards. To confirm the identification, authentic samples of methyl palmitate, methyl oleate and methyl linoleate were used as internal standards. It had been previously determined that this procedure effected complete conversion of fatty aldehydes to the corresponding fatty acid methyl esters. A 1/4 in. o.d. x 4 ft column packed with 5% LAC-446 on 60-80 mesh Chromosorb P was used at 170 C. The carrier gas was He, flowing at 80 ml/min. A flame ionization detector was employed. Relative amounts of fatty acid esters present were estimated by calculating the ratio of the area of each peak to the sum of areas of peaks identifiable as due to fatty acid esters.

Observation of Spectra

NMR spectra were obtained on a Varian A60A spectrometer, using a Varian C-1024 time averaging computer to generate 350 scans. IR spectra of the purified extracts were obtained on a Beckman IR-10 spectrophotometer as thin films on NaCl plates.

Chemical Properties

The olefinic linkage in an enol ether such as II would be expected to be very susceptible to catalytic hydrogenation. The reduction product would not liberate aldehydes on acid hydrolysis. The purified extract was therefore hydrogenated in dilute methanol solution over 50 mg. PtO_2 using 2 atm pressure at 25 C. The residue obtained after filtration and evaporation of the solvent was spotted on paper and sprayed with $HgCl_2$-containing fuchsin alde-

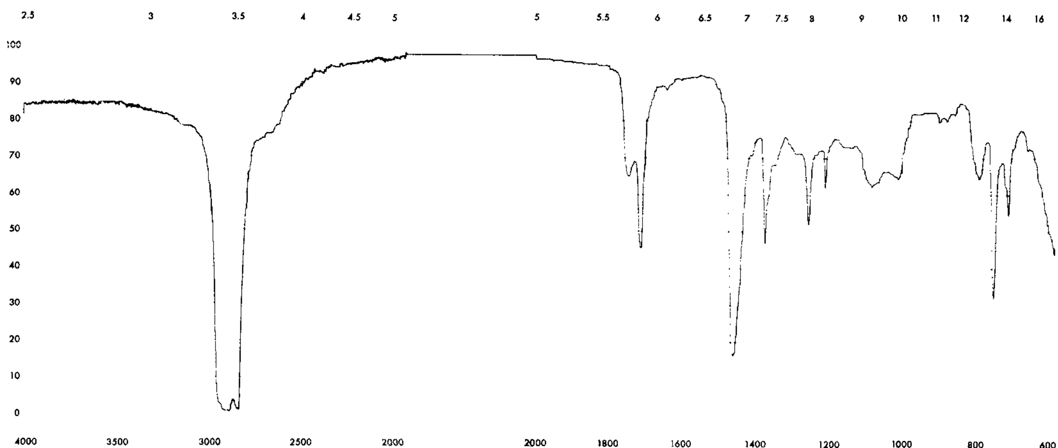


FIG. 4. Infrared spectrum of kidney phospholipid extract following hydrogenation.

hyde reagent (6). The reduced extract was also heated on a steam bath 1 hr with 3 N HCl, and the mixture extracted with ether. The IR spectrum of the residue obtained after drying and evaporation of the ether solution was observed, in order to determine the presence of aldehydes by their strong C=O stretching vibration at 1740 cm^{-1}.

RESULTS

The gas chromatograms of fatty acid methyl esters obtained from Darmstoff and the rabbit kidney phospholipid are shown in Figure 1 and 2 respectively. Peaks A, B and C in the Darmstoff chromatogram were identified as methyl palmitate, methyl oleate and methyl linoleate, respectively. Peak D was tentatively identified as methyl linolenate based on published retention data (8). Peaks A, B and C in Figure 2 were identified as due to the same substances as the corresponding peaks in Figure 1. A shoulder is observable on peak A in each case, which may result from the presence of a small amount of methyl palmitoleate. Methyl stearate is conspicuously absent, but this was verified several times. The relative amount of the aldehydes present in the two natural extracts is shown in Table I.

TABLE I

Aldehydes in Lipid Extracts

Aldehyde	Darmstoff, %	Kidney lipid, %
Palmitaldehyde (16:0)	17	32
Oleyl aldehyde (18:1)	49	55
Linoleyl aldehyde (18:2)	20	13
Linolenyl aldehyde (18:3)	14	

The IR spectra of the two extracts were essentially identical, and that of the kidney extract is shown in Figure 3. The spectrum is characterized by absorption maxima due to C–H stretching of vinylic (3000 cm^{-1}) and aliphatic (2920, 2840 cm^{-1}) C–H bonds, P=O stretching (1260 cm^{-1}), C–O–C stretching (1100, 1000 cm^{-1}), and C–H bending for the *cis*-olefinic side chain linkages (685 cm^{-1}). The peak at 1740 cm^{-1} is regularly observed, but its widely varying relative intensity indicates that it is due to the presence of a varying amount of aldehyde (C=O stretch) formed while the spectrum was being run. The peak near 3300 cm^{-1} in Figure 3 is apparently due to the presence of water. The intense band near 1550 cm^{-1} is difficult to assign. The frequency appears to be too low even for a conjugated olefinic linkage, but the fact that only this band and the 3000 cm^{-1} C–H stretch disappear upon catalytic hydrogenation (q.v.) suggests that the 1550 cm^{-1} band is due to unsaturation in the fatty chains. The band is possibly shifted to this unusual location due to interaction between the olefinic linkages and the phosphate moiety, such interactions being known to exert profound effects upon electronic spectra of olefins (9a). It is noteworthy that bands are absent near 1750, 1667 and 965 cm^{-1}. If the plasmalogen-type structure II obtained, these would be found, due to ester carbonyl stretching, C=C stretching in enol ether, and C–H bending for the *trans* olefinic linkage in the enol ether, respectively. All the observed bands would be expected if I were the best structural representation, and I is thus in best agreement with the IR data.

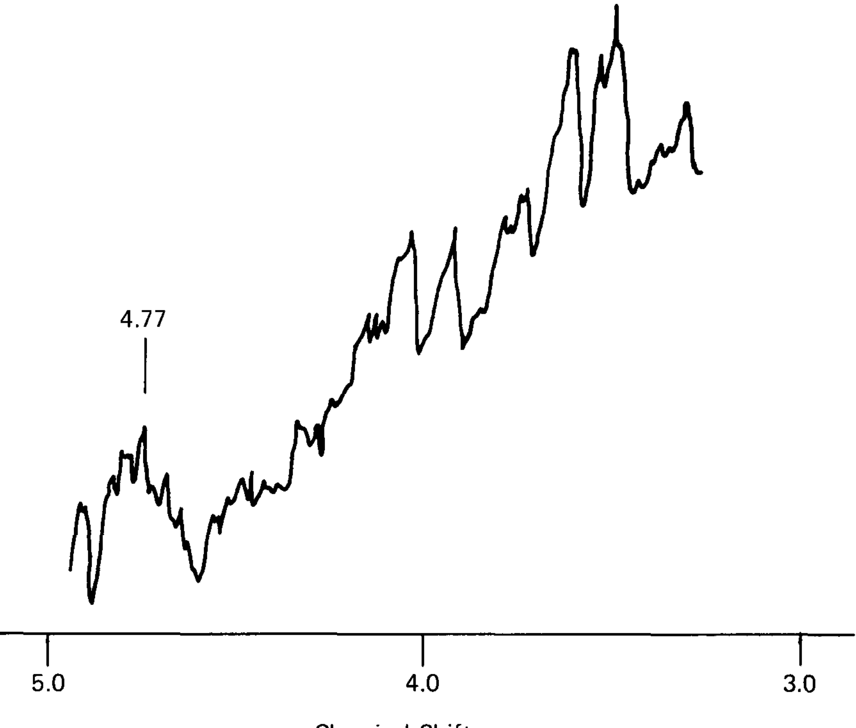


FIG. 5. 60 MHz nuclear magnetic resonance spectrum of the purified kidney phospholipid extract (3.35 to 5.0 ppm region). Resultant of 350 scans, obtained at -12°.

The spectrum of the kidney extract after catalytic hydrogenation is shown in Figure 4. It is seen that the 1550 cm^{-1} band due to side chain unsaturation and the 3000 cm^{-1} vinylic C–H stretching band are absent, but no other significant changes are found. The reduced material remained labile to acid hydrolysis, in which aldehydes were shown to be liberated both by the IR and fuchsin spray reagent methods described above, which data also militate for structure I and against II.

The NMR spectrum of both extracts was identical, and in addition to the expected peaks for CH_3 and CH_2 at δ0.9 and δ1.35 respectively, exhibited the following absorptions: δ2.10 (broad), -CH_2CH=; δ2.80 (broad), =CHCH_2CH=; δ3.30-4.20 (multiplet), ring protons; δ4.75 (broad), -OCH_2R-O-; and δ5.35 (triplet), -C*H*=. All except the δ3.30-4.20 multiplet are readily assignable to the fatty aldehyde side chains. The δ3.30-4.20 region is of considerable interest, since it had been hoped to obtain from the NMR data for these protons definitive information concerning ring type and stereochemistry. Since only small amounts of material were available, it was necessary to scan 350 times to obtain the usable spectrum for the kidney extract shown in Figure 5. The presence of an apparent absorption near δ4.75 is of particular interest. It has been shown (Wiley and Sumner, unpublished results) that the methine proton at position 2 in the four model cyclic acetals formed from butanal and glycerol absorbed as follows: *cis*-dioxolane, δ4.77; *trans*-dioxolane, δ4.87; *cis*-dioxane, δ4.44; and *trans*-dioxane, δ4.34. These model compounds are not phosphates but it has been shown (9b) that phoryphorylation of alcohols causes only very slight ($<$0.1 ppm) changes in proton chemical shifts. Thus the presence of absorption in the kidney extract below δ4.5 clearly identifies it as a dioxolane I rather than a dioxane III, and is suggestive that it is *cis* rather than *trans* substituted. Confirmation of this point must await additional data.

The evidence presented indicates that Darmstoff and the Walaszek kidney extract are essentially identical, based on chromatographic, chemical and spectral behavior, despite the different isolation procedure. Further, possible structures II and III are clearly excluded, and both extracts are shown to possess structure I, in which R is derived from a mixture of 16- and 18-carbon aldehydes. These data do not

TABLE II

Microanalytical Data

Compound	Formula	C Calc.	C Found	H Calc.	H Found
7b	$C_{21}H_{40}O_3 \cdot C_2H_5OH$	71.46	71.44	11.99	11.77
7c	$C_{21}H_{38}O_3$	74.51	74.78	11.31	11.53
1a, diphenylester	$C_{31}H_{47}O_6P$	68.19	68.43	8.68	8.83
1a	$C_{19}H_{39}O_6P$	57.77	57.60	9.95	9.76

exclude the possibility that both Darmstoff and the Walaszek extract are artifacts arising from isolation procedures.

SYNTHETIC METHODS

It was necessary to synthesize the individual acetal phosphatidic acids which comprise the natural extracts; the synthetic route is that shown Scheme I. Reduction of the commercially available acid chlorides 4a and 4b by the Rosenmund procedure (10) yielded the aldehydes 5a and 5b, which were immediately converted to the corresponding acetals 6. IR and NMR data confirm that no double bond isomerization occurred in compound 6b. The Rosenmund procedure did cause reduction of unsaturation in 4c, obtained by the procedure of Bauer (11). Reduction of 4c using lithium tri(*t*-butoxy)aluminum hydride according to Rao et al. (12) proceeded smoothly to yield 5c, which was immediately converted to the dimethylacetal 6c by treatment of the aldehyde in dry methanol with 100 mg of *p*-toluenesulfonic acid.

Synthesis of the cyclic acetals 7 was

Synthetic Route*

$R-C(=O)-Cl$ (4a—c) → [RCHO] (5a—c) → $RCH(OCH_3)_2$

Glycerol, H^+ → CH_2-O-, $CH-O-$ (cyclic, $>C<^{H}_{R}$), CH_2OH (7a-c) → CH_2-O-, $CH-O-$ (cyclic, $>C<^{H}_{R}$), $CH_2-OPO_3H_2$ (1a-c)

* a, $R = C_{15}H_{31}$
b, $R = C_{17}H_{31}$
c, $R = C_{17}H_{29}$

Scheme 1

accomplished by the transacetalation procedure of Piantadosi et al. (11,14) which had been determined (13) to yield exclusively the dioxolane shown rather than the corresponding dioxane-type acetals 3 if the reaction time were short. NMR spectra of the three acetals 7a-c confirm this observation. Microanalytical data for all new compounds prepared in this study are shown in Table II. All dioxolane acetals and phosphates were waxes which melted near room temperature. The melting range observed was dependent upon experimental conditions to such a marked degree that these melting points are considered meaningless and therefore not reported.

Phosphorylation of the pentadecyl dioxolane 7a was carried out by the procedure of Brigl and Mueller (14). This method involves the use of diphenylphosphochloridate to form the acetal-phosphatidic acid diphenyl ester, which could be purified and characterized. The diphenyl ester then undergoes hydrogenolysis over platinum catalyst to yield the desired phosphate in 95% yield. This was analytically pure and homogenous to TLC analysis. Unfortunately this method could not be employed with the other two acetals 7b and 7c, since their side chain unsaturation was labile to the hydrogenolysis conditions.

Phosphorylation of the unsaturated derivatives 7b and 7c was carried out by the method of Edgerton and Malkin (15), using phosphorous oxychloride in chloroform with a stoichiometric amount of pyridine, followed by hydrolysis. The resulting phosphates, like the natural extracts but unlike the saturated derivative 1a, were extremely labile toward hydrolysis of the acetal linkage, which made their purification a difficult challenge. Further, thin layer analysis of the reaction mixture according to the methods set forth above for the natural extracts revealed, in addition to the desired dioxolane phosphate (R_f 0.50) a second closely related phosphate (R_f 0.35). This was apparently due to the

dioxane phosphate analogous to 3. These two materials were inseparable by preparative chromatographic methods. Eventually, the substances were separated by manual countercurrent distribution (16) performed on the ammonium salts. This procedure yielded small quantities of material homogenous to TLC analysis, which was, however, too unstable to permit elemental microanalysis. The compounds were identifiable by their behavior on thin layer and paper chromatography, by the fact that they reacted with all three spray reagents in a manner identical with the natural extracts described above, the fact that the starting materials for the phosphorylation reaction were rigorously characterized and, perhaps the ultimate test, that 1b, but not 1a (fully characterized) or 1c, displayed full biological activity.

EXPERIMENTAL PROCEDURES

2-Pentadecyl-4-hydroxymethyl-1,3-dioxolane, Diphenyl Phosphate Ester (1a, Diphenylester)

2-Pentadecyl-4-hydroxymethyl-1,3-dioxolane (3.14 g, 0.01 mole) was dissolved in 15 ml of anhydrous pyridine. Diphenyl phosphochloridate (3.0 g, 0.011 mole) in 5 ml of pyridine was then added dropwise with stirring while the mixture was cooled in a bath of ice-salt-water. Atmospheric moisture was excluded from the reaction.

The mixture was stored overnight at 5 C. Ice (0.5 g) was added to the cold solution. After standing 20 min the mixture was added to 200 ml of ice and water. After neutralization with 3 N HCl the aqueous solution was extracted with petroleum ether.

The petroleum ether was dried ($MgSO_4$) and evaporated to give crude material. Filtration through Wolem neutral Alumina with the aid of petroleum ether afforded 5.25 g (96%) of the ester (Table II).

2-Pentadecyl-4-hydroxymethyl-1,3-dioxolane, Dihydrogen Phosphate Ester (1a)

The diphenyl phosphate ester (1.0 g, 1.8 mmole) was reduced in EtOH over 200 mg of prereduced PtO_2 at 1 atm H_2 pressure at room temperature. The reaction was complete after 8 hr, the catalyst was removed by filtration and the EtOH evaporated. The residue was washed with a minimum of petroleum ether and dried to give 0.715 g (95%+) of the phosphate: IR, 2920, 2860 (C–H), 2400 (w) (P-OH), 1250 (P=O), 1100, 1000 (C–O–C), 715 [-$(CH_2)_4$]. (Table II).

Neutralization equivalent: Calculated for 197, Found 189. The material was soluble in aqueous base and insoluble in aqueous acid.

2-Cis-Δ^8-Heptadecenyl-4-hydroxymethyl-1,3-dioxolane Dihydrogen Phosphate Ester (1b), and 2-cis, cis-$\Delta^{8,11}$-Heptadecadienyl-4-hydroxymethyl-1,3-dioxolane Dihydrogen Phosphate Ester (1c)

The phosphorylation of the unsaturated derivatives was accomplished by the procedure of Edgerton and Malkin (15) without modification except that it was run under nitrogen atmosphere. The isolation procedure was adapted for the monophosphate. After the reaction of $POCl_3$ and 700 mg of alcohol was complete, the $CHCl_3$ was evaporated, the flask cooled and 50 ml of cold H_2O added. The solution was acidified with 3 N HCl and extracted with 300 ml of Et_2O. To the Et_2O, 40 ml of concentrated NH_4OH was added and evaporated to provide the crude NH_4^+ salt.

Attempted recrystallization from MeOH and from petroleum ether was unsuccessful because of co-precipitation of compound and impurities. Countercurrent extraction of the material using MeOH-petroleum ether and 18 tubes led to purification of the material, which was found in tubes 1-3.

Identification was based on the R_f value on silica gel, identical to that observed for the phospholipids isolated from tissue, the fact that the starting material was rigorously characterized, and reactions with color reagents described above.

PHARMACOLOGY

The pharmacological activity of both the natural and synthetic products was tested on strips of guinea pig ileum. Two strips of ileum were suspended in 10 ml of Tyrode solution and the contractile activity measured in each. Prior to testing, each sample and each concentration a stable dose-response curve to acetylcholine was established. Following pharmacological testing, the solutions used were subjected to analytical TLC to insure that no decomposition had occurred during the testing process. In each case, the substances were found to be homogenous after testing.

The natural products produced contractions in the guinea pig ileum qualitatively comparable to those produced by acetyl-choline. These contractions were characterized by a rapid onset of action and prolonged duration. The sodium salt of the kidney extract product was shown to be active in concentrations of 5 x 10^{-6} g/ml. Concentrations of this order were shown to produce contractions of the same

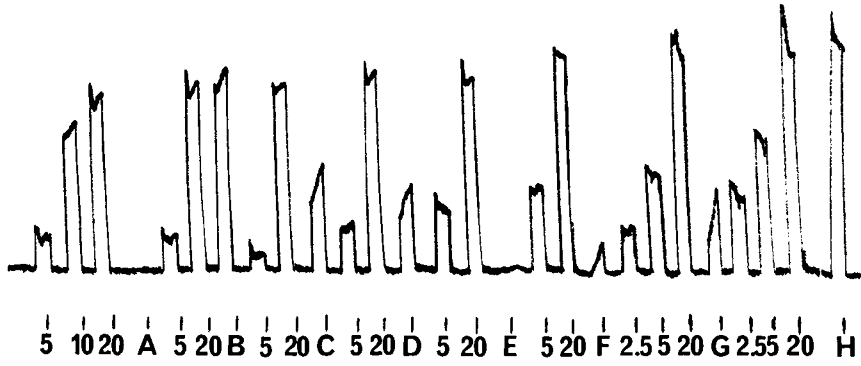


FIG. 6. Response of guinea pig ileum to synthetic glyceryl acetal phosphates. Arabic numbers indicate concentration of standard (acetylcholine) in g/ml x 10^{-9}. A, palmityl acetal 1a 5 x 10^{-6} g/ml; B, oleyl acetal 1b 6 x 10^{-6} g/ml; C, oleyl acetal 1b 3 x 10^{-6} g/ml; D, oleyl acetal 1b 1.5 x 10^{-6} g/ml; E, linoleyl acetal 1c 3 x 10^{-6} g/ml; F, linoleyl acetal 1c 6 x 10^{-6} g/ml; G, linoleyl acetal 1c 1.2 x 10^{-5} g/ml; H, oleyl acetal 1b 6 x 10^{-6} g/ml.

magnitude as 2 x 10^{-8} g/ml of acetylcholine.

It should be noted that Vogt reports the Darmstoff threshold concentration for contraction in the rabbit duodenum to be 10^{-7} g/ml (17). In the guinea pig ileum the threshold value has been reported by Gray to be 10^{-6} g/ml (18). This is comparable to the values observed with the material isolated in this laboratory.

The phosphorylated glyceryl acetal of palmitaldehyde, 1a, was tested at concentrations of 5 x 10^{-5} g/ml, at which there was no evidence of contractions.

The phosphorylated glyceryl acetal of oleaaldehyde (16) was found to produce contractions identical to those of the natural product. When used at 6 x 10^{-6} g/ml, this material was found to exhibit contractions equal to those produced by 2 x 10^{-8} g/ml of acetylcholine. The phosphorylated glyceryl acetal of linoleaaldehyde, 1c, was found to produce contractions qualitatively and quantitatively different from those of the oleyl acetal 1b. The onset was delayed with respect to that of the latter compound and the contractions developed slowly. Quantitatively, 1.2 x 10^{-5} g/ml of the linoleyl derivative produced a contraction equivalent to 2 x 10^{-9} g/ml of acetylcholine. The tracing obtained from one of the intestinal strips is shown in Figure 6.

In an analysis of these data it should be remembered that in all three cases the compounds were present as mixtures of *cis* and *trans* isomers with respect to substituent orientation about the ring. Because of the specificity exhibited by the side chain one might expect similar results with respect to ring stereochemistry and side chain geometrical isomerism. To determine the effects of these variables further study would be needed.

These data indicate that the activity of the natural product lies primarily with the oleyl glyceryl acetal phosphate. Any contribution by the linoleyl glyceryl acetal phosphate is minimized by its lower potency and its low concentration in the natural product. The presence of the palmityl acetal 1a in the natural product is unexplained. If it were present as a synthetic precursor one would expect to find stearic acid derivatives as well as the others observed. Continuing studies on side chain specificity and ring stereochemistry are anticipated in this interesting class of compounds.

ACKNOWLEDGMENTS

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Cyanolipids of *Cardiospermum halicacabum* L. and Other Sapindaceous Seed Oils[1]

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ABSTRACT

A number of sapindaceous seed oils have been investigated with respect to their cyanolipid constituents. All but one of the oils have this new class of lipids in amounts ranging from 13% to 55%. These cyanolipids are of four different types, but all consist of long-chain fatty acids esterified with an unsaturated isoprenoid hydroxy- or dihydroxynitrile. The large amounts of C_{20} acids usually found in these oils indicate an appreciable cyanolipid content because such acids are preferentially incorporated in nitrile-containing fractions. *Cardiospermum halicacabum* L. seed oil was shown to contain 49% of a diester having two fatty acid moieties esterified with 1-cyano-2-hydroxymethylprop-2-ene-1-ol and 6% of another diester derived from 1-cyano-2-hydroxymethylprop-1-ene-3-ol. Treatment of the latter diester with methanolic hydrogen chloride produces methyl 4,4-dimethoxy-3-(methoxymethyl) butyrate from the dihydroxynitrile moiety.

INTRODUCTION

Cyanolipids were probably first observed in *Schleichera trijuga* seed oil (1-6), although the nature of the nitrile moiety was not established. Seed oils from four different plants have been shown (7-11) to contain major amounts (30-40%) of cyanolipids, which consist of 18- and 20-carbon fatty acids esterified with a mono- or dihydroxynitrile moiety. One of these plants, *Cordia verbenacea*, is a borage; its cyanolipids are diesters (I, Fig. 1) containing two fatty acid moieties esterified with 1-cyano-2-hydroxymethylprop-2-ene-1-ol (7,8). The other three plants belong to the Sapindaceae. *Stocksia brahuica* (9) and *Koelreuteria paniculata* (10) seed oils contain cyanolipids (III) having one long chain fatty acid esterified with 1-cyano-2-methylprop-1-ene-3-ol. Diester cyanolipids (II) having two fatty acids esterified with 1-cyano-2-hydroxymethylprop-1-ene-3-ol are also present in *Koelreuteria* oil (10). *Ungnadia speciosa* seed oil contains a cyanohydrin ester (IV) composed of a fatty acid esterified with 1-cyano-2-methylprop-2-ene-1-ol (11).

We have now investigated seed oils from additional sapindaceous plants with respect to their cyanolipid content. The nitrile-containing substances have been isolated and identified by comparison of their NMR, IR, UV and thin layer chromatographic (TLC) characteristics with those of cyanolipids we described previously (7-10). Since each cyanolipid fraction is a mixture of esters which differ only in fatty acid constituents, each fraction (I-IV, Fig. 1) has been treated here as though it were homogeneous.

We also wish to report that 4,4-dimethoxy-3-(methoxymethyl)butyrate, which was

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             CH2
             ||
Acyl—O—CH2—C—CH—C≡N
                |
                O—Acyl
            [I]

               CH2—O—Acyl
               |
Acyl—O—CH2—C=CH—C≡N
            [II]

               CH3
               |
Acyl—O—CH2—C=CH—C≡N
            [III]

        CH2
        ||
  CH3—C—CH—C≡N
          |
          O—Acyl
         [IV]
```

Acyl = predominantly octadecenoyl, eicosenoyl

FIG. 1. Structures of cyanolipid fractions.

[1]Presented at the AOCS-ISF Joint Congress, Chicago, September 1970.

[2]No. Utiliz. Res. Dev. Div., ARS, USDA.

TABLE I

Cyanolipid-Containing Seed Oils

Plant identity	Oil in seed, %	C_{20} acids, %[a]	Cyanolipid Content			
			I in oil, %	II in oil, %	III in oil, %	IV in oil, %
SAPINDACEAE						
Allophyllus edulis St. Hil.	59.7	44	30	Trace[b]	---	---
Cardiospermum halicacabum L.	18.6[c]	47	49	6	---	---
Litchi chinensis Sonner.	1.3	0.4	---	---	---	---
Nephelium lappaceum L.	34.2	39	---	21	---	---
Paullinia meliaefolia Juss.	54.8	72	53	---	---	---
Sapindus mukorossi Gaertn.	35.4	21	---	13	---	---
Urvillea uniloba Radlk.	46.5	50	19	4	---	---
Koelreuteria paniculata Laxm.[d]	41.9	46	---	25	17	---
Stocksia brahuica Benth.[d]	19.4[c]	42	---	Trace[b]	35	---
Ungnadia speciosa Endl.[d]	63.2	48	---	Trace[b]	Trace[b]	29
BORAGINACEAE						
Cordia verbenacea DC.[d]	38.4[c]	43	35	---	---	---

[a]Area per cent of methyl ester by GLC.

[b]Based on TLC analysis; not isolated.

[c]Seed plus hull analyzed.

[d]Investigated previously; see References 7-11.

identified by Hopkins et al. (12) in methyl esters prepared from *Cardiospermum halicacabum* seed oil, is derivable from one of the cyanolipids present in this oil.

EXPERIMENTAL PROCEDURES

Spectrometry

NMR spectra were obtained with a Varian HA-100 spectrometer; either $CDCl_3$ or a mixture of $CDCl_3$ and C_6D_6 (ca. 10:1) were the solvents used. Chemical shifts were measured from internal tetramethylsilane (τ 10.0). IR analyses were performed on 1% solutions in $CHCl_3$ with a Perkin-Elmer Model 137 spectrophotometer, and a Beckman DK-2A instrument was used to determine UV spectra.

Oil Recovery and Methyl Ester Formation

Oil was recovered from finely ground seeds by a 16 hr extraction with petroleum ether (bp 30-60 C) in a Soxhlet apparatus or by a 6 hr extraction in a Butt apparatus. Methyl esters were prepared by refluxing the oils 3 hr with 3% H_2SO_4 in methanol and were recovered by ether extraction. Analyses of these ester samples by gas liquid chromatography (GLC) were performed essentially as described by Miwa et al. (13).

Thin Layer Chromatography

Analytical TLC was done on 0.25 mm layers of Silica Gel G with either benzene or ether-hexane (1:3); choice of solvent depended on which components were being separated. Spots were visualized in two ways: first, by exposure to iodine vapor and then by charring with a sulfuric acid-dichromate spray reagent.

Silica Gel G layers, 1 mm thick, were used for preparative TLC separations. When the seed oils were being fractionated, about 200 mg of oil, as a 50% solution in $CHCl_3$, was spotted on each 20 x 20 cm plate. The solvents were benzene or ether-hexane (1:2). Major bands were detected by viewing developed plates over an incandescent lamp in a darkened room, and minor components were detected by spraying the plates with an alcoholic solution of 2′,7′-dichlorofluorescein and viewing them under UV light. Desired constituents were recovered from the silica by standard procedures and the purity of the fractions was checked by analytical TLC. Amounts of cyanolipids reported in these various seed oils are the actual yields isolated by preparative TLC.

RESULTS AND DISCUSSION

General

Table I lists data on various sapindaceous seed oils. The last four listed (one is a borage) were examined previously (7-11) and are included for comparison purposes. Oil content of the seeds (Table I) is quite variable and ranges from 1.3% for *Litchi* to 63.2% for *Ungnadia*. The values for *Cardiospermum* and *Stocksia* are probably somewhat low because entire seeds (including hulls) were analyzed.

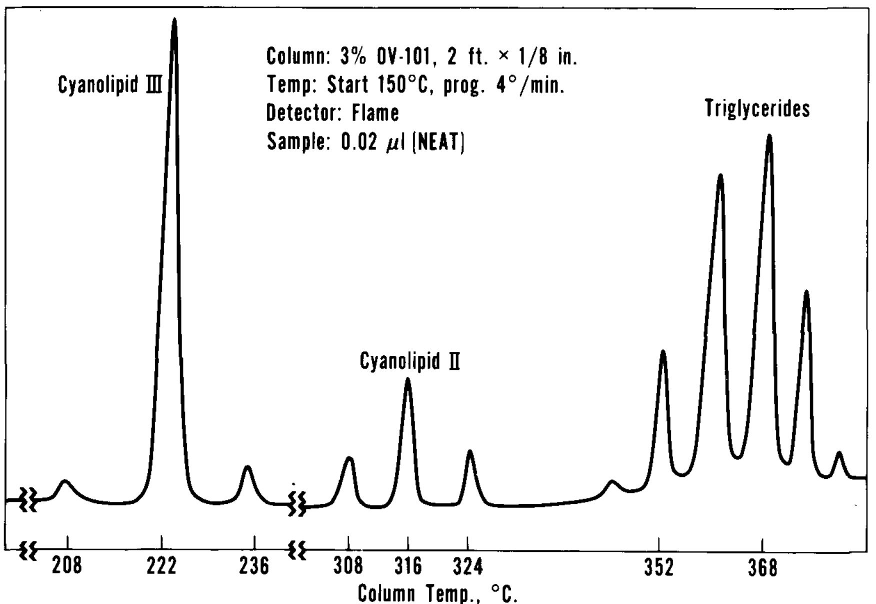

FIG. 2. GLC of *Koelreuteria paniculata* seed oil.

IR spectra of these seed oils generally have two, very weak, broad bands in the 1010 and 920-965 cm^{-1} regions. These bands are probably associated with allylic ester and terminal methylene groupings in the cyanolipids and are weak enough to be overlooked easily. Of the new species investigated, only *Nephelium* oil showed a nitrile band (weak) at 2230 cm^{-1}.

TLC analysis of the oils was more revealing than their IR spectra. Oils that contain cyanolipid fractions I or IV (Fig. 1) are best analyzed by TLC in benzene. Fraction I has R_f 0.70 and IV, R_f 0.77, while the triglycerides and other cyanolipids, if present, migrate as an elongated spot, R_f 0.53. When the ether-hexane solvent system is used, triglycerides along with cyanolipids I and IV form a single spot, R_f 0.91. Cyanolipids II and III (Fig. 1) migrate at R_f 0.70 and 0.58, respectively.

Under certain conditions (8-10) useful GLC data can be obtained by analysis of whole seed oils. This statement is exemplified by Figure 2, which shows the GLC analysis of *Koelreuteria* seed oil. Cyanolipids II and III, which occur in this oil, are widely separated. The three peaks observed for each fraction are due to the different fatty acid residues in the esters. Cyanolipid I would appear in the same region as II, and the GLC characteristics of IV have not been determined in our Laboratory.

Partial decomposition of the diester (II) under GLC conditions causes the percentage of II calculated from Figure 2 to be considerably less than is actually present in the oil. GLC analysis of this oil with a different instrument, but with other variables similar to those shown in Figure 2, caused essentially a complete breakdown of II. Monoester III appears to be more resistant to decomposition.

An interesting feature of sapindaceous seed oils, previously noted by Hopkins and Swingle (14), is that most of them contain large amounts of C_{20} fatty acids. *Litchi chinensis* (Table I) is the only exception we found, and, significantly, this oil is the only one examined that did not contain cyanolipids. It does, however, have an unusual fatty acid composition resembling that of *Euphoria longana* seed oil (15). Generally the C_{20} acid mixture is primarily monoenoic with lesser amounts of the C_{20} saturated (arachidic) acid– from 1% in *Stocksia* to 13% in *Cardiospermum*. *Nephelium* seed oil, however, has 39% of C_{20} acids and most of this (33%) is arachidic acid.

The results in Table I reveal that the amount of cyanolipid in an oil is often (but not always) directly related to the amount of C_{20} acids. Previous work demonstrated that the C_{20} fatty acid content of a cyanolipid fraction is much higher than that of the triglyceride fraction (8,10) isolated from the same seed oil. Thus it may be possible to predict roughly the cyanolipid content of sapindaceous seed oils by determining their C_{20} acid content.

FIG. 3. Proposed mechanism for transformation of cyanolipid II to methyl 4,4-dimethoxy-3-(methoxymethyl)butyrate

Cyanolipid Structures

Previous reports describe in detail the structure elucidation of cyanolipids I (7,8), II (10), III (9) and IV (11). Therefore, a comparison of the IR, TLC and NMR data for the cyanolipids isolated in our current study with those reported earlier was sufficient for structural identification. The IR spectrum of each cyanolipid is distinctive. For example, cyanolipid I shows IR maxima (weak, broad bands at 940 and 1010 cm^{-1}) not found in spectra of ordinary triglycerides. In the spectrum of II, the 940 cm^{-1} band is absent and sharp, medium-intensity bands are observed at 1600 and 2230 cm^{-1} (cyano group). The 1010, 1600 and 2230 cm^{-1} bands also appear in the IR spectrum of III, but the fourth band is shifted slightly to 965 cm^{-1}. The spectrum of IV shows medium-intensity bands at 920 and 1010 cm^{-1}, which also contain some fine structure. The absence of nitrile absorption (2230 cm^{-1}) in the spectra of cyanolipids I and IV is due to the quenching effect of the oxygen atom on the same carbon as the cyano group (16).

NMR spectra reported for I (7,8), II (10), III (9) and IV (11) provided the most conclusive structural evidence for these substances. All the cyanolipids isolated from the seven additional oils (Table I) were also analyzed by NMR and the resulting spectra duplicated those obtained earlier with respect to proton counts, chemical shifts, multiplicities and coupling constants. Six of the seven new species investigated contained cyanolipids I or II, or both, in amounts ranging from 13% for *Sapindus* to 55% for *Cardiospermum*. No cyanolipids were detected in *Litchi* seed oil and cyanolipids III and IV were not found in any of the oils. So far, *Ungnadia speciosa* is the only species that contains cyanolipid IV (11).

Cardiospermum halicacabum Seed Oil

Hopkins et al. (12) isolated 0.5% of a low-boiling fraction from the methyl esters derived by refluxing *Cardiospermum* seed oil 16 hr with HCl in methanol. They concluded that this material was methyl 4,4-dimethoxy-3-(methoxymethyl)butyrate and presumed that it existed in the oil as part of a glyceride or glyceryl ether. From considerations of both reaction mechanism and yield, we suspected that cyanolipid II might be the precursor of Hopkins' artifact (IX, Fig. 3). Because of its greater abundance in *Koelreuteria* seed oil (10), and since its NMR and IR spectra and its TLC characteristics were identical with those of II from *Cardiospermum*, a sample of II from *Koelreuteria* oil was used to test our hypothesis. This sample was subjected to acid-catalyzed methanolysis and the product mixture was recovered under conditions described by Hopkins et al. (12).

When the mixture was analyzed on Silica Gel G developed with benzene, a small spot appeared just above the origin. The mixture was separated preparatively and the desired band near the origin was recovered as a liquid in 8% yield. IR analysis of this material indicated that it contained ether functions (1080, 1120 cm^{-1}), an ester carbonyl group (1740 cm^{-1}) and a short carbon chain (2970 cm^{-1}); this spectrum agrees with that reported (12) for methyl

4,4-dimethoxy-3-(methoxymethyl)butyrate. NMR spectra determined in $CDCl_3$ and also in benzene-d_6 revealed proton counts, chemical shifts, multiplicities and coupling constants in accord with those of the artifact reported by Hopkins et al. (12).

These data provide conclusive evidence that methyl 4,4-dimethoxy-3-(methoxymethyl) butyrate (IX) isolated from *Cardiospermum halicacabum* methyl esters is actually derived from the 6% of cyanolipid II present in the oil. The yield we obtained from the pure cyanolipid is equivalent to that isolated by Hopkins. A possible mechanism for this conversion is depicted in Figure 3. Protonation of the nitrile group of cyanolipid II would yield V. Acid-catalyzed attack of solvent (methanol) on the allylic ester grouping can reasonably be expected to yield the methyl ether VI and a fatty acid ester. Intermediate VI has an enol ester grouping which could collapse to give an aldehyde function as shown in VII. Acid-catalyzed addition of alcohols to aldehydes is well known; the reaction yields an acetal (VIII), presumably via an intermediate hemiacetal. Conversion of the cyano group of VIII to a carbomethoxy group under conditions of rigorous acid hydrolysis is expected, and should yield IX. The mechanism outlined in Figure 3 is merely an attempt to account for the various aspects of this remarkable transformation. The various chemical events we have portrayed may occur in an entirely different sequence or along a multiplicity of pathways.

One observation of Hopkins et al. (12) is not explained by our results. They reported that their sample of IX was optically active, $[\alpha]_D^{25}$ C +3.3° (in methanol). We did not measure the rotation of our product because of insufficient sample, but we would not expect an optically active transformation product from II since it has no asymmetric center.

This discrepancy led us to suspect that perhaps IX might also be produced by comparable treatment of cyanolipid I, which occurs (49%) in *Cardiospermum halicacabum* seed oil and is optically active. Accordingly, cyanolipid I was treated with refluxing HCl-methanol for 16 hr and the mixture of products was isolated. This mixture was separated by preparative TLC into an ordinary methyl ester fraction and a more polar, crystalline fraction entirely different from IX. This fraction will be the subject of a future communication. Compound IX, however, was not detected in the reaction mixture.

Additional Considerations

The presence of large amounts of cyanolipids in seed oils of the Sapindaceae is extraordinary. With the exception of *Cordia verbenacea* (7,8), a borage, this plant family is the only one known to produce seed oils containing this new class of lipids.

Significantly, the hydroxy- or dihydroxynitrile moiety in all four cyanolipids has an isoprenoid skeleton; this permits numerous possibilities for its biogenesis. Since other natural cyano compounds often seem to be derived from amino acids or their precursors (17,18), we should note that decarboxylation of L-leucine would give the requisite saturated carbon and nitrogen skeleton for these nitriles.

The monoester cyanolipids, III and IV, have structures which could be derived by partial hydrogenolysis of the diesters II and I, respectively. However, the monoester which would be produced by hydrogenolysis of the cyanohydrin ester grouping of I has not been detected in any of these oils. This monoester (with its double bond saturated) is the principle product obtained by catalytic hydrogenolysis of I (8).

A curious feature of these cyanolipid-containing seed oils is their high content of C_{20} acids and the preferential incorporation of these acids into cyanolipids rather than into the accompanying triglycerides. This preference is probably related to the observation that *Litchi* seed oil, which has insignificant amounts of C_{20} acids, also contains no cyanolipids. Additional sapindaceous seed oils that are low in C_{20} acids should be examined for confirmation of this hypothesis.

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Lipids of Human Atheroma VI. Hydrocarbons of the Atheromatous Plaque

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ABSTRACT

The higher hydrocarbons of the atheromatous plaque have been examined and the major compounds present have been found to be a series of *n*-alkanes (C_{17}-C_{31}), squalene, cholest-5-ene and a cholestadiene. Identification of these compounds was based on chromatographic correlations and mass spectrometry.

INTRODUCTION

In the past, hydrocarbons have frequently been considered to be without biological significance in human tissues; however, renewed interest in the biosynthesis of saturated hydrocarbons (1-3) has stimulated recent work on hydrocarbons from human sources (4-6). Although a large number of sites have been examined, only limited consideration has been given to the study of hydrocarbons in the pathological deposits associated with atherosclerosis. Early work in this field was carried out by Blankenhorn et al. (7), who demonstrated an increase in the quantity of carotenoids present in the aorta with an increase in the severity of the disease. Other communications on the subject relate to the identification of squalene in the aorta (8-10), to the characterization of octadecane in femoral arterial plaques (11) and to the demonstration of the ability of isolated human arterial segments to synthesize squalene (12). The present communication describes an extension of the study of human aortic plaques in which we identified squalene (8,9). The availability of gas chromatography-mass spectrometry has enabled each analytical sequence to be restricted to plaque tissue from one aorta.

MATERIALS AND METHODS

Isolation of a Hydrocarbon Fraction

In a typical analytical sequence 1-3 g of lipid, obtained as described in a previous communication (9), was chromatographed on a silicic acid column (13) using 1 g of silicic acid per 10 mg of lipid. Hydrocarbons were eluted using hexane and 1% chloroform in hexane, and all fractions eluted before the appearance of cholesteryl esters were combined and concentrated to small volume (400-500 μl).

Separation of Saturated and Unsaturated Hydrocarbons

The total hydrocarbon material obtained as described above was chromatographed preparatively on one 20 x 20 cm thin layer chromatographic (TLC) plate using an 0.25 mm layer of 5% w/w AnalaR silver nitrate: MN-Kieselgel G-HR (Macherey, Nagel and Co., Düren) and hexane-benzene (97:3 v/v) as mobile phase.

Osmium Tetroxide Oxidation of Olefins (14)

Olefins (10-100 μg) were dissolved in

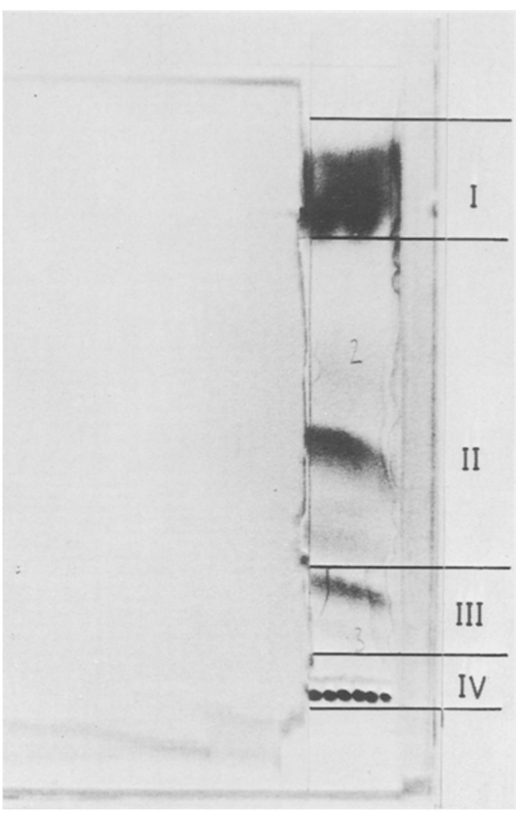
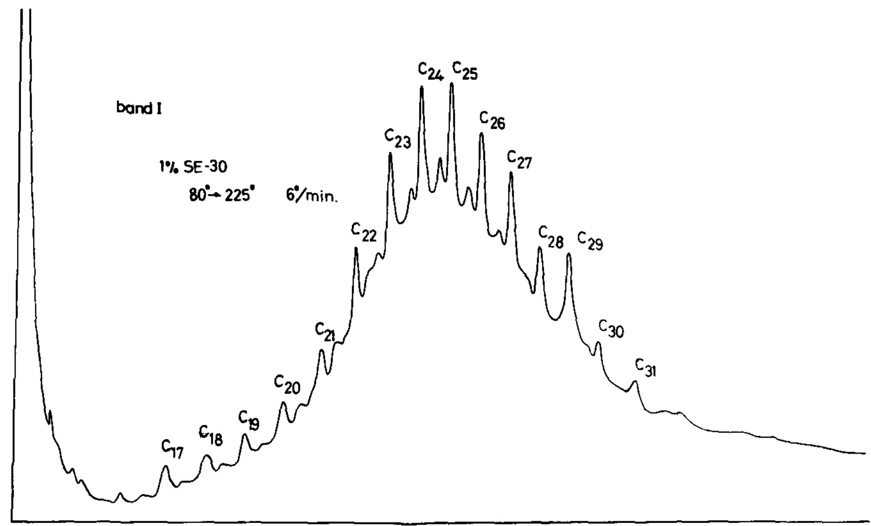


FIG. 1. The right hand margin of a preparative thin layer chromatogram of plaque hydrocarbons on silica gel impregnated with silver nitrate (5% w/w). Bands I, II, III and IV correspond to areas removed from the chromatogram. Mobile phase, hexane-benzene (97:3 v/v); Spray, aqueous sulphuric acid (1:1 v/v).



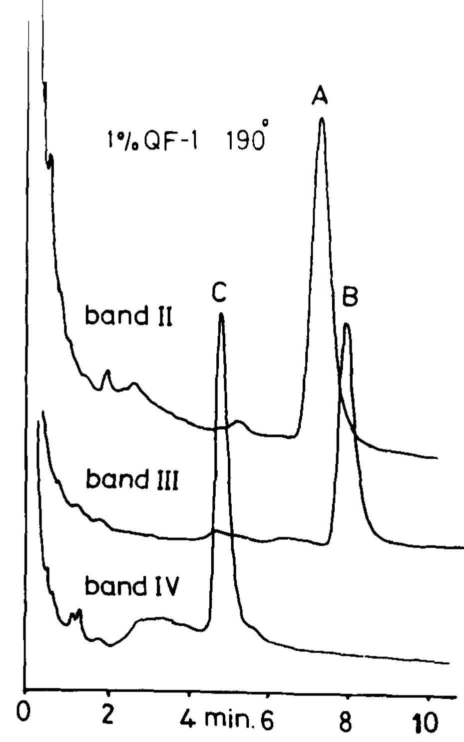


FIG. 2. Gas liquid chromatograms of the hydrocarbons in extracts of the four bands isolated by preparative silver nitrate thin layer chromatography. (a) Band I on 5 ft 1% SE-30 with temperature programming from 80 C to 225 C at 6 C/min. (b) Bands II, III and IV on 5 ft 1% QF-1 at 190 C.

sodium-dried ether (100 μl) and dry pyridine (20 μl). This solution was treated with 50 μl of a solution of osmium tetroxide (5 mg) in dry ether (1 ml). The mixture was set aside in a stoppered test-tube for three days at room temperature. The solution was then reduced to dryness in a stream of nitrogen and the residue dissolved in chloroform (1 ml) and shaken with 1 ml of an aqueous solution of potassium hydroxide (5 mg) and mannitol (5 mg) for 2 hr. The chloroform layer was then removed, washed with water and dried over anhydrous sodium sulphate.

Epoxidation and Isomerization of Olefins

Epoxides were prepared in sodium carbonate-buffered methylene chloride using pertrifluoroacetic acid (15). The epoxides thus formed were dissolved in benzene and isomerized with two drops of boron trifluoride etherate as described by Henbest and Wrigley (16). The products were extracted with ether, washed successively with 10% sodium bicarbonate solution and water, and dried over anhydrous sodium sulphate. Purification was accomplished by preparative TLC using benzene-ethyl acetate (20:1 v/v) as mobile phase.

Formation of Trimethylsilyl Ethers

Trimethylsilyl ethers were formed in pyridine solution using a mixture of hexamethyldisilazane and trimethylchlorosilane (10:1 v/v) at room temperature for 1 hr. Reagents were removed in a stream of nitrogen, the residue was extracted with ethyl acetate and insoluble material was removed by filtration or centrifugation.

Gas Chromatography-Mass Spectrometry (GC-MS)

Mass spectra were obtained using an LKB model 9000 gas chromatograph-mass spectro-

TABLE I

Chromatographic Data for Plaque Hydrocarbons

	Thin layer chromatography[a]		Gas liquid chromatography[b]		
Compound	R_f[a] $SiO_2/AgNO_3$	R_f[a] SiO_2	I SE-30	I QF-1	I OV-17
A	0.48	0.82	2755	2950	2935
B	0.25	0.75	2790	2995	2984
C	0.00	0.47	2800	2825	2935

[a]Adsorbents: $SiO_2/AgNO_3$, 5% w/w $AgNO_3$/MN-Kieselgel G-HR; SiO_2, MN-Kieselgel G-HR. Mobile phase: hexane-benzene (97:3 v/v) in both cases.

[b]Gas liquid chromatography was carried out using a Pye 104 chromatograph and the columns were as follows: 5 ft 1% SE-30 on 100-120 mesh Gas Chrom Q (Applied Science Laboratories) at 200 C; 5 ft 1% QF-1 on 100-120 mesh Gas Chrom Q (Applied Science Laboratories) at 190 C; and 5 ft 1% OV-17 on 80-100 mesh Chromosorb G-HP (Supelco Inc.) at 210 C.

meter (LKB-Produkter AB, Stockholm). The ionizing voltage was 70 eV, accelerating voltage 3.1 kV and the helium flow rate 30 ml/min.

RESULTS

The saturated and unsaturated hydrocarbons were separated using silica gel layers impregnated with 5% w/w of silver nitrate and hexane-benzene (97:3 v/v) as mobile phase. After development, four areas, corresponding to four bands indicated by analytical silver nitrate TLC, were scraped from the chromatogram as is shown in Figure 1: Band I, R_f 0.68-0.94; Band II, R_f 0.29-0.68; Band III, R_f 0.11-0.29; Band IV, R_f 0.00-0.11.

The hydrocarbons isolated in this manner were eluted from the adsorbent using hexane-chloroform (10:1 v/v). The separated hydrocarbons were examined by TLC and GLC under a variety of conditions (Fig. 2 and Table I).

Band I

Chromatographic evidence suggested that Band I contained a complex mixture of straight chain and branched chain alkanes. The main components of this fraction appear from their retention times to be a homologous series of *n*-alkanes between C_{17} and C_{31} with no particular preference for odd or even carbon numbers. The base line hump in this chromatogram is frequently encountered in the examination of saturated hydrocarbons of biological origin (17,18) and is thought to be due to a complex mixture of unresolved isomeric hydrocarbons, some of which form a homologous series, as can be seen from an examination of the small peaks which appear between successive *n*-alkanes of the larger series (Fig. 2a). Examination of eluates obtained from the silicic acid column before loading with lipid have shown that although these appear to contain alkanes their quantities are insignificant when compared with those obtained after loading of the column. Alkanes were found to be present in plaques in quantities of the order of 700-800 μg/g of total lipid extract.

Band IV

TLC evidence suggested that squalene (compound C in Table I) and a carotene were present in Band IV. The identification of squalene in plaques was the subject of previous communications (8,9) and will not be discussed further. The second compound in this fraction had TLC mobility, color reaction and UV spectrum identical to those of β-carotene, but no further work has been carried out owing to its unsatisfactory GLC behavior.

Bands II and III

Bands II and III each appeared to contain a single major component (designated A and B, respectively, in Fig. 2b). Compounds A and B were subjected to GC-MS and line diagrams of the mass spectra obtained are shown in Figure 3. The gas chromatographic data for compound A closely resembled those of 5α-cholestane: however, TLC on layers impregnated with silver nitrate had shown that it was unsaturated, while its mass spectrum showed a molecular weight and fragmentation pattern consistent with those of a cholestene. In a similar manner compound B was shown to be a cholestadiene.

Location of the Double Bond in Compound A

The following approaches were explored in an attempt to locate the double bond in the extracted cholestene: (a) TLC on silica gel impregnated with silver nitrate; (b) GLC on

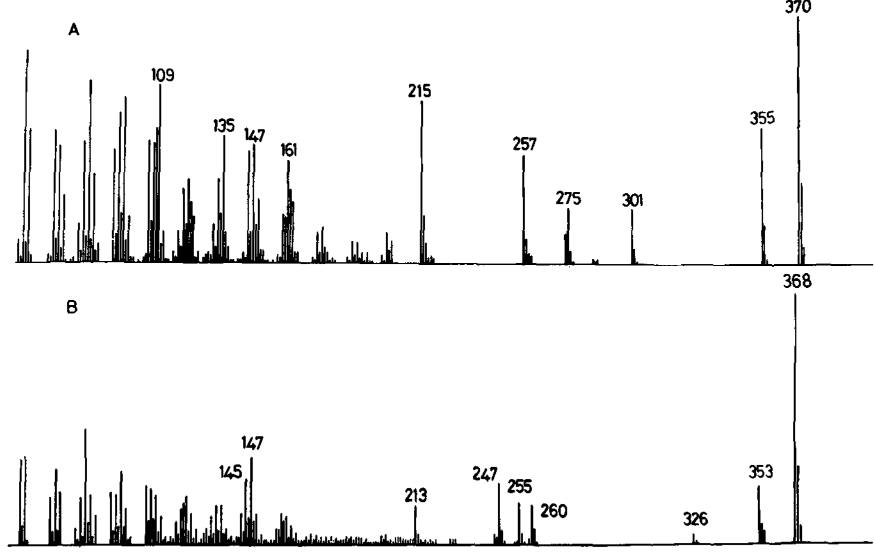


FIG. 3. Mass spectra of hydrocarbons A and B from plaque extracts.

selective and non-selective liquid phases; (c) Mass spectrometry; and (d) Specific reactions of the double bond.

(a) TLC on silica gel using hexane-benzene (97:3 v/v) as mobile phase was largely unsuccessful in separating a selection of reference cholestenes and consequently was of little use in identifying the aortic cholestene. On the other hand TLC on silica gel impregnated with silver nitrate has given a measure of characterization (Fig. 4). The extracted olefin corresponded in mobility to authentic cholest-4-ene and cholest-5-ene, but was markedly different from the $\Delta^{8(9)}$-, Δ^2- and Δ^3-isomers. (The Δ^3-isomer is not shown in Figure 4, but has the same R_f value as the Δ^2-isomer).

(b) Since no significant separations of the five available reference cholestenes could be demonstrated by GLC on the non-selective liquid phase SE-30, several selective phases, viz, QF-1, OV-17 and OV-22 were tested (Table II). Of these, OV-17 and OV-22 showed small separations useful in characterization. Especially important was the separation between the Δ^4- and Δ^5-isomers on these phases, since this pair is not adequately distinguished by silver nitrate TLC. The data recorded for the extracted cholestene were identical to those of cholest-5-ene under all of these conditions.

(c) Examination of the mass spectra of the authentic cholestenes (Fig. 5) show that most of them have distinctive fragmentation patterns and that the spectrum of the extracted material is closely similar to that of cholest-5-ene, the most characteristic ions being at m/e 275 and m/e 301. The only other compound examined having significant ions at these positions is cholest-2-ene, which has already been ruled out on the basis of evidence from silver nitrate TLC. Cholest-4-ene can be eliminated on mass spectral considerations (i.e., no ion at m/e 275 and an extremely small m/e 301).

The mass spectra of two cholestenes with unsaturation in the side chain have been examined (19) and have been shown to have fragmentation patterns distinct from each other and from all of the compounds examined in the present study.

(d) In order to substantiate the tentative

TABLE II

Retention Index Data for Authentic Cholestenes[a]

	Retention indices	
Δ	QF-1	OV-17
2	2960	2930
3	2965	2940
4	2940	2915
5	2950	2935[b]
8(9)	2950	2950

[a]Gas liquid chromatographic conditions: 5 ft 1% QF-1 on 100-120 mesh Gas Chrom Q (Applied Science Laboratories) at 185 C; 5 ft 1% OV-17 on 80-100 mesh Chromosorb G-HP (Supelco Inc.) at 210 C.

[b]The data recorded for the extracted cholestene were identical to those of cholest-5-ene. The data recorded on 1% OV-22 at 200 C were closely similar to those shown above for OV-17.

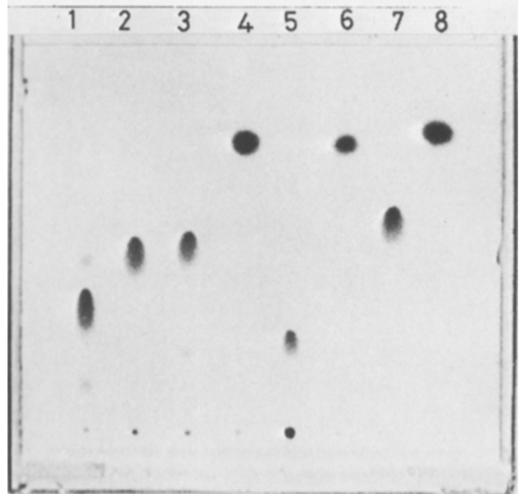


FIG. 4. Silver nitrate thin layer chromatography of reference hydrocarbons. Adsorbent, 5% w/w AnalaR silver nitrate/MN-Kieselgel G-HR. Mobile phase, hexane-benzene (97:3 v/v). Spray, aqueous sulphuric acid (1:1 v/v). 1, 5α-cholest-2-ene; 2, cholest-4-ene; 3, cholest-5-ene; 4, 5α-cholest-8(9)-ene; 5, cholesta-3,5-diene; 6, 5α-ergost-7-ene; 7, 5α-ergosta-7,22-diene; 8, 5α-ergost-8(14)-ene.

identification of cholest-5-ene, the extracted cholestene and authentic cholest-5-ene were both converted to diols using osmium tetroxide. GLC of the trimethylsilyl ethers of these diols suggested that both hydrocarbons had formed the same compound (5α-cholestane-5,6α-diol), which on examination by GC-MS proved to have formed a mono- rather than a bis-trimethylsilyl ether. Line diagrams of the mass spectra from both sources are shown in Figure 6. Incomplete etherification was not unexpected, because earlier work with 5α-cholestane-3β,5,6β-triol (20) demonstrated that the 5α-hydroxyl group is not trimethylsilylated under normal conditions. [Complete ether formation can however be accomplished under suitable conditions (21)]. This, together with the evidence cited above, indicates that the extracted olefin is cholest-5-ene.

A further reaction was carried out to confirm the identification of cholest-5-ene. Examination of the compounds obtained by isomerization of the epoxide of compound A with boron trifluoride etherate by GLC (1% SE-30 at 225 C) gave two major peaks of retention index 2970 and 3010. These were

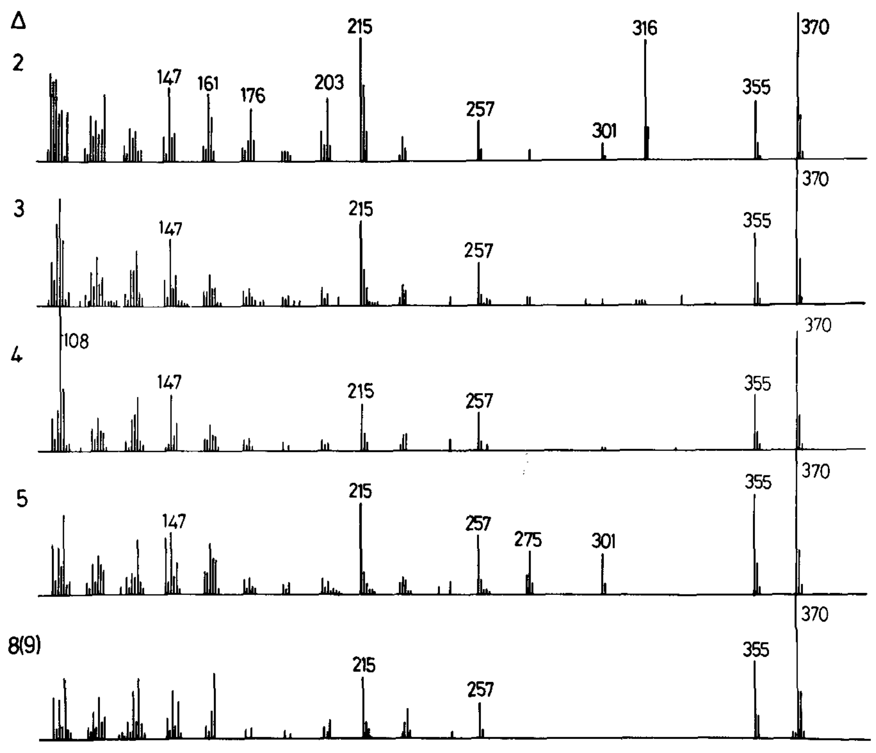


FIG. 5. High mass regions of the mass spectra of authentic cholestenes.

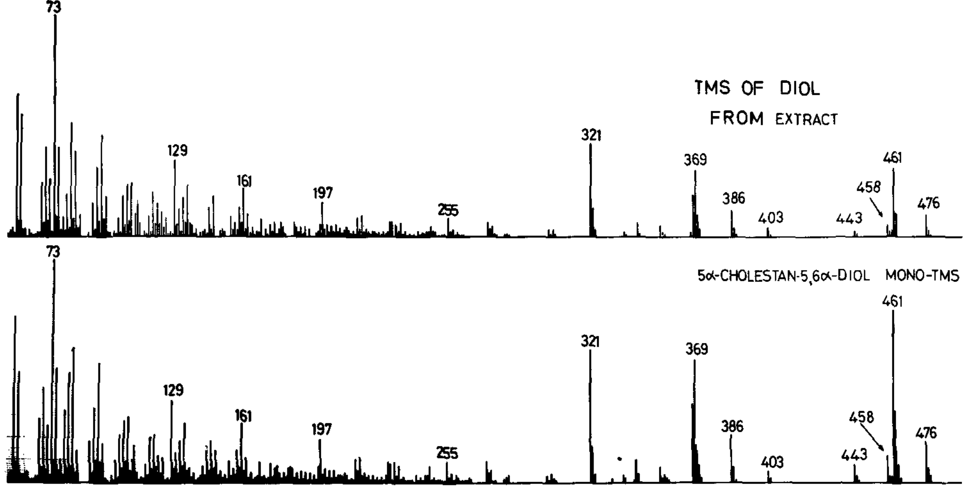


FIG. 6. Mass spectra of trimethylsilyl ethers of the diols produced from the extracted cholestene and authentic cholest-5-ene by the action of osmium tetroxide.

found to correspond in retention time respectively to 5β- and 5α-cholestan-6-one, the expected products from the epoxide of cholest-5-ene (16). The mass spectra of the two products corresponded satisfactorily with those of authentic 5α- and 5β-cholestan-6-one and also with published spectra (22).

The quantity of cholest-5-ene in plaque extracts was of the order of 30-50 μg/g of lipid.

Compound B

The chromatographic data and mass spectra recorded for compound B closely matched those of cholesta-3,5-diene. The UV spectrum of the extracted material [λ_{max} (ethanol) 228, 234 and 242-243 nm, ϵ 19,000, 21,000 and 12,000, respectively] was also in close agreement with that of cholesta-3,5-diene as recorded by ourselves and others (23).

DISCUSSION

Saturated hydrocarbons in atheroma lesions are difficult to account for since very little is known about their origin or function in human tissue. In an investigation of the possible origin of *n*-alkanes in the skin surface, Nicolaides (1) fed rats ^{14}C-labeled octadecane and demonstrated that some of this material was excreted unchanged to the skin surface via the sebaceous glands, but he also found activity in other lipid fractions, indicating that the octadecane was involved in other metabolic processes.

Evidence has been obtained that alkanes found in higher plants may arise by two different routes: via elongation of common fatty acids, or via condensation of fatty acids followed by reduction (24). It is also known that saturated hydrocarbons can be formed during the autoxidation of unsaturated lipids (25); however, these are generally of short chain length (C_1-C_5).

The presence of cholest-5-ene in the plaque is unexpected in terms of sterol metabolism. This steroidal hydrocarbon has not previously been identified in human tissue, but has been demonstrated in the plant kingdom in the leaves of *Euphorbia lateriflora* (26). The participation of cholest-5-ene in the biosynthesis of cholesterol would necessitate the postulation of a biogenetic route very different from that currently accepted (27). While non-oxidative cyclization pathways are known to exist in the case of lanosterol and the triterpene tetrahymanol (28,29), it would be difficult to account for the removal of the 4,4-dimethyl grouping in the absence of an oxygen function at C-3. We have found no evidence for the presence of lanostadiene in plaques.

An alternative and more likely explanation is that cholest-5-ene is formed reductively from oxygenated steroids, possibly by the action of infecting micro-organisms. One possible precursor might be cholesteryl sulphate which has been shown to occur in several human tissues and fluids including blood (30). Cholesteryl alkyl ethers (31), although not yet demonstrated in human tissue, are another type of lipid which might under certain circumstances yield cholest-5-ene.

In an investigation of the hydrocarbons of human hypercholesterolemic serum (C.J.W. Brooks, A. Morrison and G. Steel, unpublished results, 1968) we have again been able to iden-

tify cholest-5-ene. This result suggests the possibility that cholest-5-ene found in the atheroma plaques might have been deposited in the intima by an infiltration process.

The cholestadiene characterized in the plaque in the present work appears to be a genuine component: we have no evidence of its formation as an artefact. It should be noted, however, that cholesta-3,5-diene isolated by other workers in a study of brain lipids (32) was considered to be an artefact derived from cholesterol during treatment of a total lipid extract with boron trifluoride in methanol.

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Application of Negative Ion Mass Spectrometry to the Identification of Long Chain Aldehydes and Alcohols

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ABSTRACT

The negative ion mass spectra of a number of long chain aldehydes and alcohols are reported. The small number of fragment ions in the mass spectra of these compounds was a characteristic feature. The prominent P-1 negative ion in the mass spectra enabled identification of the molecular ions of long chain alcohols and aldehydes whose positive ion mass spectra have negligible molecular ion intensities. The potential of the method for analysis of complex mixtures of long chain aldehydes and alcohols has been demonstrated with the identification of the components of a 14 component mixture.

INTRODUCTION

The use of mass spectrometry for the determination of molecular weights, atomic compositions and molecular structure has become an indispensable tool for the chemist. Two major problems can be encountered in the use of mass spectrometry. These are sample purity and the accurate identification of the molecular ion. Positive ion mass spectrometry can be extremely sensitive but difficulty may be experienced in interpreting a mass spectrum due to ions from contaminating substances. The mass spectrometer does not differentiate from which molecules ions arise. Thermal or catalytic degradation of the sample can also contaminate an otherwise pure compound. The other problem of unequivocal identification of the molecular ion can sometimes be a difficult one in certain classes of compounds such as alcohols and aldehydes. Derivatization of these compounds (if possible) sometimes leads to a more intense molecular ion. The molecular ion needs to be positively identified before an accurate structural analysis can be made on the compound under study.

The use of negative ion mass spectrometry is a promising approach to both of the above problems encountered in mass spectrometric analysis. The mechanisms of negative ion formation as postulated by Melton (1) are as follows: (a) $AB + e \rightarrow AB^-$, resonance capture; (b) $AB + e \rightarrow A + B^-$, dissociative resonance capture; (c) $AB + e \rightarrow A^+ + B^- + e$, ion-pair formation. Process (a) occurs with electron energies of < 2 ev. The origin of interacting electrons could be either from the electron bombardment source common to most mass spectrometers or from a fragmentation process of another molecule (secondary electrons). Process (b) occurs for electron energies of 2-10 ev, whereas process (c) is in the region of > 10 ev. All three processes can occur in the mass spectrometer source, but because of their different pressure dependencies the relative abundance of ions from each process will also vary with sample pressure. Von Ardenne and associates (2,3) have worked with experimental conditions such that process (a) resulted in the major source of ions. Alpin et al. (4) obtained negative ion spectra under more conventional conditions of 70-90 ev and source pressures of 10^{-6} to 10^{-5} torr. Here processes (b) and (c) were the main source of ion formation.

Many of those who have worked with negative ion spectra (5) formed the opinion that this type of mass spectrometry did not offer a very attractive method of obtaining molecular weights or for determining molecular structure. The sensitivity of negative ion formation and detection can be as much as 10^2 to 10^4 less than that of positive ions (6). Modern instrumentation has improved these detection levels considerably. The use of negative ion mass spectrometry is enhanced by the relative simplicity of negative ion mass spectra, and this suggests that the technique might be a useful selective detector enabling the identification of certain classes of compounds (e.g., alcohols, aldehydes) in a system which has not been rigorously purified. Under conditions of approximately 60 ev and 10^{-6} and 10^{-5} torr pressures the most intense ions for alcohols (7) and aldehydes occur in the mass range of 12 to 70 and in the molecular ion region (e.g., P-1, P-3). The absence of extensive fragmentation ions (cf. field ionization mass spectrometry) does not aid structural determination but does permit the molecular ion to be determined and permits identification of compounds in a mixture. Several examples have been cited where the use of negative ion mass spectrometry has enabled the molecular ion of a molecule to be determined because in the positive ion mass spectrum it was very weak or nonexistent (8,9).

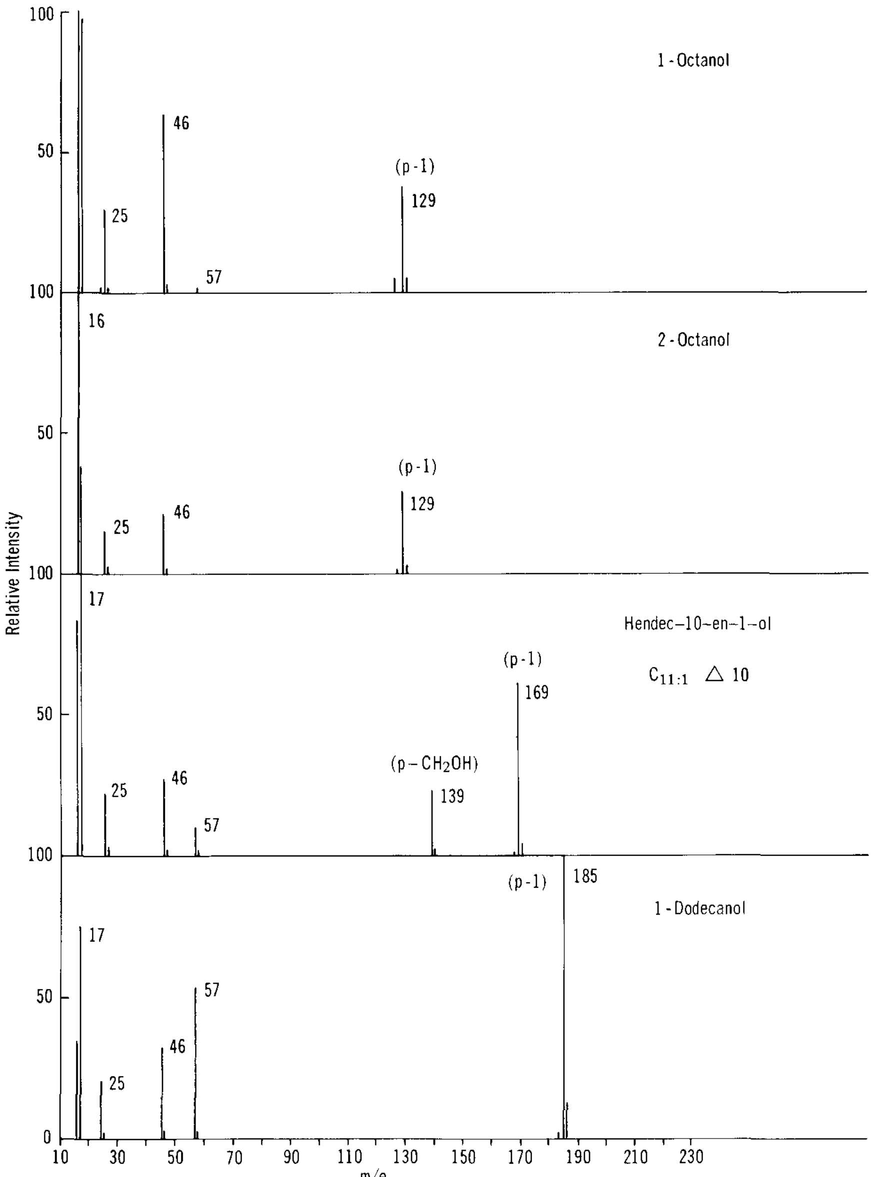

FIG. 1. The negative ion mass spectra of some long chain alcohols. Experimental conditions described in the text.

Alcohols and aldehydes are often very difficult to characterize from positive ion mass spectra because of weak molecular ion intensities (9,10). The positive molecular ions of long chain alcohols and saturated aldehydes (11) cannot be detected. A series of long chain alcohols and aldehydes, some glycols and a number of miscellaneous alcohols and aldehydes were subjected to a negative ion mass spectrometric analysis to determine the feasibility of using this technique for their identification.

EXPERIMENTAL PROCEDURES

The mass spectrometer used in this study was a Perkin Elmer-Hitachi RMU-6E which can be converted to negative ion detection simply by manipulating two switches. The first reverses the polarity of the magnet and the second reverses the accelerating voltage to a negative potential. The ionizing electron beam was operated at 55 ev, with a target current of 200-350 μ. Repeller and lens voltages were not

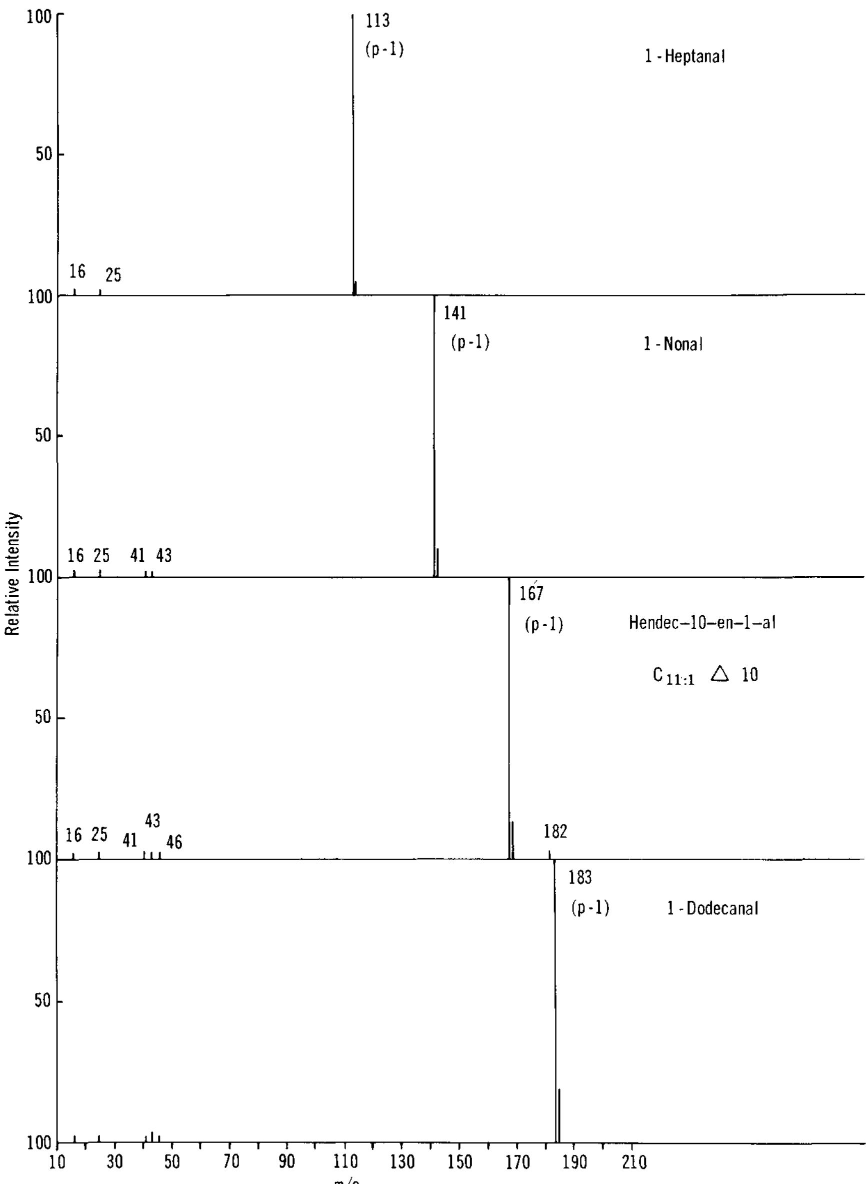

FIG. 2. The negative ion mass spectra of some long chain aldehydes. Experimental conditions described in the text.

the same as those used in the positive ion mode. These ion beam focusing voltages were very critical to obtain the maximum response possible from the electron multiplier detector. An accelerating voltage of 2.4 kv was used as this enabled a convenient mass range to be scanned by changing the magnet current.

For the determination of the nominal mass of ions in the negative ion mass spectrum, reference compounds such as perfluorokerosene (12), carbon tetrachloride and nitrobenzene were used. These three compounds typically gave intense negative ion spectra for very small sample sizes. For the same chart speed and scanning rate, positive ion and negative ion mass spectra were superimposable and this approach could also be utilized to determine the nominal mass of ions in the negative ion mass spectrum.

The samples used were analyzed by gas chromatography and found to be better than 98% pure. Liquids were placed in the mass

TABLE I

Relative Intensities[a] of Principal Peaks in the Negative Ion Mass Spectra of Some Nonaliphatic Alcohols and Aldehydes

m m/e	Cyclohexanol $P^- = 100$	Benzyl alcohol $P^- = 108$	2-phenyl-propanal $P^- = 134$	Connam-aldehyde $P^- = 132$	Benzaldehyde $P^- = 106$	3(4-isopropylphenyl)-2-methyl-propanal $P^- = 190$
16	100	100	<5	<5	<5	<5
17	51	12	---	---	---	---
25	53	50	---	---	<5	---
41	17.5	---	---	---	---	---
43	---	19	---	---	---	---
45	---	---	100	<5	---	---
46	8	---	---	---	<5	<5
49	---	9	---	---	<5	---
57	---	---	---	---	---	<5
59	---	---	---	<5	---	---
77	---	---	<5	---	<5	---
96	5	---	---	---	---	---
99	41	---	---	---	---	---
105	---	---	26	---	100	---
107	---	52	---	---	8	---
119	---	---	8.5	---	---	---
132	---	---	---	100	---	---
133	---	---	41	11	---	<5
189	---	---	---	---	---	100
190	---	---	---	---	---	15

[a]Per cent of base peak.

spectrometer via a heated glass inlet system whereas solids were manipulated on a direct insertion solids probe. Sufficient sample was used to give a source pressure of 1.5×10^{-5} to 3.5×10^{-5} torr. This high sample pressure was necessary because the electron multiplier used in the study only had a gain of approximately 2500 (measured at 2 kv accelerating voltage, with positive ions and relative to the Faraday Cup mode). With a more sensitive electron multiplier much lower sample pressures would be required.

RESULTS AND DISCUSSION

Straight Chain Alcohols

The negative ion mass spectra of the lower alcohols have been reported (7). They are typified by a strong P-1 ion. Deuterium studies have indicated that the loss of a proton occurs from the hydroxyl group (7). Other predominant ions in the spectra of the lower alcohols were P-3, P-5 and ions at 16, 17, 24, 25 and 41.

The negative ion spectra of the series *n*-heptanol to *n*-dodecanol were obtained. For comparison, hendec-10-en-1-ol, 2-octanol, and 2-ethyl hexanol were also analyzed by negative ion mass spectrometry. Figure 1 illustrates some of the typical spectra obtained. All the aliphatic alcohols were typified by ions at 16, 17, 25, 46, 57, P-5 (very weak), P-3 and P-1. The P-1 ion was > 50% of the base peak (16 or 17) except in the cases of *n*-decanol, hendecan-1-ol and *n*-dodecanol where the P-1 ion was itself the base peak. The P-3 ion arises from the loss of two hydrogen atoms on the carbon atom α to the hydroxyl group (6). It was of greatest intensity in the spectra of primary alcohols. Comparison of the spectra of primary and secondary octanols indicated that negative ion mass spectrometry does not aid in the differentiation of these isomers. The m/e 57 peak was absent from the spectrum of the secondary alcohol but it is of such low intensity in the spectrum of the primary alcohol that it can hardly be used as a means of distinguishing between the two isomers. A study (6) of the negative ion spectra of primary, secondary and tertiary butanols has indicated little change in the mass spectra other than ion intensity changes. This same study (6) and also the spectra of primary and secondary octanols reported here indicated that branching, although diminishing the intensity of the P-1 ion, did not prevent positive identification of this ion. This is certainly not true for the positive ion spectra of highly branched alcohol compounds (9,11). The exact composition of ions 46 and 57 could not be determined by mass measuring but their quite strong intensities, especially in the longer chain length alcohols, suggests they contain the oxygen atom, i.e., C_2H_6O and C_3H_5O, respectively. As a general observation, it was found that the longer chain length alcohols gave a more intense

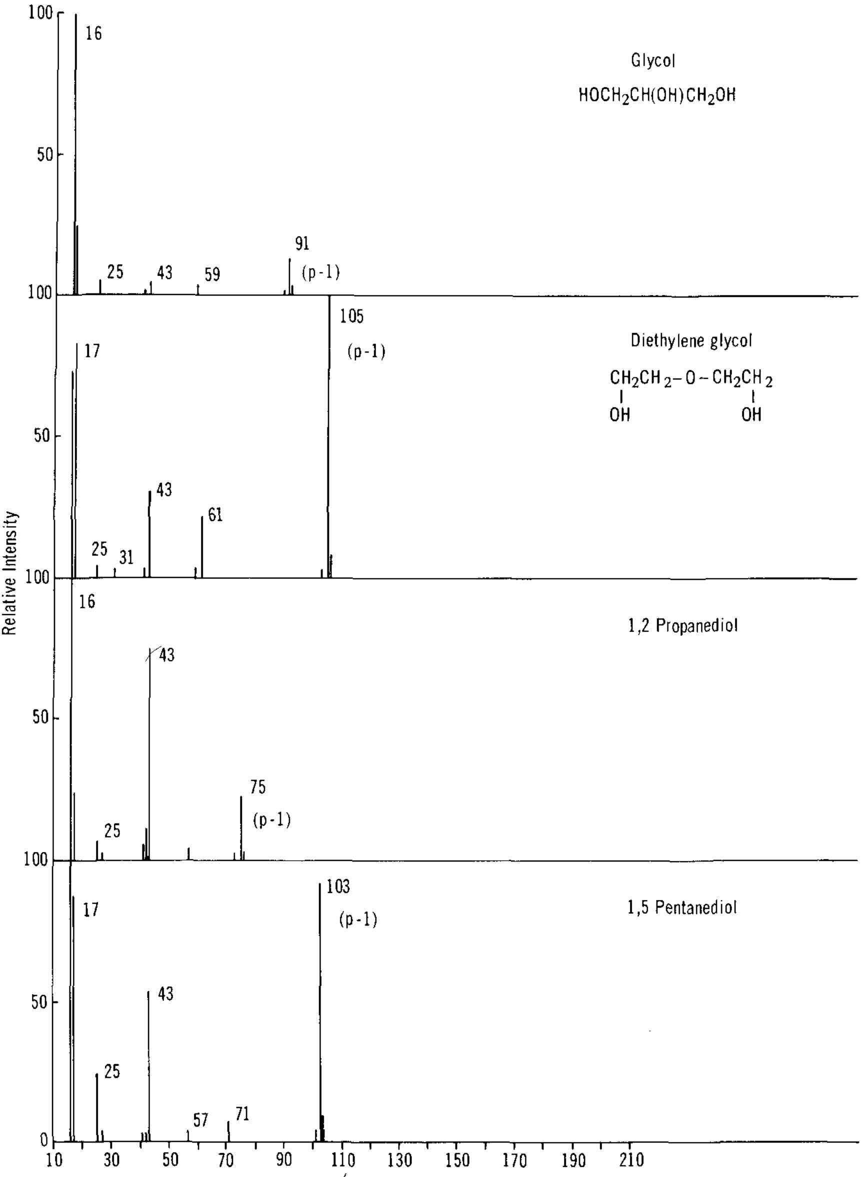

FIG. 3. The negative ion mass spectra of some glycols.

negative ion spectra for the same sample pressure than did the lower alcohols.

Straight Chain Aldehydes

The negative ion mass spectra for the series *n*-heptanal to *n*-octadecanal were obtained. Figure 2 illustrates some typical spectra. The spectra are much more simple than those of the corresponding alcohols, being dominated by the P-1 ion. The spectra indicate the ease with which the nominal molecular weight of *n*-aldehydes can be obtained. Because of the high sample pressure, ion-molecule reactions sometimes resulted in ions with a mass greater than that of the P-1 ion. The ions were of low intensity (< 1%) and would appear at [(P-1) +15] and at [(P-1) +16]. As with the alcohol spectra, the intensity of the negative ion spectra was greatest for the higher molecular weight aldehydes. Also the spectra of the aldehydes were more intense than the corresponding alcohol for the same sample pressure. The

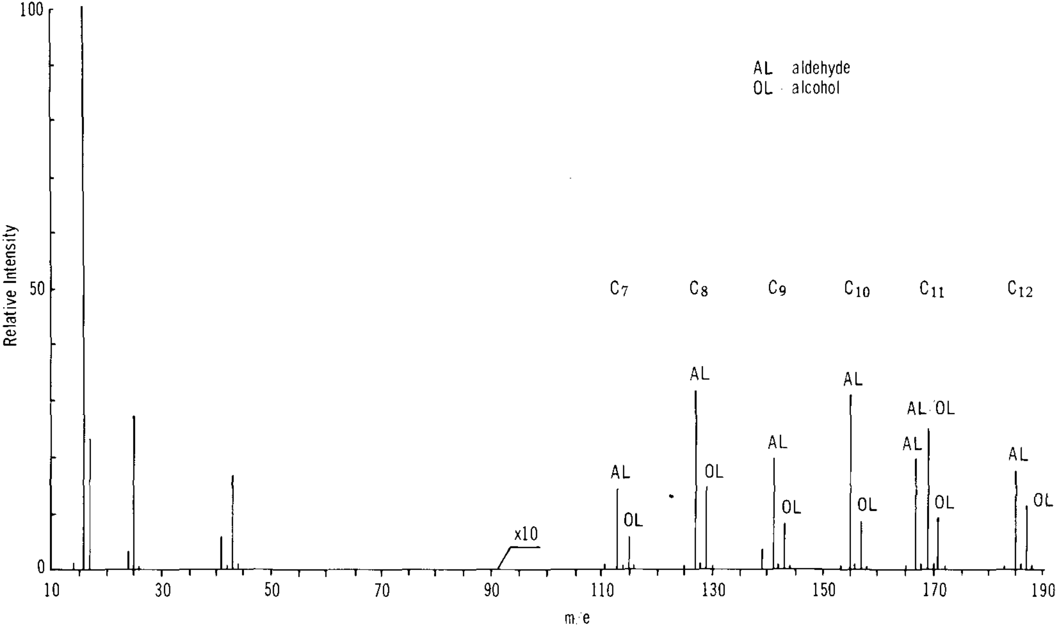

FIG. 4. The negative ion mass spectrum of a 14 component mixture of long chain aldehydes and alcohols. Note that m/e 167 is the P-1 ion of $C_{11:1}$ aldehyde, m/e 169 is the P-1 ion for both $C_{11:0}$ aldehyde and $C_{11:1}$ alcohol and m/e 171 is the P-1 ion for $C_{11:0}$ alcohol.

simplicity of the aldehyde negative ion spectra offers an excellent method for identifying components of mixtures of these aldehydes.

Glycols

This class of compounds typically has molecular ions of negligible intensity in positive ion mass spectrometry (9). Figure 3 illustrates the spectra of some of the glycols studied. Although in several cases the P-1 ion was of low intensity (e.g., glycol) it could easily be identified and enabled determination of the molecular weight of the glycol involved. Other ions in the spectra include 16 (O^-), 17 (OH^-), 25 ($C \equiv CH^-$), 43 ($C_2H_3O^-$), 57 ($C_3H_5O^-$), 71 ($C_4H_7O^-$).

Miscellaneous Alcohols and Aldehydes

To test the general applicability of negative ion mass spectrometry, a number of nonaliphatic alcohols and aldehydes were studied. Table I lists some of the results. 2-Phenyl-propanal, cinnamaldehyde, 3(4-isopropylphenyl)-2-methyl-propanal, and benzaldehyde gave the uncomplicated negative ion mass spectra which are typical of the long chain aldehydes. Except in the case of 2-phenyl-propanal, the P-1 ion was the base peak for the aldehydes with all other ions having an intensity of $< 5\%$. Cylcohexanol and benzyl alcohol had spectra similar to *n*-heptanol in that the P-1 ion was approximately 50% of the base ion m/e 16.

This study of the applicability of negative ion mass spectrometric analysis to the identification of alcohols and aldehydes has shown that the technique has promise. Although little molecular structural information can be deduced from negative ion mass spectra because of the limited number of fragment ions, the technique does enable a positive determination of the nominal molecular weight of the alcohol or aldehyde. Negative ion mass spectrometry, then, complements positive ion mass spectrometry.

Figure 4 illustrates the negative ion mass spectrum of an equi-volume mixture of alcohols and aldehydes containing heptanol through to dodecanol, heptanal through to dodecanal, hendec-10-en-1-ol and hendec-10-en-1-al. Because of the simplicity of the negative ion mass spectra of these compounds, it is possible to identify each compound (except hendec-1-al and hendec-10-en-1-ol which have the same nominal mass) in the 14 component mixture. The ion at 139 indicates the presence of hendec-10-en-1-ol (Fig. 1). The positive ion mass spectra of long chain aldehydes (10) contain too many interfering ions from fragmentation reactions to permit a similar analysis of a 14 component mixture.

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Hydroxystearic Acid Deposition and Metabolism in Rats Fed Hydrogenated Castor Oil[1]

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ABSTRACT

Groups of rats were fed diets containing corn oil, 1% hydrogenated castor oil (principal constituent: 12-hydroxystearic acid) or 10% hydrogenated castor oil. Rats were sacrificed after 4, 8, 12 and 16 weeks for determination of hydroxy fatty acids in excised abdominal adipose tissue or in lipid extracted from lyophilized carcass. Maximum content of hydroxystearic acid was 4.4% in adipose tissue of rats four weeks on the 10% hydrogenated castor oil diet. When rats on hydrogenated castor oil diets were switched to the corn oil diet, hydroxystearic acid was depleted from their tissues. 10-Hydroxypalmitic and 8-hydroxymyristic acids were characterized as metabolites of 12-hydroxystearic acid. No adverse effects of diets were observed except reduced growth in rats given 10% hydrogenated castor oil diet.

INTRODUCTION

Hydrogenated castor oil, a hard waxlike solid, mp 87 C, has the potential of imparting good functional properties to food products. It and derivatives of hydroxystearic acid, its principal constituent, are being tested as emulsifiers, as thickeners and as antistaling agents for baked goods. Determining the effects of its ingestion is thus desirable.

Considering castor oil and hydrogenated castor oil as food ingredients may seem strange. Yet castor oil has been used in China as a frying oil (1) and is occasionally used in India to adulterate peanut oil. Small doses of castor oil, taken as part of the diet, are usually noncathartic (2,3); for effective purgation it is usually taken on an empty stomach. The purgative action apparently depends on the accumulation of free ricinoleic acid in the small intestine (3,4). Hydrogenated castor oil, with ricinoleic acid converted to 12-hydroxystearic acid, is noncathartic (3). Other foods that contain hydroxy fatty acids are apples (5,6), pears (5) gooseberries and blackcurrants (6), milk fat of cows, goats, sheep and humans (7), and royal jelly (8).

Most investigations of the effects of ingested hydroxy fatty acid have utilized ricinoleic acid, triricinolein, or castor oil, but two investigations involve 12-hydroxystearic acid. Perkins et al. (9) found 6.1% hydroxy fatty acids in the carcass fatty acids of rats fed 12-hydroxystearic acid as 8.4% of their diet and 70% of their fat intake for 59 days. Their results also indicated that nonhydroxy monoenoic acids might have been directly formed from 12-hydroxystearic acid. However, Elovson (10) found no evidence for a specific dehydration of hydroxystearic acid to an unsaturated analog. Labeled, serum-bound hydroxystearic acids intravenously administered were rapidly broken down and nonhydroxy acids were then apparently synthesized from acetate (10). Perkins had recently (11) reiterated that hydroxystearic and ricinoleic acids are converted to monoenoic acids but has yet to offer substantial evidence of this.

Ingested ricinoleic acid is also rapidly metabolized. Stewart and Sinclair (2) fed adult rats a diet containing 48% castor oil (about 85% ricinoleic acid) for 25 to 40 days and found that less than 2% of the absorbed ricinoleic acid was deposited in fat. Ricinoleic acid constituted 7% of the fatty acids of adipose tissue but seemed to be absent from phospholipids of the small intestine, liver and skeletal muscle. Watson and co-workers (12,13) found that the maximum degree of ricinoleic acid incorporation in adipose tissue was about 10%, and they state that none was found in liver, brain or phospholipid. Withdrawl of castor oil from the diet caused rapid loss of ricinoleic acid from tissues (12). Further work (4,13) showed that humans can absorb castor oil and ricinoleic acid. After feeding rats ricinoleic acid or castor oil, Uchiyama and co-workers (14,15) isolated ricinoleic acid and also hydroxyhexadecenoic, hydroxytetradecenoic and hydroxydodecenoic acids from fat tissue. The latter acids were considered to be identical with metabolites of ricinoleic acid digested by *Escherichia coli*, one of which was characterized as 8-D-(+)-hydroxy-*cis*-5-tetradecenoic acid (16). 8-Hydroxytetra-

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[1]Presented at the AOCS Meeting, New York, October, 1968.

[2]Western Utiliz. Res. & Dev. Div., ARS, USDA.

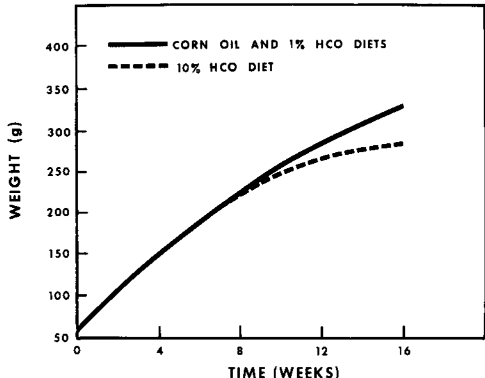

FIG. 1. Growth of rats on corn oil and hydrogenated castor oil diets.

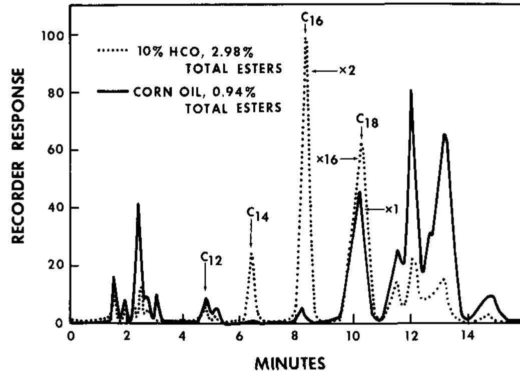

FIG. 2. GLC analysis of lipid hydroxy acid esters from rats fed corn oil and 10% hydrogenated castor oil for 16 weeks. Nearly equivalent amounts of sample were injected. Unmarked peaks represent unidentified esters.

decenoic and 6-hydroxydodecenoic acids were final products of ricinoleic acid metabolism by *E. coli*, but some strains of *Candida* degraded ricinoleic acid completely (17,18). Castor oil-fed rats grew at a rate similar to that of rats fed corn oil and showed no abnormalities at autopsy (3).

Another oxygenated fatty acid that can be deposited in adipose tissue is vernolic acid. Fat pads containing 6.1% epoxyoleic acid were found in rats fed trivernolin at the 4.8% level in the diet for 90 days (19).

From our experiments we wished to determine whether rats on diets containing hydrogenated castor oil would absorb hydroxystearic acid and incorporate it, whether rats on these diets would grow normally, and whether deposited hydroxystearic acid would be metabolized.

EXPERIMENTAL PROCEDURE

Preliminary Feeding Trial

In a 90 day subacute toxicity feeding trial, weanling female rats (three per group) were fed diets containing 0%, 5%, 10% and 20% hydrogenated castor oil (HCO). The HCO in the form of a granulated powder was added at the expense of equal amounts of a commercial rat diet. Prior to autopsy, blood samples were taken for hematological study. At autopsy, organ weights were recorded and numerous tissues were preserved in 10% formaldehyde for microscopic pathological examination. The only abnormality noted was a reduced growth rate in the rats fed diets containing 10% and 20% HCO. The HCO is probably poorly digested because of its high melting point, so poor body weight gains may be due to the lower caloric density of the diets containing 10% or more of this material. As a check on this premise, the above trial was repeated but the HCO was dissolved in corn oil before being added to the diet. The levels of HCO fed were 0%, 1%, 5% and 10% of the diet, and the amounts of corn oil added to each diet were 20, 19, 15 and 10%, respectively. The rats were autopsied after being fed these diets for 90 days, at which time growth of rats fed 10% HCO seemed equivalent to that of rats on other diets. Again, no adverse effects were detected as indicated by organ weights and results of hematological and microscopic pathological examinations.

Animals and Diets

A total of 90 young albino male rats (Slonaker substrain of Wistar strain), ranging in weight from 43 g to 83 g, were segregated into groups of six to give the same mean weight per group. Food and water were supplied ad lib. and weekly records of individual body weights and food intake by groups were maintained.

The diet fed contained either 20% corn oil (control diet) or 1% hydrogenated castor oil and 19% corn oil (1% HCO diet) or 10% HCO and 10% corn oil (10% HCO diet). Eighty per cent of each diet was Purina Laboratory Chow. The HCO was melted and mixed with the corn oil, then blended with the chow. After eight weeks of feeding, half of the groups of rats on HCO diets was given the corn oil diet until the end of the experiment at 16 weeks.

The fatty acids of our sample of HCO were 86.5% 12-hydroxystearic acid, 10.3% non-oxygenated acids, and 3.2% 12-ketostearic acid (formed during hydrogenation from 12-hydroxystearic acid).

TABLE I

Mean Hydroxy Fatty Acid Content of Rats (Eight-Week Feeding)[a]

Diet	Lipid sample	Wt per cent of carcass fatty acids[b]	Mg in rats[b]	Wt per cent of dry carcass[b]
Corn oil	Carcass lipids	.043 (.020)	7.4 (3.4)	.0113 (.0055)
1% HCO	Abdominal fat	.34		
	Carcass lipids	.27 (.04)	54.6 (19.3)	.082 (.023)
10% HCO	Abdominal fat	4.03		
	Carcass lipids	2.25 (.54)	314 (106)	.52 (.12)

[a]The hydroxy acids considered are only those whose esters have a GLC retention time equivalent to hydroxystearate, hydroxypalmitate, hydroxymyristate or hydroxylaurate.

[b]Standard deviations are given in parenthesis.

Preparation and Analysis of Lipid Samples

Either excised abdominal fat or extracted carcass lipid was analyzed. After four weeks, three rats on the 1% HCO diet were autopsied and their excised abdominal adipose tissue was combined. Three rats on the 10% HCO diet were treated similarly. At eight weeks and twelve weeks, sets of three rats on the corn oil diet were also used. At sixteen weeks, adipose tissue was not pooled; analyses were made of adipose tissue from rats on these diets: corn oil, 1% HCO, 10% HCO, 1% HCO changed to corn oil at eight weeks and 10% HCO changed to corn oil at eight weeks.

Lipids were extracted from three rats on each diet after eight, twelve and sixteen weeks. After the rats were autopsied, their hair, tails and intestinal contents were removed. The carcasses were then minced, lyophilized and ground. A 10 g portion of each was extracted with ether in a Soxhlet extractor. Extractions of duplicate samples were reproducible to within 4-5%.

Excised and extracted lipids were saponified with potassium hydroxide solution (1/2 g potassium hydroxide pellets, 1 ml water and 1 ml methanol/g of sample) at 60 C for 30 min. Nonsaponifiable lipids were extracted with ether. Acids were recovered and weighed, then esterified with 1% sulfuric acid in methanol (sixtyfold molar excess). After removal of most of the methanol on a rotary evaporator, the mixtures were partitioned between water and ether, from which methyl esters were recovered.

Esters (2-4 g) were chromatographed on a silicic acid column prepared from 50 g dried silicic acid, 100 ml benzene and 35 ml of 20% methanol in benzene (20). For elution we used 275 ml of 1% methanol in benzene, 150 ml of 4% methanol in benzene and 150 ml ether. Portions of eluate were combined to form three fractions: a fraction of nonoxygenated esters (first 200 ml, contains 94-99% of sample), a fraction that would contain any methyl ketostearate (next 190 ml) and a fraction containing hydroxy esters (remainder of eluate). The boundary between the latter fractions was usually determined by monitoring separations by use of a Silica Gel G thin layer chromatographic (TLC) strip. A strip was spotted with 3 μl of eluate from consecutive 10 ml portions, sprayed with 20% sulfuric acid solution, heated and observed under UV light. Analyses of hydroxy ester fractions were performed on a 3 ft x 1/8 in. diameter column of 10% FFAP (Free Fatty Acid Phase—a reaction product of Carbowax 20 M and 2-nitroterephthalic acid—Varian-Aerograph) on 70/80 dimethylchlorosilane-treated Chromosorb W programmed at 7.5 deg/min from 200 to 270 C with helium flow 30 ml/min in a F & M Model 720 dual column gas chromatograph. Chromatograms were obtained that showed relative amounts of hydroxy esters. Measured amounts of methyl eicosanoate were then added to the hydroxy ester fractions and chromatograms were obtained that were used to calculate the amount of methyl 12-hydroxystearate in the fraction. From this, the amounts of methyl hydroxypalmitate, hydroxymyristate and hydroxylaurate were calculated.

Characterization of Metabolites of 12-Hydroxystearic Acid

Hydroxy ester fractions from rats fed HCO were pooled and dissolved in heptane. The solution was chilled to below -50 C, then solids were filtered from it. Chromatography of the solids on a silicic acid column provided a partial

TABLE II

Mean Hydroxy Fatty Acid Content of Rats (12-Week Feeding)[a]

Diet	Lipid sample	Wt per cent of carcass fatty acids[b]	Mg in rats[b]	Wt per cent of dry carcass[b]
Corn oil	Abdominal fat	.027		
	Carcass lipids	.022 (.003)	7.5 (3.1)	.0081 (.0021)
1% HCO for 12 weeks	Abdominal fat	.36		
	Carcass lipids	.29 (.08)	110.0 (49.8)	.109 (.037)
1% HCO for 8 weeks, then corn oil for 4 weeks	Abdominal fat	.087		
	Carcass lipids	.079 (.007)	26.3 (2.9)	.028 (.001)
10% HCO for 12 weeks	Abdominal fat	2.28		
	Carcass lipids	1.81 (.10)	564 (154)	.61 (.09)
10% HCO for 8 weeks, then corn oil for 4 weeks	Abdominal fat	.87		
	Carcass lipids	.27 (.01)	85.3 (40.3)	.09 (.04)

[a]The hydroxy acids considered are only those whose esters have a GLC retention time equivalent to hydroxystearate, hydroxypalmitate, hydroxymyristate or hydroxylaurate.

[b]Standard deviations are given in parenthesis.

separation of 12-hydroxystearate from a concentrate of hydroxy esters with chain lengths shorter than that of 12-hydroxystearate. From this concentrate a compound with the retention time of methyl hydroxypalmitate was isolated by preparative gas liquid chromatography (GLC) on a 1/4 in. diameter diethyleneglycol succinate column. This was purified by saponification, reesterification, preparative TLC and treatment with decolorizing carbon whereupon it did not show an impurity on analysis by GLC or TLC. Its mass spectrum showed that it was methyl 10-hydroxypalmitate (21) and at least 99% pure. The compound with the retention time of methyl hydroxymyristate was also isolated by preparative GLC but was not further purified. Its mass spectrum showed that it was methyl 8-hydroxymyristate of 85-95% purity. No attempt was made to collect the compound that is presumably methyl 6-hydroxylaurate.

RESULTS AND DISCUSSION

Live weight data were statistically analyzed to indicate the influence of diet. Differences in initial weight were removed in order to compare live weights on the basis of the same initial weight for each rat. Weights of all rats living at a particular time were included in calculations of average weight and weight gain. The number of rats on each HCO diet living at 4, 8, 12 and 16 weeks was 33, 30, 12 and 6 rats. The corresponding number for rats on the corn oil diet are 15, 15, 12 and 6 rats. Figure 1 depicts the growth of rats on the corn oil, 1% HCO and 10% HCO diets. At four weeks and at eight weeks, live weight differences due to different diets were not significant at the 95% level. At 12 weeks, rats given the 10% HCO diet weighed significantly less than rats given either the corn oil diet or 1% HCO diet. At 16 weeks, the weight gain with the corn oil diet was significantly greater than the corresponding gain with the 10% HCO diet or with the 10% HCO diet for eight weeks changed to the corn oil diet for eight weeks. There was no statistically significant difference in weight gain of rats on the 1% HCO diet from weight gain of rats on the corn oil diet. Because of the high variability of weight data for rats in a group and the small size of our samples, we are unable to specify any other growth trends.

An explanation for the lesser growth of rats on the 10% HCO diet is that the HCO may have been difficult to absorb; both HCO and 12-hydroxystearic acid melt above 80 C. In attempts to feed rats HCO not in admixture with corn oil, most of the HCO was not absorbed. Using the rat caloric availability assay as described by Rice et al. (22), we found that

TABLE III

Mean Hydroxy Fatty Acid Content of Rats (16-Week Feeding)[a]

Diet	Lipid sample	Wt per cent of carcass fatty acids[b]	Mg in rats[b]	Wt per cent of dry carcass[b]
Corn oil	Abdominal fat	.020 (.002)		
	Carcass lipids	.023 (.016)	9.4 (6.1)	.0090 (.0061)
1% HCO for 16 weeks	Abdominal fat	.33 (.15)		
	Carcass lipids	.28 (.02)	142.7 (16.6)	.12 (0)
1% HCO for 8 weeks, then corn oil for 8 weeks	Abdominal fat	.048 (.011)		
	Carcass lipids	.042 (.038)	16.8 (16.0)	.016 (.014)
10% HCO for 16 weeks	Abdominal fat	1.67 (.08)		
	Carcass lipids	1.91 (.29)	666 (196)	.68 (.15)
10% HCO for 8 weeks, then corn oil for 8 weeks	Abdominal fat	.12 (.03)		
	Carcass lipids	.10 (.05)	25.3 (8.3)	.028 (.007)

[a]The hydroxy acids considered are only those whose esters have a GLC retention time equivalent to hydroxystearate, hydroxypalmitate, hydroxymyristate or hydroxylaurate.

[b]Standard deviations are given in parenthesis.

only 9-14% of the powdered HCO was digested and, consequently, the caloric availability value was only 18% of the value for an equivalent amount of corn oil. When a HCO-corn oil blend (1:3) was fed, the digestibility values ranged from 27-64%, depending on the level fed. It seems unlikely that the hydroxyl function in these compounds chemically inhibits growth because rats fed glycerides of ricinoleic acid as 9-10% of the diet, ad lib. or in measured amounts, gained 0.97 the weight of rats fed corn oil (3,9).

Hydroxystearic acid was deposited in abdominal fat and in other body lipids. In all cases it was accompanied by its metabolites, hydroxypalmitic acid, hydroxymyristic acid and hydroxylauric acid (Fig. 2). In rats four weeks on the 1% HCO diet, abdominal fat fatty acids were 0.90% by weight HCO-derived hydroxy acids, but this proportion decreased to about 0.35% in the 8-16 week period (Tables I-III). Acids obtained from carcass lipids (carcass fatty acids) contained a smaller proportion of HCO-derived hydroxy acids than did abdominal fatty acids—about 0.28% in the 8-16 week period. Therefore, it appears that there is a greater concentration of hydroxy acid in abdominal fat than in the rest of the body lipids. The greatest content of HCO-derived hydroxy acids in lipid was 4.4% hydroxy acids in abdominal fat of rats four weeks on the 10% HCO diet. This proportion decreased during the following weeks and at 16 weeks was less than 2% (Table III)—approximately the same as in the carcass lipids. Hydroxy acids as a percentage of the dry carcass weight increased during the 8-16 week period in rats on both diets. Using a diet comparable to our 10% HCO diet, Perkins et al. (9) found 6.1 ± 3.6% hydroxy acids in carcass fatty acids after eight weeks. Their result is apparently based on the acetyl value of unfractionated esters. However, we found that a polar ester fraction from rats on the corn oil diet, isolated by chromatography on silicic acid, constituted 0.6-2.2% of the lipid esters and consisted of hydroxylated material, as indicated by polarity, IR spectra and hydroxyl values. Similar material is part of the hydroxy ester fraction from rats fed HCO diets (Fig. 2); therefore, use of acetyl value would not measure accurately the content of HCO-derived hydroxy esters in carcass fatty acid esters.

Rats on the corn oil diet apparently con-

tained hydroxystearic acid; a component of their hydroxy ester fraction had the GLC retention time of hydroxystearate (Fig. 2). It constitutes less than 0.05% of the total esters (Table I-III). We have not tried to isolate and identify it but previous work suggests several possible identities. Kishimoto et al. (23) found a series of saturated and unsaturated 2-hydroxy fatty acids in various rat tissues. Aged liver microsomes can convert stearic acid to 18-hydroxystearic acid and 17-hydroxystearic acid (24), but such a conversion has not been demonstrated in the intact rat. Rat liver homogenates which converted stearic acid to oleic acid converted 1-2% of the stearic acid to a mixture of hydroxystearic acids, including 10-hydroxystearic acid (25). The same acid was produced from oleic acid by some microorganisms (26). 10-Hydroxystearic acid is also synthesized in the intestine and is found in human (27,28), canine (29) and rat (30) fecal lipids.

When rats were changed from HCO to the corn oil diet, the amount of HCO-derived hydroxy fatty acids in their tissues decreased rapidly. At eight weeks, rats on the 1% HCO diet had an average hydroxy fatty acid content of 54.6 mg (Table I). After the change to the corn oil diet, average hydroxy fatty acid content was 26.3 mg at 12 weeks and 16.8 mg at 16 weeks. Loss of hydroxy fatty acid from rats on the 10% HCO diet was more pronounced. The average content of 314 mg at eight weeks dropped to 85.3 mg at 12 weeks (Table II) and 25.3 mg at 16 weeks (Table III). The rate of disappearance of the hydroxy acids was roughly proportional to the amount present.

Degradation of 12-hydroxystearic acid occurs by successive losses of two-carbon units from the carboxyl end of the fatty acid chain. We have identified the metabolites 10-hydroxypalmitic acid and 8-hydroxymyristic acid by their mass spectra. Per cent composition of the HCO-derived hydroxy fatty acids in rat lipids is approximately 81% 12-hydroxystearic acid, 17% 10-hydroxypalmitic acid, 1.6% 8-hydroxymyristic acid and 0.4% 6-hydroxylauric acid. Our results parallel those of Uchiyama et al. (14,15) who fed ricinoleic acid and then found shorter chain unsaturated hydroxy fatty acids in adipose tissue.

Ketostearic acid is apparently metabolized at the same rate as hydroxystearic acid. The ratio of 12-ketostearic acid to 12-hydroxystearic acid in the diet was 0.037. In rats on the 10% HCO diet for 16 weeks, this ratio was 0.040.

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Fatty Acid Composition of the Living Layer and Stratum Corneum Lipids of Human Sole Skin Epidermis

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ABSTRACT

Lipids from the living layer and stratum corneum of human sole epidermis were extracted, saponified and the fatty acids analyzed. The proportion of fatty acids to unsaponifiables (mainly cholesterol), was higher in the living layer than in the stratum corneum. Fatty acids of the living layer and stratum corneum of human sole epidermis comprise saturates, monoenes, dienes, traces of polyenes and α-hydroxy fatty acids. Homologs of monoenoic and dienoic fatty acids for both living layer and stratum corneum lipids have a similar distribution. C_{16} and C_{18} were major components for each type of acids. There appeared to be two clusters, especially for saturates of both living layer and stratum corneum acids. One of these clusters ranged from C_{12} to C_{20} with C_{16} or C_{18} as a maximum and the other ranged from C_{21} to C_{30} with C_{24} as a maximum. The proportion of saturated acids with chain length C_{20} and above was much higher in the stratum corneum than in the living layer. Position isomers of the monoenoic fatty acids for both the living layer and stratum corneum show a predominance of ω9 acids, due to the overwhelmingly large amount of oleic acid. Linoleic acid was by far the major component of the dienoic acids. Homolog distribution of α-hydroxy fatty acids for the living layer was similar to that of the stratum corneum and again two clusters of acids below and above C_{20} with maxima at C_{16} and C_{24} were noticeable. Comparison of epidermal acids with those of sebaceous glands showed that each tissue can synthesize the same kind of acids but in widely different amounts. Oxidation of palmitate and stearate could supply the necessary energy for the late stages of keratinization.

INTRODUCTION

In a process called keratinization, epidermal cells of human and animal skin undergo a degradation with the concomitant formation of a tough, protective protein called keratin. A major function of the lipids of these cells is to serve as membrane constituents. These lipids are synthesized by cells of the inner part of the epidermis (living layer), and are probably modified as the cells proceed outward into the stratum corneum (dead layer). Ultimately the end products of this process contribute to skin surface lipids.

Epidermal cells can also differentiate to form sebaceous glands. These glands produce sebum, an oily product made up of lipids with

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TABLE I

Lipid Yield From Parts of Human Sole Epidermis

Sample[a]	Fraction	Living layer	Stratum corneum
1	Crude lipid[b]	100% (16 mg)	100% (74 mg)
	Recovered lipid[c]	71%	80.5%
	Fatty acids	50%	33.9%
	Unsaponifiables	50%	66.1%
2	Crude lipid[b]	100% (62 mg)	100% (143 mg)
	Recovered lipid[c]	65.5%	82.6%
	Fatty acids	44.3%	29.2%
	Unsaponifiables	55.7%	70.8%

[a]For identification of samples see Figure 1, footnote a.

[b]Total yield from $CHCl_3$/MeOH extracts, see Figure 1, footnote c.

[c]Total weight of fatty acids plus unsaponifiables divided by weight of crude lipid.

TABLE II

The Unsubstituted Fatty Acids of Living Layer and Stratum Corneum Lipids of Human Sole Epidermis[a]

	Sample 1						Sample 2					
	Living layer			Stratum corneum			Living layer			Stratum corneum		
Carbon number[b]	Saturated, %	Monoene, %	Diene, %	Saturated, %	Monoene, %	Diene, %	Saturated, %	Monoene, %	Diene, %	Saturated, %	Monoene, %	Diene, %
12	Trace			.2			.1			.2		
13	.1			Trace			Trace			.1		
14	1.4	.2		1.1	.1		2.5	.4		1.9	.5	
14.7	.5	ND[c]		.5	ND		.2	ND		.7	ND	
15	3.7	.4		2.8	Trace		4.8	.3		3.3	.1	
15.6	.9	ND		.4	ND		.2	ND		.4	ND	
16	40.6	6.3	.4	24.6	7.8	.1	48.6	7.3	.3	25.1	7.7	.2
16.5	1.1	ND		.8	ND		.7	ND		1.0	ND	
16.7	.5	ND		.3	ND		.5	ND		.5	ND	
17	3.7	1.6	.1	3.6	1.2	.1	3.4	1.2	.1	4.0	1.8	.3
17.6	ND	ND		Trace	ND		ND	ND		Trace	ND	
18	34.8	86.0	96.7	18.6	83.6	97.6	33.9	86.2	96.7	19.3	80.0	96.1
18.7	.5	ND		.1	ND		.2	ND		.2	ND	
19	.4	.4	.1	2.0	.2	.1	.6	.4	.1	2.8	.5	.2
20	1.2	2.4	2.5	3.9	2.4	2.0	1.0	2.5	2.7	4.8	2.9	3.1
21	.3	.3	Trace	1.8	Trace	Trace	.1	.1	Trace	2.3	.1	.1
22	2.9	1.0	.1	7.5	1.4	.1	1.2	1.0	.1	7.8	1.8	.2
23	1.0	.1		5.6	.1		.2	Trace		5.9	.2	
24	3.7	.7	.1	14.0	2.0	Trace	1.0	.5	Trace	12.1	2.9	Trace
25	.6	.1		3.9	.1		.1	Trace		3.2	.2	
26	1.4	.2		4.7	.8	Trace	.4	.1	Trace	2.8	1.0	Trace
27	.3	.1		1.2	Trace		.1	Trace		.6	.1	
28	.8	.2		1.6	.2		.2	Trace		.7	.5	
29	.1	Trace		.6	Trace		Trace	Trace		.1	Trace	
30	.1	Trace		.2	.1		Trace	Trace		.2	.4	
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
C_{20} and below	89.8	97.3	99.8	58.9	95.3	99.9	96.7	98.3	99.9	64.3	93.5	99.7
Above C_{20}	11.2	2.7	.2	41.1	4.7	.1	3.3	1.7	.1	35.7	6.5	.3

[a]For description of Sample 1 and 2 see Figure 1.
[b]Determined by the method of Woodford and Van Ghent (10).
[c]Not detected.

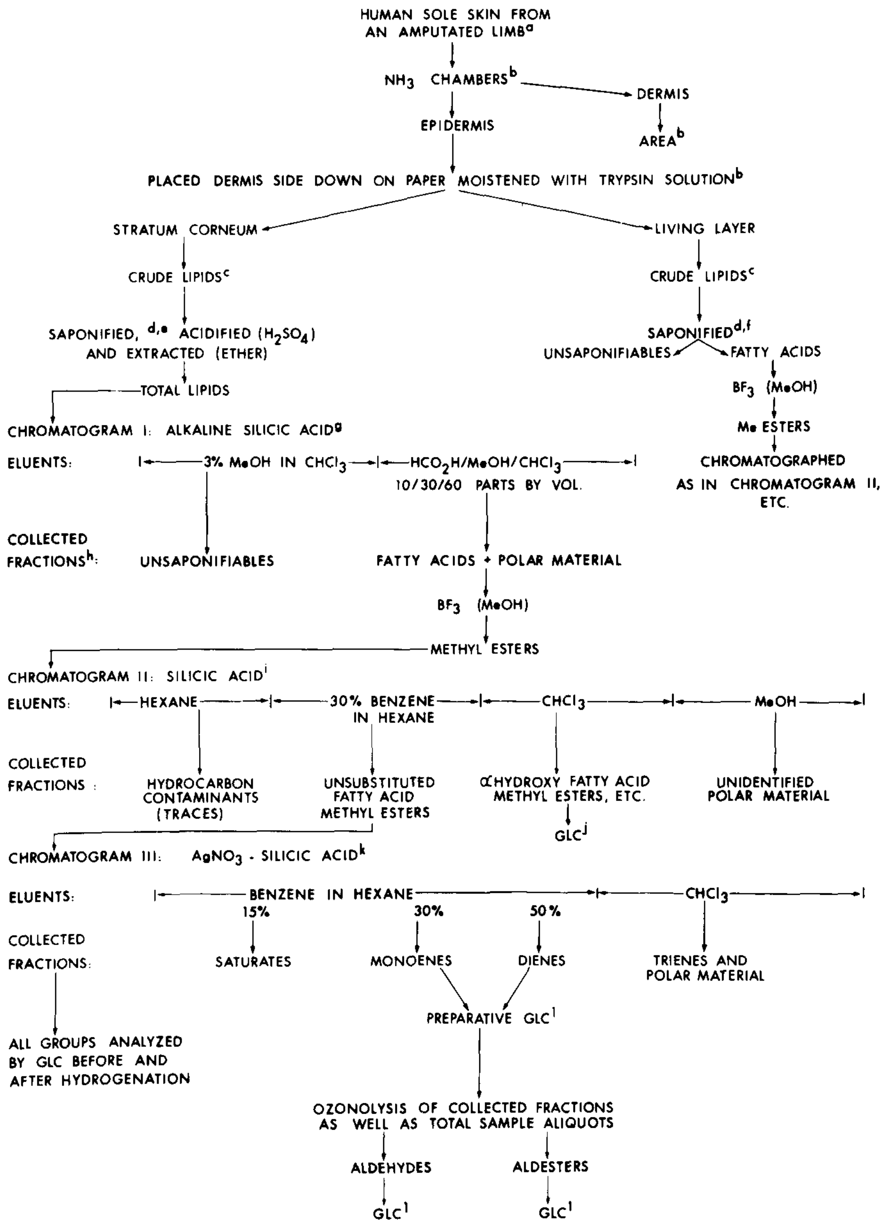

FIG. 1. Isolation and analysis of fatty acids from the living layer and stratum corneum of human sole epidermis.

[a]One sample (called Sample 1 in the Tables) from a patient with osteosarcoma of the right tibia, another (called Sample 2) from a patient with a tumor of the hip. Skin was removed from the bottom of the foot with care not to contaminate it with subcutaneous fat from the knife, wrapped in aluminum foil, then stored at -20 C until work up. Although the samples were primarily from the sole of the foot and are designated as sole skin throughout this paper, the samples included skin from the heel as well.

[b]Separation of epidermis from dermis after exposure to NH_3 fumes, determination of area, separation of living layer from stratum corneum by exposure of epidermis to trypsin, and extraction of lipids with $CHCl_3$/MeOH, 2/1 by vol were as previously described (3,4).

[c]The crude lipids were unwashed and therefore contained non-lipid material. Yields are in Table I.

[d]Saponifications were as in reference 5.

[e]For stratum corneum the unsaponifiable matter could not be separated from fatty acids by extraction with ether from the alkaline saponification mixture because of excessive emulsions, hence, the necessity of Chromatogram I.

[f]For living epidermis the unsaponifiable matter could be separated from fatty acids by ether extraction. Fatty acids were extracted after acidification in the normal manner. They contained small amounts of polar material removed by subsequent chromatography.

[g]As described in references 5 and 6.

[h]Completeness of separation of unsaponifiables from fatty acids verified by TLC. Plates of silica gel plain/$MgSiO_3$ 9/1 (Ref. 5) spotted then developed to $R_f = 0.5$ with ether/HA_c, 99/1 then dried 10 min and redeveloped to $R_f = 1.0$ with hexane/ether 95/5.

[i]Column 1 x 14 cm packed with 4.5 g silicic acid (Unisil 100-200 mesh Clarkson Chem. Co., Williamsport, Pa.). Loading factor 7 mg (or less) methyl esters per gram of Unisil.

[j]Besides TLC data these acids were identified as α hydroxy fatty acids by GLC as in reference 7.

[k]The adsorbent was made from 100 g of Unisil (100-200 mesh) added to a beaker containing 7 g $AgNO_3$ (Matheson Coleman and Bell, Rutherford, N.J.), dissolved in 100 ml water. The slurry was heated with gentle mixing until it was free flowing then dried in an oven at 115 C for 24 hr, cooled and while still warm transferred to a brown bottle and tightly stoppered. Just prior to packing the column, 5 g of the adsorbent was deactivated by adding 1 ml of water and mixing thoroughly in a small brown bottle. The adsorbent was packed into a column 1 x 14 cm i.d. as a slurry in hexane and the column protected against light with aluminum foil. Loading factor was 5 mg (or less) methyl esters per gram of adsorbent.

[l]As described in references 8 and 9.

many unusual features (1). Since the stratum corneum normally soaks up sebum, it is difficult to obtain epidermis free of sebum. However, if the skin sample is taken from either the palms or soles where sebaceous glands are absent, contamination with sebum is minimized. To obtain epidermis from skin it must first be separated from dermis, and there are a number of techniques for doing this (2). Recently a technique has also become available for separating the living epidermis from the stratum corneum (2,3). Thus, if these separations are applied to sole skin, the lipids synthesized by epidermal cells can be studied. This may help not only in understanding what lipids are contributed to the skin surface film by epidermal cells, but may assist us in understanding what role the lipids may have in the keratinization process itself. In this paper we report the fatty acid composition of the living epidermis and the stratum corneum of human sole skin with these objectives in mind.

EXPERIMENTAL PROCEDURES

Figure 1 shows how we prepared the skin samples and obtained and analyzed the fatty acids.

RESULTS AND DISCUSSION

Table I lists the yield of lipid from the two samples of human sole epidermis. Note that the weight of fatty acids plus unsaponifiables (i.e., recovered lipid) is appreciably less than the weight of total crude lipid. It is probable that some of this loss represents non-lipid material which dissolved in the aqueous phase and some the loss of the polar moieties of the polar lipids. We do not know how these losses are distributed. The relatively large amount of unsaponifiable matter, especially in the stratum corneum, is noteworthy. Thin layer chromatography (TLC) showed that most of this was cholesterol. The proportion of fatty acids in the stratum corneum is less than that of the living layer. Thus, if both fatty acids and unsaponifiables are synthesized in the lower part of the living layer, as appears likely, these data would indicate that some of the fatty acids were metabolized by the time the cells reached the stratum corneum.

Table II gives the homolog distribution of the saturated, monoenoic and dienoic acids from the living layer and stratum corneum lipids. Although C_{16} and C_{18} are major components for each type of acid, there appear to be two clusters, especially for the saturates of both the living layer and the stratum corneum acids. One of these clusters ranges from C_{12} to C_{20} with C_{16} or C_{18} as maximum and the other ranges from C_{21} to C_{30} with C_{24} as maximum. These clusters are not unlike those found in the fatty acids of brain (11). The relative amounts of homologs in each of these clusters are totaled at the bottom of Table II. Homologs of the monoenoic and dienoic fatty acids of both stratum corneum and living layer lipids have a similar distribution, but stratum corneum has a much greater proportion of the saturated long chain acids than living layer has. These data are also consistent with the idea that some fatty acids (chiefly 16:0 and 18:0) are metabolized as cells pass from the living layer to the stratum corneum.

TABLE III

Relative Amounts of the Different Fatty Acids Types in Living Layer and Stratum Corneum Lipids of Human Sole Epidermis

	Sample 1[b]	Sample 2	
Fatty Acid Type[a]	Stratum corneum, %	Living layer, %	Stratum corneum, %
Saturated			
straight even	38.4	37.3	35.2
straight odd	11.4	3.9	10.5
branched	1.2	.8	1.3
sub total	51	42	47
Monoene			
straight even	30.5	39.2	32.9
straight odd	.5	.8	1.1
branched	Trace	Trace	Trace
sub total	31	40	34
Diene			
straight even	18.0	18.0	18.9
straight odd	Trace	Trace	.1
sub total	18	18	19
Total	100	100	100

[a]The values of this Table were computed on the basis that the total amounts of saturates, monoenes and dienes isolated comprised 100% of the fatty acids, the further subdivision into straight even, straight odd and branched being calculated from the data of Table II. In actuality these fatty acid types represent approximately 80% of the total material isolated as fatty acids in the separation of saponifiable from unsaponifiable matter. Of the remaining 20% we estimate about one-fourth to be α hydroxy fatty acids. The remaining unidentified polar matter could include oxidized polyenoic acids.

[b]An unknown portion of the living layer acids of this sample was lost.

Table III summarizes the relative amounts of the different types of fatty acids for stratum corneum and living layer lipids. Note that stratum corneum has the greatest amount of odd and branched chain acids.

Table IV lists the homolog distribution of α hydroxy fatty acids for living layer and stratum corneum lipids. Note again the two clusters of acids above and below C_{20} and that the stratum corneum of each sample has the greater proportion of acids above C_{20}.

Table V lists the position isomers of the fatty acid monoenes of living layer and stratum corneum lipids. Note that for 16:1 and 18:1 of both the living layer and stratum corneum, the $\Delta 9$ position isomer is by far the major isomer and that there is very little difference in the distribution of position isomers for all chain lengths of both living layer and stratum corneum lipids. Note also that because of the overwhelmingly large amount of 18:1 the extension pattern, as represented by $\omega 9$, is by far the predominant one. It thus appears that at least a part (if not all) of the oleic acid found in human skin surface lipid could be derived from the lipids of epidermal cells as earlier anticipated (12).

Table VI lists the position isomers of the dienoic fatty acids 18:2 and 20:2 for both living layer and for stratum corneum lipids. Although linoleic acid (18:Δ9,12) is by far the major component and presumably derived from

TABLE IV

α-Hydroxy Fatty Acids of the Living Layer and Stratum Corneum Lipids of Human Sole Epidermis

α-Hydroxy fatty acids (No. C atoms)	Sample 1		Sample 2	
	Living layer, %	Stratum corneum, %	Living layer, %	Stratum corneum, %
14	.1	.2	.1	.2
15	2.1	2.2	3.0	6.0
16	43.2	27.8	58.8	44.0
17	8.9	3.2	10.3	7.1
18	12.6	10.6[a]	17.2	18.4[a]
19	1.4	.2	1.0	1.2
20	.6	.8	.5	1.0
21	.1	.1	.1	.1
22	1.7	2.0	1.8	2.3
23	3.9	2.9	1.0	1.7
24	14.2	23.1	4.4	7.0
25	3.4	11.1	.3	3.3
26	7.8	15.8	1.5	6.7
Total	100.0	100.0	100.0	100.0
C_{20} and below	68.9	45.0	90.9	78.9
Above C_{20}	31.1	55.0	9.1	21.1
Total	100.0	100.0	100.0	100.0

[a]Includes ~10% unsaturates.

TABLE V

Position Isomers of Fatty Acid Monoenes of Living Layer and Stratum Corneum Lipids of Human Sole Epidermis[a]

Structures found[b]	Living layer: Per cent this chain length is of total monoenes	Living layer: Position isomer distribution, %	Stratum corneum: Per cent this chain length is of total monoenes	Stratum corneum: Position isomer distribution, %	Possible mode of formation			
16:Δ6		1		1		Δ6		
16:Δ7	7.3	4	7.7	5			Δ7	
16:Δ8		1		1				
16:Δ9		94		93				Δ9
18:Δ7		2		2	Δ7	↓ $+C_2$ Δ8		
18:Δ8		1		1				
18:Δ9		92		93			↓ $-C_2$ Δ9	
18:Δ10	86.2	1	80.2	1				
18:Δ11		4		3				↓ $+C_2$ Δ11
18:Δ12		1		Trace				
18:Δ13		Trace		Trace				
20:Δ9		20		4	↓ $-C_2$ Δ9			
20:Δ11	2.5	70	2.9	93			↓ $+C_2$ Δ11	
20:Δ13		10		3				↓ $+C_2$ Δ13
22:Δ11		2		2	↓ $+C_2$ Δ11			
22:Δ13		90	1.8	95			↓ $+C_2$ Δ13	
22:Δ15		8		3				↓ $+C_2$ Δ15
24:Δ13	.5	ND[c]	2.9	5	↓ $+C_2$ Δ13			
24:Δ15				85			↓ $+C_2$ Δ15	
24:Δ17				10				↓ $+C_2$ Δ17
C_{26}:Δ15	.1	ND	1.0	5	↓ $+C_2$ Δ15			
C_{26}:Δ17				95			↓ $+C_2$ Δ17	
ω series					ω11	ω10	ω 9	ω 7

[a]Sample 2 of Table II.

[b]By reductive ozonolysis as shown in Figure 1.

[c]Not determined.

TABLE VI

Dienoic Fatty Acids of the Living Layer and Stratum Corneum Lipids of Human Sole Epidermis[a]

Carbon number				
Hydrogenated	Original	Deduced structures	Isomer distribution, %	Possible mode of formation
18:00	18.52	18:Δ6,9	Trace	18:Δ9-2H → 18:Δ6,9
		18:Δ8,11	Trace	18:Δ11-2H → 18:Δ8,11
		18:Δ9,12	100	Linoleic acid from diet
20:00	20.50	20:Δ8,11	5	18:Δ9+C_2-2H → 20:Δ8,11
		20:Δ11,14	95	18:Δ9,12+C_2 → 20:Δ11,14

[a]Both living layer and stratum corneum gave the same values.

the diet, the other isomers of the Table appear to be synthetic products of skin and can be explained by known patterns of desaturation and chain extension of monoenes. For living layer lipids traces of material were seen which corresponded to 18:3 on the basis of gas liquid chromatography (GLC) on polyester phase before and after hydrogenation.

Although Coon et al. have reported (13) that fatty acids between C_{20} and C_{30} occur in significant amounts in the free fatty acid fraction of barrier zone lipids of normal and psoriatic skin, the pattern of homologs found by them does not remotely resemble that found by us. Furthermore, in a later report (14) Wheatley et al. found, after extensive purification of their free fatty acid fractions, only traces of acids with chain lengths longer than C_{20}. They attributed the earlier findings of long chain material to contaminants and methylation artifacts.

In a study of labeled acetate incorporation into fatty acids of isolated epidermal cells grown in tissue culture, Wilkinson (15) found a distribution of the even chain homologs remarkably similar to that found by us for living layer lipids (Table II). We recalculated his results for direct comparison with ours (Table VII). These data suggest that the cells grown by this technique of tissue culture produce lipids very similar to those found in living human epidermis.

Since the epidermis is an avascular tissue it is dependent upon diffusion of nutrients from capillaries of the dermis. However, as cells leave the basal layer of the living epidermis on their way towards keratinization, available nutrients decrease. Furthermore, as keratinization proceeds, cell organelles undergo dissolution (16). Thus, the fatty acids released from the lipids of the membranes of these organelles could serve as a source of energy, and, indeed, there is other evidence that this does occur (17). The persistence of some mitochondria even in late stages of keratinization supports this concept (18). These mitochondria could perform the fatty acid oxidation and produce ATP necessary for the final stages of keratin synthesis.

Thus in summary, the data of this paper are

TABLE VII

Comparison of the Composition of Fatty Acids From Epidermal Cells Grown in Tissue Culture With Those From the Living Layer of Human Sole Epidermis

	Fatty acids of epidermal cells grown in tissue culture[a]			Fatty acids of the living layer of human sole epidermis[b]		
Carbon number	Saturates, %	Monoenes, %	Dienes, %	Saturates, %	Monoenes, %	Dienes, %
14	2.4			2.2	.3	
16	45.2	10.2		51.0	6.9	.4
18	33.6	84.2	98.1	39.4	88.4	96.8
20	2.6	2.8	1.9	1.3	2.6	2.6
22	4.4	2.8		2.4	1.0	.1
24	5.9			2.7	.6	.1
26	5.9			1.0	.2	

[a]Data from Wilkinson (15).

[b]Data from Table II, but only the straight even numbers considered for comparison.

entirely consistent with the following interpretation. In the basal layer, blood glucose provides the primary source of energy and C_2 units for lipid synthesis required for the membranes of rapidly dividing cells. As keratinization proceeds in the upper portion of the living layer and the cell organelles are disrupted, the oxidation of palmitate and stearate occurs to supply the necessary ATP for the late stages of keratinization. Hence, the relative decrease of these acids in the stratum corneum as compared to those of the living layer. The longer chain fatty acids at about C_{24}, both straight and hydroxy (as reported here), may then be incorporated into the sphingolipids which, together with the free cholesterol, make a tight type of plasma membrane (16).

Since epidermal cells have the capability of either producing keratin or differentiating into sebaceous glands that produce sebum, it was of some interest to see whether the fatty acids of the living layer had any resemblance whatsoever to those of sebum. Sebum has significant amounts of a variety of fatty acids not ordinarily found in most living tissues (19,20), e.g., those with odd chain lengths (~10%), branched (mainly iso and anteiso, ~15%), and especially $\omega 10$ monoenes (~40%). Living layer produces ~5% odd, ~1% branched, and ~40% of monoenes predominantly $\omega 9$ but having a small trace of $\omega 10$ (Table III). Thus, in the main, living layer and sebaceous glands can make the same kinds of fatty acids, although they do so in widely differing amounts. Also, the odd and branched acids accumulate in the stratum corneum presumably because of the utilization of the biologically more valuable C_{16} and C_{18} acids, as noted above, leaving the less valuable odd and branched acids (as is also true in the case of sebum) to arrive at the surface lipid film. Although it has not yet been proved, it is quite conceivable that the microflora contacting the skin surface may have greater difficulty in metabolizing these acids than they would palmitic and stearic acid.

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Phosphoلipid Oxidation in Emulsions[1,2]

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ABSTRACT

The autoxidation of purified phosphatidyl ethanolamine (PE) and phosphatidyl choline (PC), extracted from egg and soybean lipids, was followed by oxygen uptake measurements in emulsified systems. All emulsified phospholipid fractions had comparable activation energies. Measurement by various physico-chemical tests was made of specific changes in the phospholipid molecule during autoxidation. PE oxidized more rapidly and absorbed more oxygen than PC. Higher 2-thiobarbituric acid test and diene and triene conjugation absorbance values were observed for PE than for PC. Of the two major polyunsaturated fatty acids in egg phospholipids, arachidonic acid disappeared at a more rapid rate during oxidation while the concentration of linoleic acid decreased to a level that was relatively constant. Although typical unsaturated fatty acid oxidation appeared to occur in all phospholipid fractions, oxidation in aqueous emulsions was only partly a function of fatty acid composition. The nitrogen moieties, ethanolamine and choline influenced the induction period for the oxidation of PE and PC respectively.

INTRODUCTION

Phospholipids are among the more labile constituents of food and play a role in the oxidative deterioration which can arise during the processing and storage of such food products as fish (1), dehydrated eggs (2), poultry (3), and dairy products (4). Some of the factors influencing the oxidation rate of phospholipids have been reported (1-4).

Since these lipids contain phosphorus, a nitrogen containing component such as choline, ethanolamine or serine, and unsaturated fatty acids, they offer a system far more complicated for oxidation than the neutral triglycerides. The oxidation of unsaturated lipids can be followed in a number of ways. These may include determination of lipid peroxides or the total consumption of oxygen (5). Changes in the concentration of specific unsaturated fatty acids (3,6) and in the free amino group (7) as well as thin layer chromatography (TLC) spotting (8,9), are other methods which have been successfully utilized to measure changes in lipids due to oxidation.

A major objective of this study was to characterize the role of the nitrogen moiety *during oxidation* in order to learn whether the oxidation of PE and PC is related to the degree of unsaturation of the fatty acids or to the nitrogen containing component. Although published reports have indicated that unsaturated fatty acids and the nitrogen moiety are factors affecting the oxidation of phospholipids (1,3,4,7), further study was needed to determine the relative importance of unsaturation and the nitrogen component on phospholipid oxidation. The autoxidation of phosphatidyl ethanolamine (PE) and phosphatidyl choline (PC), emulsified in borate buffer, was followed manometrically. Measurement by various physico-chemical tests was made of specific changes in the phospholipid molecule.

EXPERIMENTAL PROCEDURES

Separation and Purification of Phospholipids

Phospholipids extracted from egg yolks (10) and a commercial soybean phosphatide sample (Centrolex P, Central Soya, Chicago) were separated into fractions by silicic acid (Mallinckrodt) column chromatography (11). Resolution of individual phospholipids was improved with a five stage multibore column (12).

Nitrogen gas was introduced at the top of the column to minimize opportunities for oxidation during preparative work, and dry ice was used to create an inert atmosphere in and around the collection tubes or separation was achieved with a fraction collector in a cold room at 2 C. The space above the solvent in each tube was blanketed with nitrogen after collection was complete. Screw-on caps were fastened and the tubes were stored in the freezer at -20 C.

The purity of individual fractions was as-

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[2]Presented in part at the AOCS Meeting, Chicago, October 1967.

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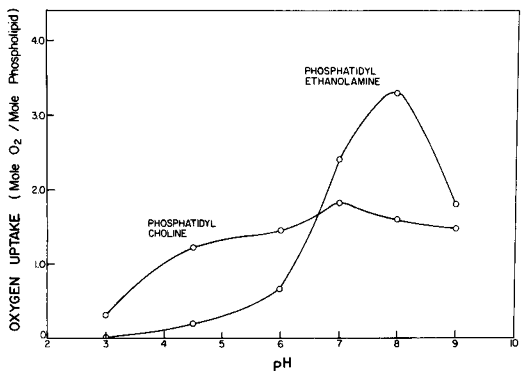

FIG. 1. The effect of pH on oxygen consumption of egg phosphatidyl ethanolamine (PE) and phosphatidyl choline (PC) in 0.1 M borate buffer after 50 hr. Temperature of reaction: PE, 25 C; PC, 40 C.

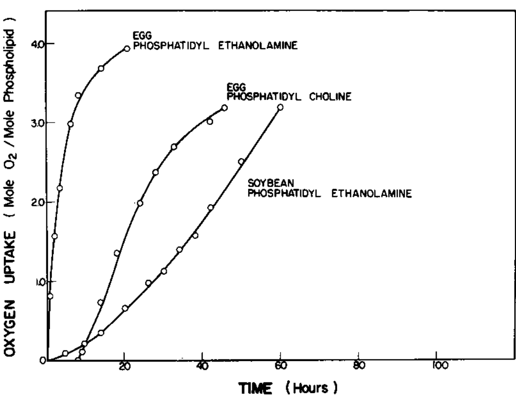

FIG. 2. The oxygen consumption of egg and soybean phospholipids emulsified in 0.1 M borate (pH 7.0), Tween 20 buffer at 25 C.

certained by TLC on Silica Gel G (Brinkman Instruments Inc., Westbury, N.Y.). Ninhydrin (0.2% ninhydrin in *n*-butanol-aqueous acetic acid 10%, 95:5 v/v), molybdate reagent (13) and sulfuric acid (50%) were used to detect components and impurities. The developing solvent mixture was chloroform-methanol-water (65:25:4 v/v/v).

Preparation of Phospholipid Emulsions

Phospholipids were emulsified in three ways. In the first method, purified PE or PC, dissolved in chloroform-methanol, was pipetted into cell homogenizer flasks. After the chloroform-methanol was evaporated from the homogenizer flasks by a stream of nitrogen, 50 ml of 0.1 M borate (pH 7.0) buffer [Boric acid was dissolved in redistilled deionized water and adjusted to pH 7.0 with 0.1 N NaOH.] and 0.1 ml of Tween 20 (polyoxyethylene sorbitan monolaurate) were added to the dry residues which remained in the flasks. The atmosphere within the flasks was purged with nitrogen and emulsification was achieved with a Braun Cell Homogenizer (Bronwill Scientific, Inc., Rochester, N.Y.) at a speed of 4,000 rpm for 1-2 min. Carbon dioxide liquified under pressure was employed to keep the contents of the flask below room temperature while the emulsion was being formed. A control consisting of equivalent amounts of borate buffer and Tween 20 was prepared in the same manner.

A second method of emulsification was adopted for studies of the optimum pH for oxidation. Because of difficulties in dispersing PE over a rather wide range of pH, even with the aid of Tween 20, PE and PC, dissolved in chloroform-methanol in amounts corresponding to 40-50 μg phosphorus (14), were introduced directly into respirometer flasks. Solvents were removed by nitrogen and 4 ml of 0.1 M borate buffer [Boric acid was dissolved in redistilled deionized water and adjusted to the desired pH with 0.1 N NaOH or 0.1 N HCl.] was added.

A third technique was necessary because Tween 20 contained palmitic, stearic and oleic acids in addition to lauric acid which interfered with subsequent GLC determinations of the changes in the fatty acids of PE and PC during oxidation. The addition of 10% PC to PE was observed to reduce the time required to disperse PE. Accordingly, 10% PC was added to each preparation of PE utilized in the studies of phospholipid change during oxidation. A homogeneous emulsion was needed to minimize pipetting errors for physico-chemical determinations. Egg PC was used with egg PE and soybean PC with soybean PE.

Physico-Chemical Tests

Oxidation of PE and PC was studied by measuring oxygen uptake on phospholipid emulsions in a Differential Respirometer (Model GR 14, Gilson Medical Electronics, Middleton, Wis.). Each respirometer flask contained 0.2 ml of 5% KOH in its center well. A minimum of four replicates were run against three to four controls containing buffer and Tween 20. Experiments were conducted at temperatures ranging from 15 to 50 C in an atmosphere of air with a shaking rate of 150 oscillations per minute.

Methylation of the fatty acids in the phospholipids was performed by a low temperature method (15) modified by deletion of sulfuric acid. This eliminated any problem which might arise from dimethyl acetal formation from plasmalogens. Gas liquid chromatography was accomplished with a Beckman GC-5 equipped

TABLE I

Fatty Acid Composition of Purified Phospholipids and Energy of Activation for the Autoxidation of Phospholipids Emulsified in 0.1 M Borate (pH 7.0), Tween 20 Buffer

Fatty acid	Egg PC[a], %	Egg PE[b], %	Soybean PC, %	Soybean PE, %
C_{14}	Trace	Trace	1.2	4.4
C_{16}	30.2	19.5	21.7	18.8
$C_{16:1}$	1.5	3.6	---	---
C_{18}	16.4	30.0	4.1	1.9
$C_{18:1}$	32.1	16.9	17.0	8.2
$C_{18:2}$	16.0	16.6	50.4	56.0
$C_{18:3}$	Trace	Trace	5.7	10.6
$C_{20:4}$	3.8	13.3	---	---
Polyunsaturates	19.8	29.8	56.1	66.1
Activation energy K cal./mole	12.2	11.7	12.8	11.8

[a]PC, phosphatidyl choline.
[b]PE, Phosphatidyl ethanolamine.

with a flame ionization detector; 6 ft x 1/8 in. (O.D.) stainless steel coiled columns were packed with 20% diethylene glycol succinate (DEGS) and 1% phosphoric acid on 80-100 mesh acid-washed chromosorb W. The detector was maintained at 250 C while the column temperature was 184 C. Methyl ester peaks were identified by comparison with those from standards.

UV absorbance, 2-thiobarbituric acid (TBA) tests and determinations of the free amino group were based on aliquots containing 5 μg phosphorus as assayed by the procedure of Rouser et al. (14). Malonaldehyde formation was measured by a TBA test designed to accomodate emulsions of polyunsaturated fatty acids (5). Conjugation of oxidized and nonoxidized PE and PC was determined by diluting 1.0 ml of emulsified sample with 2 ml of Fisher certified American Chemical Society spectranalyzed methanol. UV absorption spectra were recorded with a Beckman model DU spectrophotometer at 232 mμ and 268 mμ against 1.0 ml of borate buffer similarly diluted with methanol. The free amino group of PE was quantitated by reaction with trinitrobenzene sulfonic acid (16).

RESULTS AND DISCUSSION

The Effect of pH on Oxygen Consumption

Preliminary oxygen uptake measurements on phospholipids in organic solvents, in aqueous dispersions and in a dry state indicated that the rate of oxidation could be most readily determined under controlled pH in emulsified systems. An additional reason for measuring oxidation under these conditions was that any changes that occurred would be more typical of changes occurring in a number of natural food systems.

Figure 1 illustrates the influence of pH on the oxygen consumption of egg PE and PC. Oxygen consumption after 50 hr was greatest for PC at pH 7.0 and PE at pH 8.0. Mattsson and Swartling (4), working with butter phospholipids in phosphate buffers, reported that PE was reluctant to take up oxygen except at pH values in the alkaline range, while PC had a maximum probably below pH 2 and reacted fairly slowly around pH 7.0

Subsequent oxygen uptake determinations were conducted at pH 7.0 since it represented the maximum pH for the less reactive PC, and because it approximated the physiological pH of many products used for food. Consequently, studies on the oxidation of phospholipids could possibly provide information on the mode of autoxidation of foods during processing and storage.

Activation Energies of PE and PC

The rates of oxygen uptake at temperatures ranging from 15 to 50 C were utilized to determine activation energies for the autoxidation of PE and PC from egg and soybean. The oxidation rate for a phospholipid was measured at 4-5 temperatures and was obtained from the straight line portion of the uptake curve after the onset of initiation and other early autoxidation reactions. Thus the rates reflect the oxidizability of the unsaturated fatty acids of PE and PC. The activation energies presented in Table I were not grossly different from each other, which suggests that the mode of oxidation, at least during the period of steady state

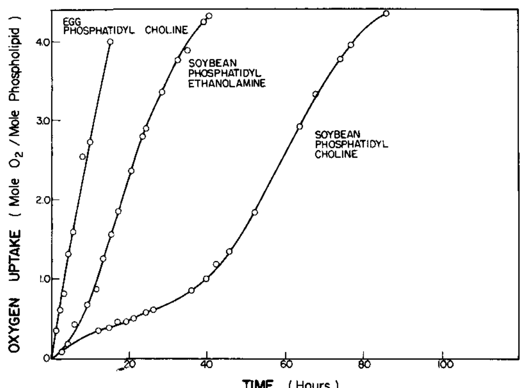

FIG. 3. The oxygen consumption of egg and soybean phospholipids emulsified in 0.1 M borate (pH 7.0), Tween 20 buffer at 40 C.

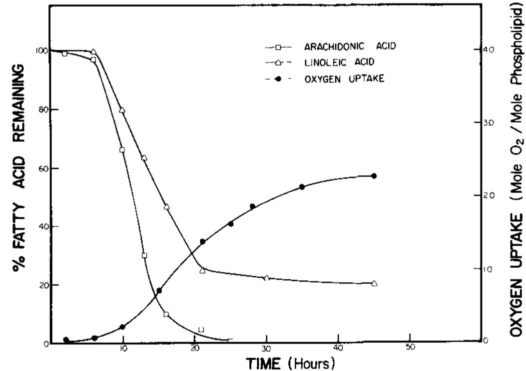

FIG. 4. Relationship between the disappearance of the two major polyunsaturated fatty acids of egg phosphatidyl choline (PC) emulsified in 0.1 M borate (pH 7.0) buffer and oxygen uptake at 40 C.

oxidation, was the same for the egg and soybean phospholipids utilized in this study. The values obtained were approximately one half of the values reported for the autoxidation of methyl linoleate and linoleic acid emulsions (17) indicating a greater reactivity for the phospholipids.

Effect of Fatty Acid Unsaturation on Phospholipid Oxidation

The oxidation of both PE and PC was temperature dependent, increasing with increasing temperatures. Egg PC reacted with oxygen to the extent of 4 moles O_2 per mole PC in approximately 45 hr at 25 C (Fig. 2) whereas at 40 C it required approximately 15 hr (Fig. 3). A mole of soybean PE reacted with 3 moles O_2 in 60 hr (Fig. 2) at 25 C while 4 moles O_2 were consumed in 40 hr at 40 C (Fig. 3). The PE from each source reacted much more rapidly with oxygen than the PC from the same source (Fig. 2,3). These observations are consistent with other reports. Lea (2) reported that thin films of egg PE oxidized rapidly at 37 C, while PC oxidized at a much slower rate. Roubal (1) observed that PE from the flesh lipids of codfish exhibited a high rate of oxidation while PC required the addition of hemoglobin to oxidize at a rapid rate.

Variations in experimental techniques among those studying the oxidation of phospholipids make it difficult to compare results with regard to the relative reactivity of phospholipids from different sources. When phospholipids from egg and soybean were compared by the same method of oxidation, egg phospholipids were found to be more reactive. Egg PE and PC oxidized more rapidly than did soybean PE (Fig. 2), and egg PC oxidized more rapidly than soybean PE and PC.

A partial explanation for the differences in oxidation of egg and soybean PE and PC can be traced to their fatty acid compositions. Lea (2) concluded that a relationship existed between the oxidation rate and the degree of unsaturation of individual phospholipids. Hawke (18) reported that egg PE contained a higher percentage of polyunsaturated fatty acids than egg PC. The fatty acid composition of the phospholipids used in this study is presented in Table I. The concentration of arachidonic acid is markedly higher in egg PE than egg PC while the amount of linolenic acid in soybean PE is nearly twice the amount present in soybean PC.

The greater reactivity of egg phospholipids compared to soybean phospholipids cannot be explained solely by the percentage of polyunsaturated fatty acids since the degree of unsaturation is higher for soybean phospholipids. Of perhaps greater significance is the presence of arachidonic acid in egg phospholipids. Holman and Elmer (19) reported that the rate of oxidation of methyl arachidonate is about twice that of ethyl linolenate. These workers concluded that the introduction of one additional double bond into a fatty acid at least doubled the oxidation rate. Thus, egg phospholipids containing arachidonic acid would be expected to be more susceptible to oxidation than soybean phospholipids which have linolenic acid as the most highly unsaturated component.

Measurement of Phospholipid Autoxidation by Physico-Chemical Tests

Since various products are produced by the autoxidation of phospholipids, an attempt was made to correlate oxygen absorption with specific changes in the phospholipid molecule as evaluated by various physico-chemical tests.

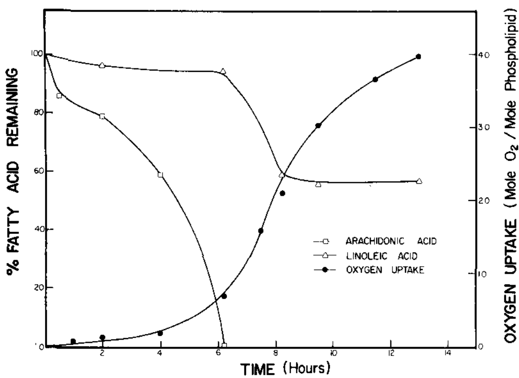

FIG. 5. Relationship between the disappearance of the two major polyunsaturated fatty acids of egg phosphatidyl ethanolamine (PE) emulsified in 0.1 M borate (pH 7.0) buffer and oxygen uptake at 25 C.

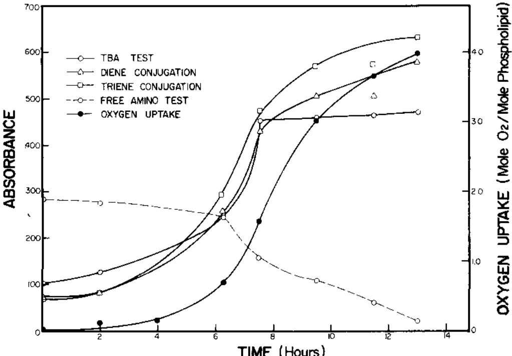

FIG. 7. Alterations of egg phosphatidyl ethanolamine (PE) emulsified in 0.1 M borate (pH 7.0) buffer during oxidation at 25 C as evaluated by various physico-chemical tests.

Figures 4 and 5 illustrate the susceptibility of arachidonic and linoleic acids in egg PE and PC to oxidative attack. Oxygen uptake and the percentage of each unsaturated fatty acid remaining at various stages of the period of oxidation are plotted as functions of time. The pattern was similar for both phospholipids. The arachidonic acid concentration was reduced at a more rapid rate and was no longer detected by the time the studied period of oxidation reached midpoint. The amount of linoleic acid decreased to a minimum level that remained relatively constant. During the 45 hr reaction period for PC and the 13 hr reaction period for PE, the concentration of the other unsaturated fatty acids, 16:1 and 18:1 and the saturated fatty acids, 16:0 and 18:0 remained fairly constant relative to each other. They increased in amount concurrent with the reduction in the percentage concentration of arachidonic and linoleic acids. Similar results have been reported for the autoxidation of cod PE (1) and human red cells (6).

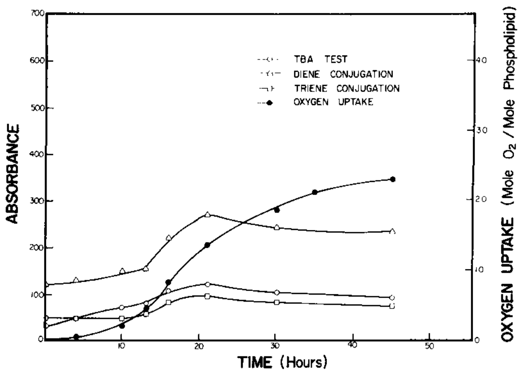

FIG. 6. Alterations of egg phosphatidyl choline (PC) emulsified in 0.1 M borate (pH 7.0) buffer during oxidation at 40 C as evaluated by various physico-chemical tests.

The arachidonic and linoleic acid curves in Figures 4 and 5 support and, in part, explain the differences in reactivity for PE and PC. Although the arachidonic acid concentration is much higher in PE than in PC (Table I), the time required for the total disappearance of arachidonic acid was approximately 6 hr at 25 C for PE and about 25 hr at 40 C for PC. The arachidonic acid concentration was reduced so rapidly and selectively in PE that none was detected after 6 hr, while almost all of the linoleic acid was still present. However, the reduction of linoleic acid followed more closely the decrease of arachidonic acid in PC and reached its minimum for the period of oxidation at about the same time that the percentage of arachidonic acid dropped to zero. The difference in the decrease of linoleic acid in PC and PE could have resulted because of a higher rate of reaction at the higher temperature. Also the longer time and higher temperature (40 C) that were required to completely eliminate arachidonic acid from the PC reaction mixture may have allowed products of the oxidation of arachidonic acid more opportunity to catalyze the oxidation of linoleic acid.

Considerable oxygen was absorbed, especially by PE but also by PC, after arachidonic acid was no longer detectable in the system and linoleic acid had reached its minimum level. This probably is related to the manner in which lipids oxidize. The primary products of the reaction of oxygen with unsaturated lipids are hydroperoxides, while alcohols, aldehydes and ketones are secondary products formed by degradation of primary hydroperoxi-

TABLE II

Fatty Acid Composition of Three Egg PC Fractions Separated on a Silicic Acid Column

Fatty acid	First fraction, %	Second fraction, %	Third fraction, %
C_{14}	Trace	Trace	Trace
C_{16}	26.5	34.6	34.0
$C_{16:1}$	1.4	1.9	3.3
C_{18}	28.8	16.5	11.3
$C_{18:1}$	18.9	28.4	37.7
$C_{18:2}$	12.0	14.4	12.4
$C_{18:3}$	Trace	Trace	Trace
$C_{20:4}$	12.4	4.2	1.3

des. The latter compounds, particularly if they are unsaturated, are highly susceptible to further oxidation (20). Lillard and Day (21) have reported that certain unsaturated aldehydes oxidize at a much faster rate than either methyl linoleate or linolenate. The degradation compounds from the autoxidation of PE and PC would be expected to be highly reactive and as a consequence capable of increasing the total amount of oxygen consumed by the reaction mixture.

The relationship between oxygen uptake and the development of alterations in egg PC, egg PE and soybean PE as measured by the TBA test, UV absorbance of diene and triene conjugation, and changes in the amount of the free amino group of ethanolamine are summarized in Figures 6-8. The TBA test and development of diene and triene conjugation follow a similar pattern in egg PC (Fig. 6). These attained maximum levels when the rate of oxygen consumption began to decrease. This resembled the observation of Tarladgis and Watts (22) who measured malonaldehyde production with the TBA test during the oxidation of unsaturated fatty acids. They noted that malonaldehyde production followed oxygen uptake, reaching a peak at the same time that oxygen uptake started to decline and they concluded that the malonaldehyde precursor was not accumulating as a stable end-product since, after reaching the peak, more malonaldehyde precursor was destroyed than produced. The relationship between oxygen uptake and the extent of the UV absorption of autoxidized methyl linoleate has been shown by Privett and Blank (9). At the end of the induction period, the UV absorption of methyl linoleate increased sharply with the formation of stable (conjugated diene) hydroperoxides (9).

Higher TBA test and diene and triene conjugation values for egg PE (Fig. 7) reflect the more vigorous oxidation of PE as compared to

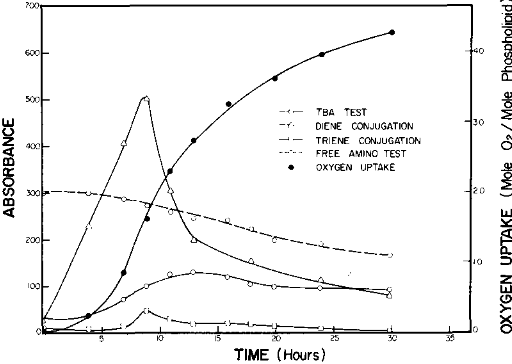

FIG. 8. Alterations of soybean phosphatidyl ethanolamine (PE) emulsified in 0.1 M borate (pH 7.0) buffer during oxidation at 40 C as evaluated by various physico-chemical tests.

PC. These values increased with oxygen uptake but then continued to increase even after the rate of oxygen consumption had decreased. Triene conjugation was slightly higher than diene conjugation for egg PE. The greater amount of arachidonic acid in PE as compared to PC (Table I) probably accounted for the higher triene conjugation in the PE. Diene conjugation increased rapidly in oxidizing soybean PE (Fig. 8) during the early phase of oxidation as measured by oxygen uptake but only a slight amount of triene conjugation was observed. TBA absorbance reached a maximum soon after diene and triene conjugation values were at their highest levels.

The free amino test was performed on PE to determine if changes in its concentration might be related to the reactivity of PE. [The concentration of ethanolamine can be obtained from a standard curve once the absorbance between trinitrobenzene sulfonic acid and PE has been determined (16).] Lea (7) reported that the free amino group of PE disappeared during oxidation approximately proportional to the amount of oxygen absorbed. The free amino absorbance value of egg PE was significantly reduced during the period of oxidation (Fig. 7). The disappearance of the free amino group of soybean PE (Fig. 8) was not as pronounced as that for egg PE, even though both phospholipids consumed nearly equivalent amounts of oxygen. During oxidation, a brownish yellow color developed in egg and soybean PE emulsions. The development of the discoloration and the decrease in the concentration of the free amino group are evidence for the occurrence of Maillard-type browning reactions. Malonaldehyde and other 2-thiobarbituric acid-reactive substances, produced during the oxidation of polyunsaturated fatty

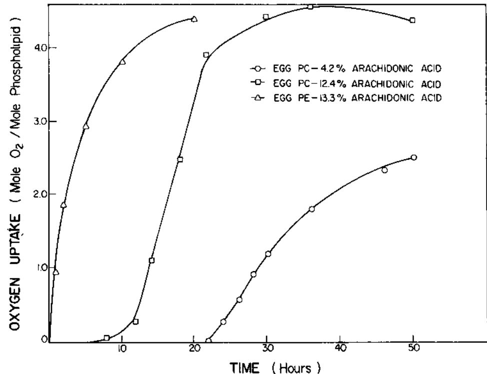

FIG. 9. The effect of arachidonic acid composition on the oxygen consumption of egg phospholipids emulsified in 0.1 M borate (pH 7.0), Tween 20 buffer at 40 C.

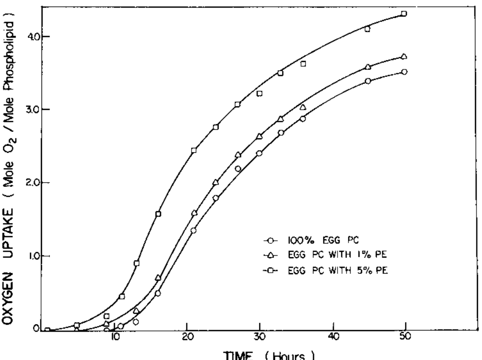

FIG. 10. The effect of phosphatidyl ethanolamine (PE) contamination on the oxygen consumption of egg phosphatidyl choline (PC). PC was emulsified in 0.1 M borate (pH 7.0), Tween 20 buffer and oxidized at 40 C.

acids, can react with amino acids and proteins (3). The decrease in the free amino group of PE probably resulted from its interaction with specific autoxidation products of unsaturated fatty acids such as carbonyl compounds (2). Hydroperoxides, epoxides and aldehydes have also been mentioned as substances which could contribute to the disappearance of the free amino group of PE (7).

Effect of the Free Amino Group on Phospholipid Oxidation

Silicic acid column chromatography (24,25) and thin layer chromatography (26) have been used to obtain natural phosphatidyl choline with varying degrees of unsaturation. The faster-moving PC subfractions were found to have higher proportions of stearic and arachidonic acids than slower moving fractions. The fatty acid composition of PC fractions that were initially recovered from the column or that moved the farthest on TLC resembled the fatty acid composition of PE (24,26). When egg PC was chromatographed with chloroform-methanol (25-35% methanol by volume) on a silicic acid column, the first fractions contained a higher degree of unsaturated fatty acids, particularly arachidonic acid, than did later fractions (Table II). In fact, the percentage of arachidonic acid in egg PC first recovered from the column was almost identical with the percentage of arachidonic acid in egg PE (Table I).

The oxygen uptake of the PC containing the higher arachidonic acid content was compared with PC from succeeding fractions and with egg PE (Fig. 9). This PC fraction with higher arachidonic acid was more reactive than pure unfractionated PC which has approximately 4% of its fatty acid composition as arachidonic acid; however, it was less reactive than PE. The oxygen uptake pattern of the third PC fraction is not shown in Figure 9 because its induction period was more than double the time scale used for the other phospholipid fractions. The third PC fraction containing 1.3% arachidonic acid did not begin to absorb any measurable amount of oxygen at 40 C until 116 hr had elapsed.

The greater reactivity of PE as compared to the PC containing the high arachidonic acid content was characterized by a shorter induction period. The maximum rates of oxidation for the high arachidonic acid PC and PE were found to be almost equal. The similarity in rates for the high arachidonic acid PC and PE suggest that the straight line portion of the oxygen uptake curve (representing the maximum rate of oxidation) for a particular phospholipid is regulated primarily by the composition of unsaturated fatty acids. Since the arachidonic acid concentration of the first egg PC fraction was almost identical with the arachidonic acid concentration of egg PE, an additional factor, such as the influence of the nitrogen moiety, could explain the different induction periods for PE and the PC with the high arachidonic acid level. To test this hypothesis, egg PC was purposely contaminated with 1% and 5% PE (Fig. 10). The rate of oxidation of PC was not altered appreciably by the addition of a small amount of PE, which was not unexpected in view of the observation that the straight line portion of the oxygen uptake curve is regulated primarily by the unsaturated fatty acid composition. However, there was a marked shortening of the induction period with the greatest reduction occurring for the PC with 5% added PE.

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SHORT COMMUNICATIONS

In Vitro Studies on Cholesterol Metabolism in the Blood Fluke *Schistosoma mansoni*

ABSTRACT

Adult *Schistosoma mansoni* were axenically maintained in NCTC 109 culture media containing ^{14}C labeled acetate or mevalonate. Dibromide derivatives prepared from purified blood fluke cholesterol exhibited a lack of significant radioactivity on examination by liquid scintillation spectrometry. Autoradiographic electron microscopy of tissues from flukes maintained in the presence and absence of labeled acetate and especially fixed to retain cholesterol revealed a similar random distribution of nonspecific grains. However, when particulate labeled cholesterol was supplied, a count of 6,832 dpm was found in the dibromide derivative. Under the above maintenance conditions *S. mansoni* adults do not synthesize cholesterol from acetate or mevalonate but most probably obtain this sterol from the host animal.

INTRODUCTION

The ability to synthesize sterols from short chain carbon compounds is not ubiquitously distributed among the animal phyla. Investigations of sterol synthetic ability among the crustaceans revealed that neither of the two species investigated could synthesize cholesterol (1,2).

Fagerlund and Idler (3) reported that two species of pelecypod molluscs, the clam (*Saxidomus giganteus*) and the mussel (*Mytilus californianus*), could incorporate acetate into digitonin precipitable material. The gastropod, *Helix pomatia*, apparently has been shown to synthesize cholesterol from acetate (4). However, the oyster (*Ostrea grypha*) did not incorporate 2-^{14}C-mevalonate or radioactivity from methyl-^{14}C-L-methionine into squalene or sterols (5). The sea urchin (*Parecentrotus lividus*) could not incorporate acetate into squalene or sterols (5).

Among the platyhelminth parasites investigated, neither *Spirometra mansonoides* (6) nor *Hymenolepis diminuta* (7,8) can synthesize cholesterol. Rothstein (8) reported that free living nematodes could not synthesize cholesterol from acetate or mevalonate.

This paper reports the lack of significant incorporation of either mevalonate or acetate into cholesterol by axenically maintained adult *Schistosoma mansoni*.

TABLE I

Radioactivity in Cholesterol Dibromide From Free Sterol of Adult *Schistosoma mansoni* Maintained in NCTC 109 Containing 1-^{14}C-Sodium Acetate

No. of flukes	Wt. of free sterol, mg	^{14}C as labeled acetate μc/ml	Cholesterol dibromide, dpm
164 pairs 273 males	0.2	0.05	20
198 pairs 195 males	0.3	0.05	0
138 pairs 228 males	0.2	0.05	21
233 pairs 16 males	1.6	0.15	30
168 pairs 68 males	0.9	0.15	25

TABLE II

Radioactivity in Cholesterol Dibromide From Free Sterol of Adult *Schistosoma mansoni* Maintained in NCTC 109 Containing 2-^{14}C-DL-Mevalonic Acid

No. of flukes	Wt of free sterol, mg	^{14}C as labeled acetate μc/ml	Cholesterol dibromide, dpm
200 pairs 42 males	0.2	0.05	20
103 pairs 186 males	1.0	0.05	142
260 pairs 50 males	1.9	0.05	12

MATERIALS AND METHODS

Adult flukes obtained by dissection from mesenteric venules of experimentally infected mice were washed by repeated suspension in sterile physiological saline containing antibiotics (penicillin, 500 units/ml, streptomycin sulfate, 100 μg/ml). Schistosomes were then maintained for four days in National Cancer Institute Tissue Culture Medium 109 (NCTC 109, Difco Laboratories, Detroit) (9) containing 0.05 μc/ml (2 mg/liter) or 0.15 μc/ml (6 mg/liter) of 1-^{14}C-sodium acetate or 0.05 μc/ml (2 mg/liter) of 2-^{14}C-DL-mevalonic acid-N,N' dibenzylethylenediamine salt) or 0.09 μc/ml (3.5 mg/liter) of permanently suspended, particulate 4-^{14}C-cholesterol (10). Acetate, mevalonate and cholesterol were obtained from New England Nuclear Corp., Boston.

Cultures were examined and media were changed daily. Tubes containing dead worms or showing contamination with bacteria or higher fungi were discarded.

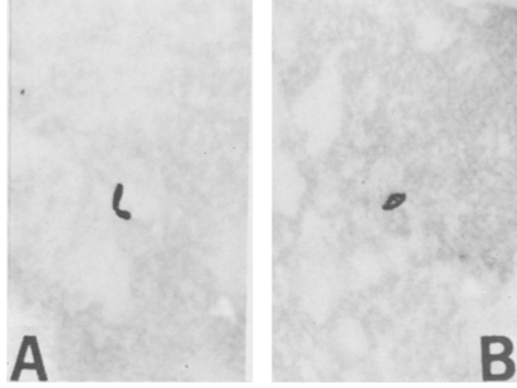

FIG. 1. Autoradiographic electron micrographs of a control schistosome (A) and one maintained in NCTC 109 containing 0.05 μc/ml of 1-^{14}C-sodium acetate (B). The cytoplasm of both shows random nonspecific grains. [(A) 15,960 X; (B) 20,520 X.]

Following removal from maintenance media containing either 2-^{14}C-mevalonate or 1-^{14}C-acetate flukes were washed in sterile saline. Flukes removed from maintenance media containing particulate cholesterol were washed in sterile saline and maintained for an additional day in isotope-free NCTC 109 to allow expulsion of particulate radioactive cholesterol from the caeca. Flukes were then washed by repeated suspension in 10, 10 ml changes of sterile saline. The final saline wash was evaporated to dryness and the residue extracted with chloroform-methanol (2:1 v/v) (11). The chloroform extract was transferred to a liquid scintillation vial, solvents evaporated, and a toluene scintillation system (12) added. Examination by liquid scintillation spectrometry revealed an absence of radioactivity in the final wash. After tissue lipid extraction (11), fluke free sterols were separated from other lipid components by ascending thin layer chromatography (TLC) on 20 x 20 cm glass plates coated with a 250 μ Silica Gel G adsorbent layer using two hexane-diethylether-acetic acid solvent systems (90:10:1 and 30:70:1 v/v/v) (13). Adequacy of separation at this point was monitored by IR spectrophotometry using potassium bromide micro-pellets containing fluke free sterol. Authentic cholesterol (Fisher Scientific Co., Atlanta) which had been subjected to repeated TLC was used as a standard.

Cholesterol dibromide was prepared from fluke free sterol and separated from trace contaminants by TLC in benzene-ethanol (95:2 v/v) (14,15) using the dibromide prepared from the above cholesterol as a standard.

Silica gel bands containing fluke cholesterol dibromide were scraped into scintillation vials and the toluene solvent scintillation system containing 4% Cabosil (Packard Instrument Co., Downers Grove, Illinois) was added (12). Each vial was shaken to suspend the silica gel and coat the vial wall. Radioactivity was determined

by liquid scintillation spectrometry. Counting efficiencies of 74-76% were obtained and counts expressed as disintegrations per min (dpm).

Prior to autoradiographic electronmicroscopy, tissue from flukes maintained in NCTC 109 with and without radioactive acetate were fixed in 10% formalin, soaked in 0.5% digitonin in 50% aqueous ethanol for 1 hr, fixed in Veronal acetate-buffered osmium tetroxide (pH 7.4) (16) containing 0.25 molar sucrose and embedded in Epon 812 (17). Ultrathin sections (90-100 mμ), cut with diamond knives, were mounted on parlodion coated stainless steel grids, and the grids were then dipped into Gevaert emulsion. At one week intervals, grids were developed and examined in a model 300 electron microscope (Philips Instrument Co., Mt. Vernon, N.Y.).

RESULTS AND DISCUSSION

Previous color tests, thin layer and gas liquid chromatographic studies revealed that adult *S. mansoni* free sterols were composed of at least 90% cholesterol (18). In the present study, strip chart recordings of IR spectra of fluke free sterol and authentic cholesterol were identical in all respects.

The insignificant amount of radioactivity in fluke cholesterol dibromide samples shown in Tables I and II and the equal distribution of random nonspecific grains seen in autoradiographs (Fig. 1) of cells from control flukes and those maintained in media containing 1-^{14}C-acetate clearly show that under the maintenance conditions detailed above adult *Schistosoma mansoni* are incapable of incorporating significant quantities of either acetate or mevalonate into body cholesterol.

When cholesterol dibromide obtained by derivatization of 1.5 mg of free sterol from 213 pair + 17 male flukes maintained in media containing particulate 4-^{14}C-cholesterol was examined by liquid scintillation spectrometry, a count of 6,832 dpm was obtained at a counting efficiency of 76%. This represented 0.09% of the total radioactivity supplied in the media. Incorporation of exogenous cholesterol into the fluke body is further evidence that adult *Schistosoma mansoni* obtain this sterol from host animal blood.

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Coenzymes Q in Lemon Shark Liver

ABSTRACT

Earlier studies have shown that some extracts of nonpolar lipids from lemon shark liver are able to stimulate the reticuloendothelial system (RES) of experimental animals. This communication reports the identification of the coenzymes Q as the constituents of a fraction from lemon shark liver lipid which showed intense biological activity.

by liquid scintillation spectrometry. Counting efficiencies of 74-76% were obtained and counts expressed as disintegrations per min (dpm).

Prior to autoradiographic electronmicroscopy, tissue from flukes maintained in NCTC 109 with and without radioactive acetate were fixed in 10% formalin, soaked in 0.5% digitonin in 50% aqueous ethanol for 1 hr, fixed in Veronal acetate-buffered osmium tetroxide (pH 7.4) (16) containing 0.25 molar sucrose and embedded in Epon 812 (17). Ultrathin sections (90-100 mμ), cut with diamond knives, were mounted on parlodion coated stainless steel grids, and the grids were then dipped into Gevaert emulsion. At one week intervals, grids were developed and examined in a model 300 electron microscope (Philips Instrument Co., Mt. Vernon, N.Y.).

RESULTS AND DISCUSSION

Previous color tests, thin layer and gas liquid chromatographic studies revealed that adult *S. mansoni* free sterols were composed of at least 90% cholesterol (18). In the present study, strip chart recordings of IR spectra of fluke free sterol and authentic cholesterol were identical in all respects.

The insignificant amount of radioactivity in fluke cholesterol dibromide samples shown in Tables I and II and the equal distribution of random nonspecific grains seen in autoradiographs (Fig. 1) of cells from control flukes and those maintained in media containing 1-^{14}C-acetate clearly show that under the maintenance conditions detailed above adult *Schistosoma mansoni* are incapable of incorporating significant quantities of either acetate or mevalonate into body cholesterol.

When cholesterol dibromide obtained by derivatization of 1.5 mg of free sterol from 213 pair + 17 male flukes maintained in media containing particulate 4-^{14}C-cholesterol was examined by liquid scintillation spectrometry, a count of 6,832 dpm was obtained at a counting efficiency of 76%. This represented 0.09% of the total radioactivity supplied in the media. Incorporation of exogenous cholesterol into the fluke body is further evidence that adult *Schistosoma mansoni* obtain this sterol from host animal blood.

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ABSTRACT

Earlier studies have shown that some extracts of nonpolar lipids from lemon shark liver are able to stimulate the reticuloendothelial system (RES) of experimental animals. This communication reports the identification of the coenzymes Q as the constituents of a fraction from lemon shark liver lipid which showed intense biological activity.

Previous communications from this Institute have presented evidence of the biological activity (reticuloendothelial system stimulation) of some extracts of lemon shark (*Negaprion brevirostris*) liver lipids (1). These extracts were obtained by different extractions and purification methods, but the biological activity resided always in the nonpolar fraction of the neutral lipids. In some species of shark, the oil content of the liver constitutes about 90% of the weight of the liver (2). The main constituents of these oils are: hydrocarbons (such as squalene), triglycerides, alkoxyglyceryl diesters, higher alcohols, fatty acids, vitamin A and its esters, and other lipids in trace amounts (2,3). The class composition of neutral lipids from lemon shark liver was studied by dry-column chromatography and found to contain almost 97% triglycerides and 2.3% of unsaponifiable material (4). In this fractionation technique the biological activity was associated with the hydrocarbon fraction which contained unidentified materials that traveled along the column with the hydrocarbon components of the extract, possibly as molecular complexes. These unidentified materials have since been found to be cholesterol, carotenes and coenzymes Q. It has been observed that when relatively large amounts of sterols and carotenes are present in the unsaponifiable fraction of tissue extracts, the coenzymes Q tend to accompany these materials during crystallization (14). Moreover, in the presence of sterols and carotenes, the coenzymes Q tend to be eluted, in column chromatography with the less polar fractions (15). Since the active principle was presumably present in very small amounts and since we had evidence of its thermal and light lability (5), it became necessary to develop a very mild large-scale fractionation that would allow separation in high concentration prior to identification.

This paper reports the isolation and identification of the coenzymes Q as principal components of a highly active fraction obtained through a large-scale extraction method. The biological activity of this shark liver extract and of pure commercially available coenzymes Q, namely their ability to stimulate the phagocytic rate of rats and the primary hemolytic antibody response in mice, has been reported by us elsewhere (6,7).

Neutral lipids from lemon shark liver were obtained as reported earlier (4). This crude hexane extract was further fractionated by solvent partition in a mixture of previously equilibrated 2.5 liter of hexane and 2.5 liter of methanol per 100 g of crude lipid, at -50 C for 3 hr with frequent shaking. (All solvents were purified and distilled prior to use.) The layers were separated and re-extracted as follows: the hexane layer was extracted with 2.5 liter of methanol for 1 hr at -50 C; separation of the layers and evaporation of the solvents at 30 C in a rotary evaporator gave two residues, H-1 (97 g) from the hexane layer and M-2 (0.45 g) from the methanol layer, with almost no biological activity. The primary methanol solution was re-extracted with 2.5 liter of hexane at -50 C for 1 hr. After separation of the layers, evaporation of each solvent in a rotary evaporator at 30 C gave two residues, M-1 from the methanol layer and H-2 from the hexane layer. The secondary hexane extract, H-2 (0.6 g) showed intense biological activity.

Preparative thin layer chromatography (TLC) (Silica Gel G, 1 mm thick, petroleum ether-ethyl ether-acetic acid, 60:40:2, used as development solvent) of fraction H-2 gave several distinctive bands, all of which were eluted, isolated and bioassayed. The dark orange semisolid, obtained from the fifth band from the origin, with R_f 0.7 had good biological activity. This material was recrystallized several times from a minimum of hot ethanol to give orange crystals, mp 37-40 C. These crystals showed strong biological activity.

The identity of the constituents of the red crystals was established on the basis of the following criteria: (a) The UV spectrum of the mixture in absolute ethanol (λ_{max} 275 mμ which disappears on reduction with $NaBH_4$) was in good agreement with that reported for the coenzymes Q (8). (b) The IR spectrum (bands at 1650, 1600, 1260 cm^{-1})had all the typical bands of the coenzymes Q (8). (c) The mass spectrum showed a prominent peak at m/e 235, which is the dominant feature on the mass spectra of the coenzymes Q homologous series (9). (d) On vaseline impregnated paper, developed with dimethyl-formamide-water, 97:3, a method reported by Linn et al. (10) for separation of the individual members of the coenzyme Q homologous series, the components of the red crystals migrated with R_f values identical to those of coenzyme Q_9 and coenzyme Q_{10} used as standards, with no other material present. It was estimated spectrophotometrically, by using the characteristic differential in UV absorption for the quinone and hydroquinone forms of the two coenzymes (8), that coenzyme Q_9 constituted about 85% of the mixture.

The presence of coenzymes Q in the liver of a few shark species has been reported. Nazir and Magar (11) found coenzyme Q_6 in the liver of the shark *Carcharias ellioti Day*. Gale et al. (13) reported the identification of coenzymes

Q_9 and Q_{10} in tiger shark (*Galeocerdo cuvieri*) liver, and Diplock and Haslewood (12) found coenzyme Q_{10} in dogfish (*Squalus acanthias*) liver and discussed the possibilities of its being of dietary origin. If the coenzymes Q found in the liver of these animals is mainly exogenous, this would be consistent with the variety of homologues observed in the different classes of shark.

Clearly the presence of coenzymes Q_9 and Q_{10} in lemon shark liver is not unique. However, the study reported here, and the biological activity of this shark liver fraction and pure coenzyme Q reported elsewhere offers an explanation for the earlier observations of biological activity produced by nonpolar lipid fractions of shark liver.

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Uptake and Release of Fatty Acids by Rat Adipose Tissue: Last in – First Out?

ABSTRACT

Fatty acid labeled chyle was administered iv to ad lib fed rats. At intervals from 1 hr to 50 days later rats were killed and pieces of their parametrial adipose tissue were incubated in vitro with norepinephrine. The specific radioactivity of the mobilized free fatty acids was compared to that of tissue glycerides and that of tissue free fatty acids. The results indicate that fatty acids taken up by the adipose tissue do not mix immediately with the bulk of tissue lipids and that the mobilized free fatty acids do not pass through the tissue free fatty acid pool.

Several investigators have shown that there are at least two glyceride compartments in adipose tissue (1-3) one of which has a rapid turnover. In vitro experiments indicate that fatty acids which enter the tissue are first incorporated into the pool of glycerides that has a rapid turnover (1) and can be released preferentially compared to the bulk lipid of the cell (1). In the present paper we show that this is also true for fatty acids which are taken up by the adipose tissue in vivo from chylomicra.

To prepare labeled chyle, the thoracic duct of a male rat was cannulated and the rat then given via a stomach fistula a micellar solution of 9-10-^{3}H-oleic acid (The radiochemical Centre, Amersham, England), monoolein and tauro-

Q_9 and Q_{10} in tiger shark (*Galeocerdo cuvieri*) liver, and Diplock and Haslewood (12) found coenzyme Q_{10} in dogfish (*Squalus acanthias*) liver and discussed the possibilities of its being of dietary origin. If the coenzymes Q found in the liver of these animals is mainly exogenous, this would be consistent with the variety of homologues observed in the different classes of shark.

Clearly the presence of coenzymes Q_9 and Q_{10} in lemon shark liver is not unique. However, the study reported here, and the biological activity of this shark liver fraction and pure coenzyme Q reported elsewhere offers an explanation for the earlier observations of biological activity produced by nonpolar lipid fractions of shark liver.

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TABLE I

Ratio of Specific Radioactivity in Released Free Fatty Acids and in Tissue Free Fatty Acids to That in Tissue Glycerides[a]

	Norepinephrine stimulation		No stimulation
Time after injection	Mobilized FFA (tissue glycerides)	Tissue FFA (tissue glycerides)	Tissue FFA (tissue glycerides)
1 hour	3.76 ± 1.50	1.76 ± 0.82	1.08 ± 0.28
1 day	0.84 ± 0.09	0.65 ± 0.06	0.59 ± 0.24
4 days	0.77 ± 0.25	0.47 ± 0.06	0.26 ± 0.07
50 days	0.76 ± 0.18	0.54 ± 0.13	0.44 ± 0.21

[a]Pieces (usually 0.7 - 1.0 g) of parametrial adipose tissue were obtained from rats at various times after injection of labeled chyle and were incubated in vitro with or without norepinephrine (see text for details). Each value for norepinephrine-stimulated and control fat pad pieces is the mean ± SD for 8-10 and 4-5 observations respectively. To obtain the specific radioactivity for glycerides, 849 g was considered equivalent to 3 moles of fatty acid.

cholic acid (4). Labeled chyle was collected for 12 hr and was used for injections within the following 12 hr.

Ad lib. fed female Sprague Dawley rats (Anticimex, Stockholm, Sweden) weighing 150-200 g were used. They were anesthetized with ether and the labeled chyle was injected ($84 \cdot 10^6$ cpm per rat) into an exposed neck vein. Groups of animals were killed at intervals varying from 1 hr to 50 days after the injection. Their parametrial adipose tissue was removed, extensively washed, divided into three pieces and incubated at 37 C in a total volume of 5 ml Krebs-Ringer bicarbonate buffer (5), pH 7.35, containing 5% bovine serum albumin (fatty acid poor, Pierce Chemical Company, Rockford, Ill.) Two of the fat pad pieces were incubated with 1 μg norepinephrine per milliliter to stimulate mobilization of fatty acids, whereas one fat pad piece served as control and was incubated without norepinephrine. After 2 hr the fat pad pieces were removed from the medium, washed and then homogenized in 15 ml chloroform-methanol (2:1 v/v). The homogenates were filtered and 6 ml 2% KH_2PO_4 in water added. The lower, chloroform phases were dried over anhydrous Na_2SO_4. Phospholipids were removed from glycerides and fatty acids by adsorption on a column of 0.5 g silicic acid that was eluted with 20 ml chloroform. The eluate was evaporated and the residue dissolved in 8 ml petroleum ether. To separate free fatty acids from glycerides the method of Borgstrom (6) was used. The petroleum ether solution of the lipids was extracted twice with 8 ml 0.1 N KOH in 50% aqueous ethanol and was then transferred to a counting vial. This fraction contains the glycerides, which were quantified by weighing. The combined ethanol-water fractions containing the free fatty acids were washed three times with petroleum ether to remove remaining glycerides and were then acidified and extracted twice with petroleum ether to obtain the free fatty acids. Their amount was determined by titration according to Dole and Meinertz (7). To recover the fatty acids for determination of their radioactivity, the titration medium was acidified and extracted twice with 3 ml of heptane which was then transferred to a counting vial. Model experiments showed that the glyceride contamination of the fatty acid fraction was less than 0.001% of the original amount of glycerides in the tissue extract.

The incubation medium was extracted according to Dole and Meinertz (7) and the amount of free fatty acids determined by titration. The alkaline ethanol-water phase containing the fatty acids was then extracted twice with heptane to remove glycerides. It was then acidified and again extracted twice with heptane to obtain the free fatty acids for determination of their radioactivity. Media incubated without a fat pad piece were extracted in the same manner. They contained less than 1.7 μeq free fatty acid. This value was used as a blank and was subtracted from the values obtained after incubation of fat pad pieces.

Radioactivity was determined in a Packard liquid scintillation spectrometer. Quenching was corrected for by the use of an internal standard.

Part of the radioactivity in the fractions might derive from lipids of trapped blood. To estimate the extent of such contamination the volume and the radioactivity of the blood was determined. The volume was determined by the injection of ^{131}I-albumin. The amount of blood in each fat pad piece never exceeded 0.02 ml. The amount of radioactivity in free fatty acids per milliliter of blood was less than 0.02% of the injected dose at 1 hr after the injection

and still less at later times. Therefore, a maximum of 4% of the radioactivity found in free fatty acids in the medium after incubation of a fat pad piece could derive from blood free fatty acids.

The fat pad pieces incubated with and without norepinephrine, respectively, released means of 4.6 and 1.0 μeq fatty acid per gram of wet weight. With fat pad pieces from the rats killed 1 hr after the injection of the labeled chyle, the specific radioactivity in the released fatty acids was higher than that in tissue glycerides. With fat pad pieces obtained from rats killed one day or more after the injection of the labeled chyle, the specific radioactivity of the released free fatty acids was lower than that of the tissue glycerides. This suggests that the fatty acids taken up by the tissue did not immediately mix fully with the bulk of the tissue lipids. Thus, at 1 hr the radioactive fatty acids were preferentially released upon stimulation with norepinephrine, whereas at one day or more nonradioactive fatty acids were mobilized somewhat in preference to the bulk of tissue lipids. Presumably, these nonradioactive fatty acids had been recently taken up by the tissue or recently synthetized in the tissue.

If the source of the mobilized free fatty acids were the tissue free fatty acids, the specific radioactivity of the latter should be as high as or higher than that of the former. In our experiment, the specific radioactivity of the tissue free fatty acids, with and without stimulation, was less than that of the mobilized fatty acids. This suggests that the major part of the mobilized fatty acids did not pass the tissue free fatty acid pool. This conclusion is in concord with that of Dole (8) from experiments with epididymal adipose tissue which was induced to take up and to mobilize free fatty acids in vitro.

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Mass Spectrometry of Triglycerides: I. Structural Effects[1]

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ABSTRACT

Mass spectra of several triglycerides of specific structure or with specific deuterium labeling have been measured with a low resolution mass spectrometer. With saturated triglycerides the abundances of ions characteristic of the component acids, $[M-RCO_2]^+$, increase with increasing chain length, and $[M-RCO_2CH_2]^+$ decrease with increasing chain length. Unsaturation in the acyl moiety causes the abundant formation of $[RCO-1]^+$. Structures have been suggested for a number of the main peaks obtained from saturated triglycerides, and high resolution spectra of one triglyceride agree with the postulated structures. The peaks, $[RCO+74]^+$, $[RCO+115]^+$ and $[RCO+128+14n]^+$, represent structures which contain the glyceryl portion of the triglyceride, since in case of the replacement of its hydrogens with deuteriums, these peaks are shifted accordingly. Evidence which indicates the possibility of determining the location of unsaturation by the interruption of homology of the $[RCO+128+14n]^+$ series, brought about by the addition of deuterium to the unsaturated linkages, is introduced. Further evidence is also presented, which indicates that the $[M-RCO_2CH_2]^+$ ions arise from the positions 1 and 3 and, in agreement with earlier studies from other laboratories, it is thus possible to identify the acyl groups attached to the 1 and 2 positions of the glyceryl moiety.

INTRODUCTION

Ryhage and Stenhagen (1) and Barber et al. (2) have shown that the spectra of triglycerides are characterized by major peaks, which are readily interpreted. One of these major peaks results from the loss of an acyloxy group from the parent molecular ion, and in the case of mixed triglycerides, peaks corresponding to the loss of each acyloxy group are obtained. An acyl ion results from each fatty acid residue present in the mixed triglyceride. Peaks with mass numbers 74 and 128 higher than each acyl ion are also prominent, but structures have not been previously postulated for them.

The present study revealed an additional type of ion $[RCO+115]^+$ and a homologous series of ions $[RCO+128+14n]^+$ of which $[RCO+128]^+$ is the simplest member. This series is of special interest because it presents the possibility to determine the location of unsaturation in triglycerides, for its homology is interrupted by unsaturation or substituents in the acyl chains.

The studies of Dinh-Nguyen et al. (3,4), dealing with isotopically substituted normal long chain methyl esters demonstrated that different mechanisms exist for the formation of the methoxycarbonyl ions, $[CH_3-CO-O-(CH_2)_n]^+$ where $n > 1$. One perhaps unexpected source of this type of ion involves the extrusion of part of the hydrocarbon chain and therefore militates against the possibility of locating the position in the chain at which the homology is interrupted. Hydrogen-deuterium exchange reactions also complicate the problem. Dinh-Nguyen has delineated the behavior of the methyl ester of octadecanoic acid by utilizing isotopically-substituted preparations (deuterium and ^{13}C) and has discussed the applicability of this method for locating double bonds in long chain methyl esters. Our results with triglycerides appear to parallel the behavior of the long chain methyl esters as established by these studies.

EXPERIMENTAL PROCEDURES

The mass spectra were measured using two Hitachi RMU 6D instruments operated at 70 eV. Samples of triglycerides of short chain acids were introduced through the liquid sample insertion system and less volatile triglycerides were directly inserted. The results with the two instruments were consistent. High resolution mass spectra were measured on AEI MS-9 double focussing mass spectrometer.

Glyceryl triacetate, tripropionate, tributanoate, tripentanoate, trihexanoate and trioctanoate were obtained from Eastman Organic Chemicals. Glyceryl tridecanoate, tridodecanoate, tritetradecanoate, trihexadecanoate, tri-

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[1]Presented in part at the AOCS Meeting, New Orleans, April 1970. Part V of a series on Mass Spectrometry of Lipids. For IV see Lipids 4:421-427 (1969).

octadecanoate, trihexadec-9-enoate, trioctadec-6-enoate, trioctadec-9-enoate and trioctadeca-9,12-dienoate were obtained from The Hormel Institute, Lipids Preparation Laboratory. These triglycerides were found to be better than 95% pure by thin layer chromatography (TLC). The saturated diacid triglycerides used in this study were obtained from E.S. Lutton of the Procter and Gamble Co., and from R.E. Jensen of the University of Connecticut. The preparations of perdeuterio-glyceryl trioctadecanoate and 2-deuterio-glyceryl trioctadecanoate are described in a subsequent paper (5).

Methyl-6,7-dideuterio-octadecanoate was prepared according to the method of Rohwedder et al. (6). One milliliter of hydrazine-d_4 solution prepared from 5.6 g hydrazine hydrate-d_6 and 1.6 g of deuterium oxide was added every 12 hr to a solution of 313 mg methyl octadec-6-enoate in 20 ml dry dioxane. Dry oxygen was bubbled continuously through the mixture, which was maintained at 60 C. After 64 hr, the solvent was removed, water was added to the residue and the ester was extracted with petroleum ether. Gas liquid chromatography (GLC) revealed that less than 1% of the starting material remained. The ester was crystallized from petroleum ether [235 mg, 74%, mp 37.5 C Lit. (7) 37.78 C methyl octadecanoate].

The mass spectrum showed a strong parent ion at m/e 300 and major ions at m/e 269 $[M-OCH_3]^+$, 257 $[M-CH_2CH_2CH_3]^+$ and 256 $[M-CHDCH_2CH_3]^+$. The latter was stronger than that at m/e 257.

6,7-Dideuterio-Octadecanoic Acid

The methyl ester (235 mg) was hydrolyzed by refluxing 2 hr in a solution of potassium hydroxide (2 ml, 90%) and methanol (20 ml). The reaction mixture was acidified with dilute hydrochloric acid and then extracted with ether. Removal of the solvent left 171 mg of a solid with a yield of 76%. The impure acid was crystallized, once from petroleum ether and twice from acetone, to yield 121 mg of a product which melted at 69.5 C [Lit. (8) 69.42 C for octadecanoic acid]. Isotopic purity: 86% d_2, 13.7% d_1. The mass spectrum showed a parent ion at m/e 286, and ions at m/e 242 and 243 corresponding to $[M-CHDCH_2CH_3]^+$ and $[M-CH_2CH_2CH_3]^+$, respectively, the former being the more abundant.

Glyceryl Tri-6,7-Dideuterio-Octadecanoate

A mixture consisting of 89 mg 6,7-dideuterio-octadecanoic acid, 6 mg glycerol and 9 mg *p*-toluenesulfonic acid was heated at 80 C at 1 mm for 8 hr. The triglyceride was purified by preparative TLC and recrystallizations from petroleum ether yielding 16 mg of a crystalline product. The triglyceride which was chromatographically indistinguishable from authentic glyceryl trioctadecanoate melted at 72 C [glyceryl trioctadecanoate (9): 72 C]. Isotopic purity of RCO^+ ion: 87% d_2, 13% d_1.

Glyceryl tri-9,10-dideuterio-hexadecanoate and -tri-9,10-dideuterio-octadecanoate were prepared as described above using methyl hexadec-9-enoate and methyl octadec-9-enoate, respectively, as starting materials.

2-Deuterio-Glyceryl Tributanoate

Thirty-seven milligrams of 2-deuterio-glycerol (5), 20 ml butanoic acid and 2 mg *p*-toluenesulfonic acid were heated at 115 C for 39 hr. After removing the excess acid by distillation, the residue was fractionated by TLC. Homogeneous 2-deuterio-glyceryl tributanoate was obtained (77 mg, 64%).

1,3-Dihexanoyloxyacetone was prepared by stirring 1 g dihydroxyacetone and 13.3 g hexanoyl chloride in 25 ml anhydrous benzene plus 2 ml dry pyridine at room temperature for 60 hr. Water was added and the mixture extracted with chloroform. The acid was removed by distillation under vacuum. The remaining yellow oil was crystallized from petroleum ether at 0 C. The crystals, 802 mg (32%), melted at 52 C. The IR spectrum exhibited at intense absorption at 1740 cm^{-1} (CO-stretch) and no absorption in the 3500 cm^{-1} region (OH-stretch). The product had an appropriate NMR spectrum displaying a singlet ((4 H) at 4.78 ppm, a triplet (4 H) at 2.45 ppm, a multiplet (12 H) in the region 1.9–1.1 ppm and a triplet (6 H) at 0.91 ppm. Major peaks in the high mass region of the mass spectrum were m/e 171 $[M-C_5H_{11}CO_2]^+$, 157 $[M-C_5H_{11}CO_2CH_2]^+$, 141 and 99 $[C_5H_{11}CO]^+$. The compound could not be detected on TLC charred with sulfuric acid indicating facile hydrolysis to volatile products. It reacted, however, with 2,4-dinitrophenylhydrazine to give a yellow spot.

2-Deuterio-glyceryl 1-hexanoate was prepared from 100 mg 1,3-dihexanoyloxyacetone by stirring with 30 mg of sodium borodeuteride in 2 ml of ethanol-d_1 for 1 hr at room temperature. Water was added and the mixture extracted with ether. Removal of the solvent left 80 mg which was preparatively chromatographed on a thin layer plate, yielding 17 mg of chromatographically pure material. Its mobility corresponded to a monoglyceride. Important peaks in its mass spectrum were m/e 160 $[M-CH_2OH]^+$ and m/e 99 $[C_5H_{11}CO]^+$.

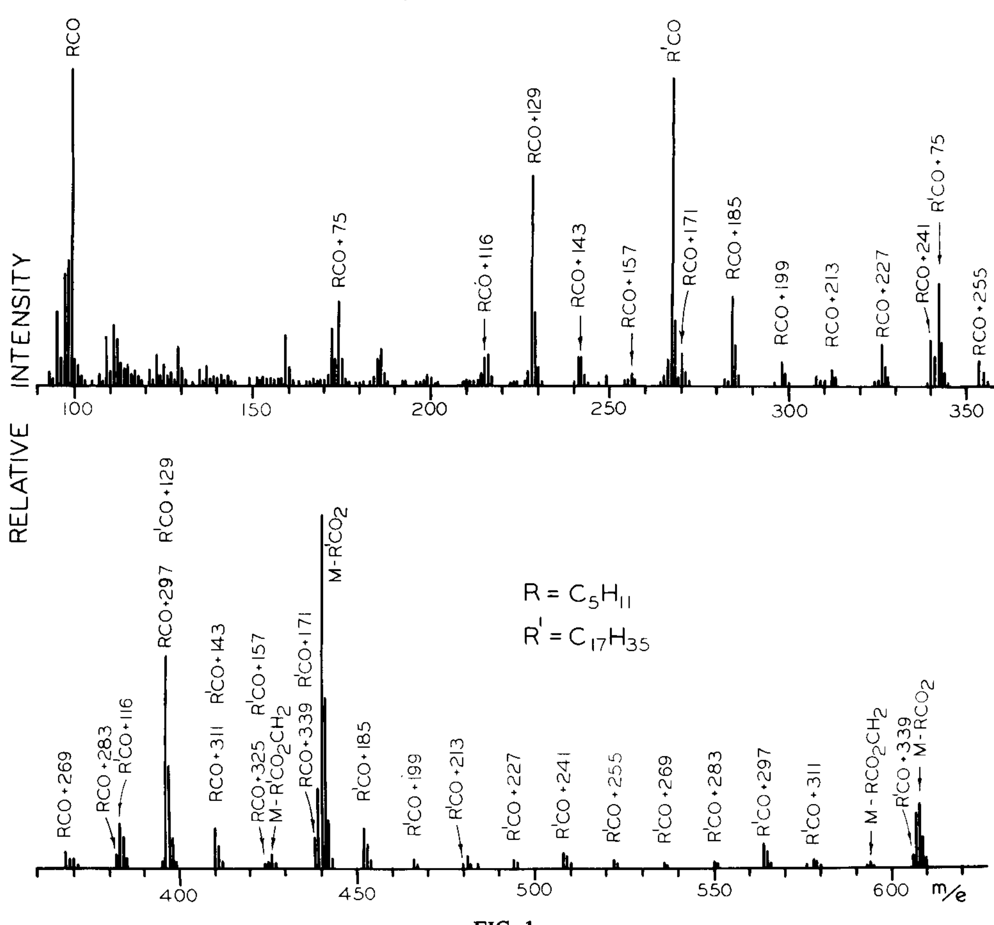

FIG. 1

2-Deuterio-glyceryl 1-hexanoate 2,3-dioctadecanoate was prepared from 17 mg 2-deuterio-glyceryl 1-hexanoate and 116 mg octadecanoyl chloride held under vacuum (ca. 1 mm) at 40 C for 6 hr. The triglyceride was purified by preparative thin layer chromatography twice, and crystallization yielded 13 mg of needles (20%) melting at 45 C. Estimated isotopic purity 83% d_1, based on RCO+74 (75), uncorrected for perhydro analog.

RESULTS AND DISCUSSION

Effect of Chain Length of Fatty Acid Upon the Mass Spectra of Triglycerides

Monoacid triglycerides of the very short chain fatty acids have no molecular ion in their mass spectra. The molecular ion generally increases with increasing chain length of the fatty acid. Even with long chain fatty acids the molecular ion is of low abundance, amounting to 0.013% of the total ionization and 0.22% of the base peak in the case of glyceryl trioctadecanoate.

The ions $[M-RCO_2CH_2]^+$ have been suggested (1) as a means of identifying the fatty acids in the 1 and 3 positions in a triglyceride. In the series of saturated triglycerides studied, the ratio of $[M-RCO_2]^+/[M-RCO_2CH_2]^+$ increased from virtually zero for glyceryl triacetate to 31 for glyceryl trioctadecanoate. Thus the chain length of the fatty acid has a profound effect upon the relative yields of these ions, and the elucidation of structure of mixed triglycerides is thus rendered more difficult.

Mixed triglycerides have mass spectra in which each acyloxy group present is manifested by an ion $[M-RCO_2]^+$ (Fig. 1). The size of this group rather than its location appears to

TABLE I

Per cent Yield of Ions $[M\text{-}RCO_2]^+$ Characteristic of Each Acid in Mixed Triglycerides

Acid	Atoms %	Atoms %	Atoms %
6:18:18	12.8	43.6	43.6
12:18:18	19.8	40.1	40.1
18:12:18	39.6	20.8	39.6
18:14:18	36.1	27.8	36.1
18:16:18	35.0	30.0	35.0
18:18:18[b]	33.3	33.3	33.3
16:12:16	37.8	24.3	37.8
16:14:16	35.4	29.1	35.4
16:16:16[b]	33.3	33.3	33.3
16:18:16	30.8	38.3	30.8

[a]Saturated acids are designated by their numbers of carbon.

[b]Assuming equivalence of the three positions.

TABLE II

Relative Intensities of $[M\text{-}RCO_2CH_2]^+$ Ions in Spectra of Saturated Triglycerides and Their 2-Deuterio-Glyceryl Counterparts

Ion	m/e	Relative intensities
Glyceryl trioctadecanoate		
	592	38
$[M - RCO_2CH_2]^+$	593	100
	594	40
Glyceryl tributanoate		
	199	12.7
	200	3.6
$[M - RCO_2CH_2]^+$	201	100
	202	14.3
	203	2.6
2-Deuterio-glyceryl trioctadecanoate		
$[M - RCO_2CHD]^+$	593	48
$[M - RCO_2CH_2]^+$	594	100
	595	37
2-Deuterio-glyceryl tributanoate		
	200	12.0
$[M - RCO_2CHD]^+$	201	3.0
$[M - RCO_2CH_2]^+$	202	100
	203	11.8
	204	1.8

determine the relative amounts of these ions. This statement is based on studies of a number of mixed triglycerides containing two different acyloxy groups. In the case of glyceryl 2-hexadecanoate-1,3-dioctadecanoate (18:16:18) the observed height of the $[M-C_{17}H_{35}CO_2]^+$, peak is 220 and that of the $[M-C_{15}H_{31}CO_2]^+$ peak is 94. Thus, if there is constant sensitivity in this region of the spectrum, the yields of these ions are 35.0% each for the two 18:0 residues and 30.0% for the 16:0. Table I is the result of similar measurements with a series of mixed triglycerides. A comparison between 18:16:18 and 16:18:16 reveals that in both cases the $[M-C_{17}H_{35}CO_2]^+$ fragment is approximately 1.2 times more abundant than the $[M-C_{15}H_{31}CO_2]^+$ fragment. The table also reveals that in two series in which two acyl groups remain constant and one changes, the yield of $[M-RCO_2]^+$ increases with chain length of the acid. This phenomenon has been observed for glyceryl 1-acyl-2,3-diacetates and glyceryl 2-acyl-1,3-diacetates (10) and in diesters of short chain diols (11).

The molecular ion presumably is formed by the loss of an unshared electron from an oxygen, and the $[M-RCO_2]^+$ may be produced from the molecular ion by the loss of one acyloxy group from the 1 or 2 position giving rise to ions Ia or Ib, or both.

Ia Ib

The larger the acyloxy group lost, the more intense is the residual ion, I.

Position of Fatty Acid in Triglyceride Molecule

Earlier studies (1,2) indicated that the acids esterified at the 1 and 2 positions in a mixed triglyceride could be distinguished on the basis of the relative populations of the two ions, $[M-R^1CO_2CH_2]^+$ and $[M-R^2CO_2CH_2]^+$ derived from positions 1 and 3 or from the 2 position, respectively. This method has been applied to a natural triglyceride of unusual structure (12). The ionic population of $[M-R^2CO_2CH_2]^+$ from the 2 position, is very small or negligible. In the case of the higher members of the series of saturated triglycerides, the ions $[M-R^1CO_2CH_2]^+$ are of low intensity, but are readily recognizable. The $[M-CH_3CO_2CH_2]^+$ ion from glyceryl triacetate is very prominent, but as the molecular weight of the triglyceride increases, the corresponding ions become much less abundant.

A comparison of the spectra of glyceryl trioctadecanoate and 2-deuterio-glyceryl trioctadecanoate revealed a maximum contribution of 10% due to $[M-C_{17}H_{35}CO_2CHD]^+$ ions (Table II). A much more significant example is given by comparison of spectra of glyceryl tributanoate and 2-deuterio-glyceryl tributanoate. In this case, unlike the preceding one, the $[M-RCO_2CH_2]^+$ peak is prominent. Table II

TABLE III

Relative Intensities of Members of the Series, $[RCO+128+14n]^+$ (% of base peak)

RCO	Glyceryl tridodecanoate	Glyceryl tritetradecanoate	Glyceryl trihexadecanoate
+128	29.0	89.0	100.0
+142	2.7	5.9	11.2
+156	1.9	1.6	7.6
+170	4.0	8.9	19.5
+184	6.9	23.7	46.0
+198	1.6	5.4	7.6
+212	5.1	3.0	11.9
+226	2.3	4.0	13.2
+240	0.5	12.1	10.5
+254	1.3	3.8	6.2
+268	---	0.6	27.0
+282	---	2.6	10.3
+296	---	---	2.9
+310	---	---	8.4

also shows that the abundance of $[M-C_3H_7CO_2CHD]^+$ ions formed from 2-deuterio-glyceryl tributanoate is within experimental error of the measurement.

The synthesis of mixed triglycerides without any rearrangement is difficult (13), and as a consequence samples regarded as pure may be contaminated with small amounts of rearranged products. Accordingly, a mixed triglyceride, ABA, may contain some AAB which could give rise to a small amount of ion $[M-RCO_2CH_2]$ corresponding to the acid B whereas the pure triglyceride ABA should not do so. It appears that the $[M-RCO_2CH_2]^+$ ions originate from the 1 and 3 positions and that little, if any, arises from the 2 position. Use of these ions to determine structure of pure triglycerides is possible if the effects of chain length of acyl groups are considered.

Effect of Unsaturation

Unsaturation in the acyl chains would be expected to interrupt the homology in the $[RCO+128+14n]^+$ series. The mass spectra of glyceryl trihexadec-9-enoate, trioctadec-6-enoate, trioctadec-9-enoate and trioctadeca-9,12-dienoate revealed that this was the case. However, the mobility of the double bonds caused formation of many ions in relatively equal abundances which obscure the interruption of homology needed for assignment of double bond position. This is analogous to the phenomenon observed with unsaturated methyl esters (14,15). In the case of unsaturated triglycerides, the RCO^+ ions are accompanied by prominent $[RCO-1]^+$ ions. This loss of one mass unit occurred in all cases studied. With glyceryl trioctadeca-9,12-dienoate, prominent ions representing RCO^+, $[RCO-1]^+$, $[RCO-2]^+$ and $[RCO-3]^+$ occurred. With glyceryl trioctadec-6-enoate, trihexadec-9-enoate, trioctadec-9-enoate and with glyceryl-1-hexadecanoate-2-octadecanoate-3-octadec-9-enoate (16:0,18:0,18:1), only the RCO^+ and $[RCO-1]^+$ ions were prominent in this part of the spectrum. The ratio of $[RCO-1]^+/RCO^+$ varied between 1.5 and 0.7. In the single case, glyceryl trihexadec-9-enoate, in which low electron voltage (15 eV) was employed, the intensity of the $[RCO-1]^+$ ion showed a considerable relative increase when compared with the value obtained at 70 eV. The loss of hydrogen might give rise to a ketene, which is an attractive possibility for the structure of this $[RCO-1]^+$ ion.

Locating Double Bond Positions via Deuteration

The $[RCO+128]^+$ peaks are very prominent in the spectra of triglycerides. In the case of glyceryl trihexadecanoate it proved to be more

TABLE IV

Composition of Ions of the Series $[RCO+128+14n]^+$ in the Mass Spectrum of Glyceryl Tritetradecanoate

$C_{13}H_{27}CO$	Ion	Calculated	Found
+128	$C_{20}H_{35}O_4$	339.2535	339.2519
+142	$C_{21}H_{37}O_4$	353.2692	353.2672
+156	$C_{22}H_{39}O_4$	367.2848	367.2808
+170	$C_{23}H_{41}O_4$	381.3005	381.2973
+184	$C_{24}H_{43}O_4$	395.3161	395.3155
+198	$C_{25}H_{45}O_4$	409.3318	409.3316
+212	$C_{26}H_{47}O_4$	423.3474	423.3470
+226	$C_{27}H_{49}O_4$	437.3631	437.3606
+240	$C_{28}H_{51}O_6$	451.3787	451.3764
+254	$C_{29}H_{53}O_4$	465.3944	465.3948
+282	$C_{31}H_{57}O_4$	493.4257	493.4254

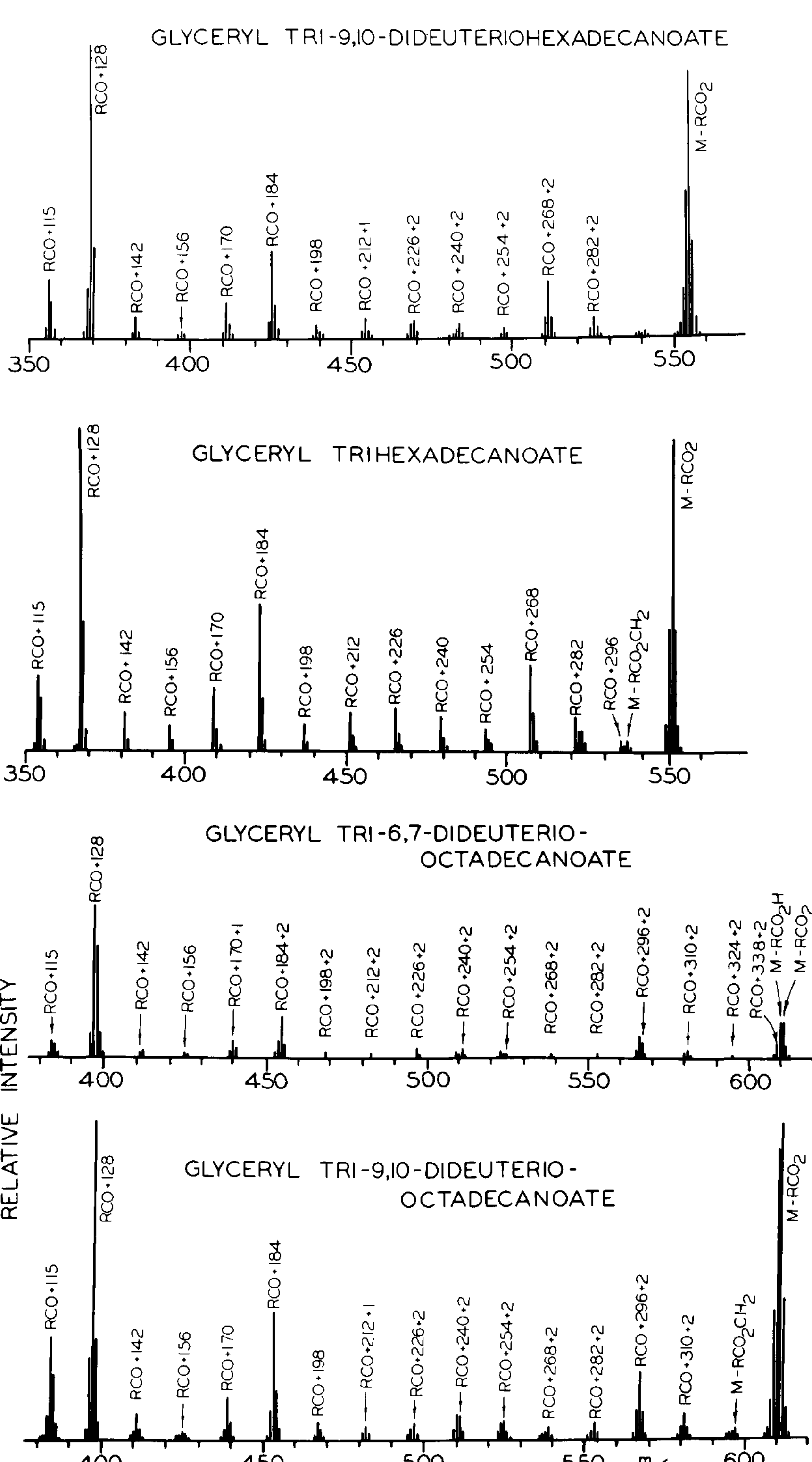

FIG. 2

intense than the RCO^+ or $[M-RCO_2]^+$ peaks under the conditions used. It is the first member of a series, the successive members of which differ by 14 mass units and which therefore can be used for locating double bonds which have been saturated by deuterium. The intensities of the members of the series vary widely, but their recognition presents little difficulty, because for each the neighboring peaks are less intense. Table III gives the uncorrected values for three typical simple triglycerides.

The composition of the ions of this series was established by high resolution studies in the case of glyceryl tritetradecanoate and these data are shown in Table IV. It is apparent that this series of fragment ions results from the molecular ion, $C_{45}H_{86}O_6$, by loss of one acyloxy ion, $C_{14}H_{27}O_2$, plus an alkane, or by the loss of $C_{14}H_{28}O_2$ plus an alkyl radical. Several structures may be assigned, but structures II and III have been adopted as a working hypothesis. These ions may also include species containing six-membered rings involving all three carbon atoms of glycerol, analogous to ion Ia. Similar cyclic structures have been suggested for ions formed from ethylene ketals (16-18), deuterated glyceryl 1,3-dioctadecanoates (19) and from 1-acyl-2-alk-1'-enyloxy ethanols (20).

CH_2-O^+, $CH-O$ (ring) $>C(CH_2)_nCH_3$; $CH_2-O-C(=O)-(CH_2)_nCH_3$ — $[M-CH_3(CH_2)_nCO_2]^+$ — II

CH_2-O^+, $CH-O$ (ring) $>C(CH_2)_n$ minus H; $CH_2-O-C(=O)-(CH_2)_nCH_3$ — $[RCO+128+14n]^+$ — III

Glyceryl trihexadec-9-enoate was deuterated with tetradeuterio-hydrazine and the spectrum of the resulting glyceryl tri-9,10-dideuteriohexadecanoate was compared with that of glyceryl trihexadecanoate. The pertinent portions of the spectra are shown in Figure 2. Figure 2 also presents similar results obtained in the cases of glyceryl tri-6,7-dideuterio-octadecanoate, and glyceryl tri-9,10-dideuterio-octadecanoate. Inspection of the series of ions, $[RCO+128+14n]^+$, in the spectra of the three deuterated triglycerides, revealed discontinuities related to the original double bond position. The first members of the series are normal in all cases, but when the first carbon atom which bore the double bond appears in the series, the ion bearing one deuterium atom is one mass too large. All subsequent members of the series are two mass units too large because they include both carbon atoms bearing deuterium. In these three cases, expulsion and exchange reactions do not constitute a series limitation (5). The method likewise seems applicable to locating double bonds in all positions in a fatty acid residue, for the series plus ion e (5) subtend all the carbon atoms of a fatty acid residue. The applicability of the method may be limited because of the relatively low intensities of the ion in which n=2 and the last member of the series and the possibility of exchange and extrusion reactions. Mixed triglycerides, such as 16:18:16, give two series; one $[C_{15}H_{31}CO+128+14n]^+$ and another, $[C_{17}H_{35}CO+128+14n]^+$, some members of which overlap.

Structures of Other Prominent Ions in Mass Spectra of Triglycerides

A McLafferty rearrangement occurs, for in the case of glyceryl tritetradecanoate a peak appears at mass 554 (calculated for $C_{33}H_{62}O_6$, 554.45465; found 554.45501), which corresponds to the loss of $C_{12}H_{24}$, leading to the replacement of the acyl group with an acetyl group. A series of such ions, in which CH_3CO is the acyl group is indeed found. The $[CH_3CO+115]^+$ peak, m/e = 158, is present as well as the series $[CH_3CO+128+14n]^+$, m/e 171, 185, 199, etc. The presence of an acetyl group, resulting from a McLafferty rearrangement manifested itself in the spectra of all of the saturated triglycerides studied. A second series of ions produced from the McLafferty product by loss of a fatty acid molecule likewise occurs. Their structures would be analogous to ion III in which the remaining acyl group is CH_3CO. When the triglycerides contained glycerol labeled in the 2 position with deuterium, all these ions shifted by one mass unit, indicating that they all include the glyceryl moiety.

$[RCO+74]^+$

This peak is a prominent one, as indicated by earlier studies (1,2). High resolution measurement of this ion in the spectrum of glyceryl tritetradecanoate indicated a composition of $C_{17}H_{33}O_3$ (calculated 285.2429; found 285.2420). The structures IVa and IVb are viewed as probable for this ion.

HO^+ (ring with CH_2 and CH) $CH-O-C(=O)-C_{13}H_{27}$ — IVa

CH_2-OH^+, CH (ring), $CH-O-C(=O)-C_{13}H_{27}$ — IVb

Its formation may involve the loss of a ketene, $C_{12}H_{25}CH=C=O$, from the $[M-C_{13}H_{27}CO_2]^+$ ion. In spectra of mixed triglycerides there is a prominent $[RCO+74]^+$

peak corresponding to each RCO in the triglyceride structure. In the cases of 2-deuterioglyceryl trioctadecanoate and perdeuterioglyceryl trioctadecanoate the peak appears at $[RCO+75]^+$ and $[RCO+79]^+$, respectively, indicating that the entire glycerol moiety is involved, consistent with the structures postulated.

$[RCO+115]^+$

The spectra, published by Ryhage and Stenhagen (1), and by Barber et al. (2), as well as those obtained in the present study show prominent $[RCO+115]^+$ peaks. In the case of glyceryl tritetradecanoate, the composition of $C_{19}H_{34}O_4$ was established by high resolution measurements (calculated 326.2457; found 326.2433). This corresponds to a loss of $C_{13}H_{27}CO_2$ plus $C_{12}H_{25}$, or to $C_{13}H_{27}CO_2H$ plus $C_{12}H_{24}$ from glyceryl tritetradecanoate, $C_{45}H_{86}O_6$.

```
        O—CH2   O              CH2—O
       ╱  |     ‖              |     +C-CH2·
·CH2-C +  CH-O-C-C13H27        CH—O
       ╲  |                    |
        O—CH2                  CH2-O-C-C13H27
                                      ‖
                                      O
           Va                           Vb
```

Formulas Va and Vb represent possible structures of this ion. In the cases of 2-deuterioglyceryl trioctadecanoate and perdeuterioglyceryl trioctadecanoate this peak is shifted to $[RCO+116]^+$ and $[RCO+120]^+$, respectively, indicating retention of the entire glycerol moiety.

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Mass Spectrometry of Triglycerides: II. Specifically Deuterated Triglycerides and Elucidation of Fragmentation Mechanisms[1]

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ABSTRACT

Deuterium labeled monoacid triglycerides were synthesized and their mass spectra were measured. The spectra provided further support for proposed (1) structures of principal ions, knowledge about the formation of $[M\text{-}18]^+$, the interexchange of hydrogen atoms between 2 and the 5, 6 or 7 positions and the expulsion of part of the alkyl chain.

INTRODUCTION

Mass spectrometry has been used to only a limited extent in characterizing glyceryl esters. Brief notes by Ryhage and Stenhagen (2) and Barber et al. (3) recognized the major ions, $[M\text{-}18]^+$, $[M-RCOOH]^+$, $[M-RCO_2]^+$, $[M-RCO_2CH_2]^+$, RCO^+, and stated that the acyl groups attached to 1, 3 and 2 positions in a mixed triglyceride could be distinguished by comparing the abundances of [M-acyloxymethylene]$^+$ ions. Johnson and Holman (4) studied the di-trimethylsilyl ethers of 1 and 2 monoglycerides and found the populations of the ion $[M-CH_2OSi(CH_3)_3]^+$ to differ greatly in the two cases. Morrison et al. (5) recently discussed the mass spectra of some deuterated glyceryl 1,3-dioctadecanoates.

Practical applications of mass spectrometry of glycerides have been found in elucidating the structure of an allene-containing triglyceride from *Sapium sebiferum* (6), quantitative analysis of triglyceride mixtures (7) and pyrolysis of phosphoglycerides (8). Of special interest is the ion $[M\text{-}18]^+$ first described by Barber et al. (3). To our knowledge this is the first observation of an ester function being involved in the formation of this ion. In the present study elucidation of the cracking pattern was attempted by deuteration on successive carbon atoms of the triglycerides.

EXPERIMENTAL PROCEDURES

Alkyl mesylates and cyanides were prepared according to the methods described by Baumann and Mangold (9,10). The procedure of Christie and Holman (11) was followed in the malonic ester syntheses. Unless otherwise stated, triglycerides were synthesized by heating glycerol, 25-50% excess fatty acid and *p*-toluenesulfonic acid as catalyst at 80-90 C and 1 mm pressure for 6-7 hr. The triglycerides were isolated by preparative thin layer chromatography (TLC) and recrystallized 2-4 times from petroleum ether. All triglycerides used in this study were chromatographically homogenous.

The mass spectra were measured in a Hitachi RMU6D instrument at 70 eV and at about 1 to 2×10^{-7} torr. The direct solid sample insertion system was used for triglycerides and the liquid sample insertion system for compounds of lower molecular weight.

2,2-Dideuterio-Tetradecanoic Acid (I)

Methyl tetradecanoate, 3.52 g, was added to a solution of 80 mg sodium in 5 ml methanol-d_1. The mixture was refluxed for 15 min. The methanol was removed under vacuum and the treatment repeated twice with two portions of 5 ml fresh methanol-d_1. The ester was hydrolized by refluxing for 1 hr after adding a solution of 265 mg sodium in 2 ml ethanol-d_1 and 3 ml deuterium oxide. The mixture was acidified with 3 ml 10 N deuterium chloride and extracted with chloroform. The solvent was distilled under reduced pressure leaving a colorless crystalline solid, 3.24 g (97%). The acid was recrystallized twice from acetone. Melting point 53.5-54 C. [Lit. (12) 54-54.1 C for tetradecanoic acid]. Important peaks in the high mass region of the mass spectrum were m/e 230 M^+, 213 $[M\text{-}17]^+$, 187 $[M-CH_2CH_2CH_3]^+$, 186 $[M-CDHCH_2CH_3]^+$ and 185 $[M-CD_2CH_2CH_3]^+$.

Glyceryl Tri-2,2-Dideuterio-Tetradecanoate (II)

Seventy-three milligrams of II was obtained from 42 mg glycerol, 404 mg I using 10 mg *p*-toluenesulfonic acid as catalyst. The labile protons of glycerol had been exchanged with deuterium by shaking with two portions of 1 ml deuterium oxide which subsequently was removed under diminished pressure. Melting point 57.5 C [Lit. (13) 57 C for glyceryl tri-

[1]Presented in part at the AOCS Meeting, New Orleans, April 1970. Part VI of a series on Mass Spectrometry of Lipids. For V see Lipids 5:861-868 (1970).

tetradecanoate]. Isotopic purity, based upon RCO^+: 90.3% d_2, 6.1% d_1, 3.5% d_0.

2,2-Dideuterio-Tetradecanol (III)

An amount of 1.66 g I was dissolved in 25 ml anhydrous ether and added slowly to a suspension of 1.7 g lithium aluminum hydride in 150 ml dry ether. The mixture was refluxed for 5 hr. Moist ether was added and the slurry filtered through celite. The solvent was removed leaving 1.35 g (87%) as a white solid. The product was chromatographically homogenous and melted at 37.5-38 C [Lit. (14) 37.62 C for tetradecanol]. No parent peak was observed in the mass spectrum but an intense peak, m/e 198, corresponded to $[M\text{-}18]^+$.

2,2-Dideuterio-Tetradecyl Mesylate (IV)

Methanesulfonyl chloride, 1.71 g, was added dropwise to a cooled (O C) solution of 1.25 g III in 15 ml dry pyridine. The mixture was stirred 5 hr at room temperature and worked up as described by Baumann and Mangold (9). Recrystallization from petroleum ether gave 1.09 g (63%) melting at 44 C [Lit. (9) 44-45 C for tetradecyl mesylate]. The TLC revealed one spot only. Its mass spectrum exhibited no parent peak but a strong peak at m/e 198 agreeing with $[M-CH_3SO_3H]^+$.

2,2-Dideuterio-Tetradecyl Cyanide (V)

A solution of 1.02 g IV in 20 ml dimethyl sulfoxide was added to 0.62 g potassium cyanide. The mixture was stirred at 120 C for 2 hr and worked up as described by Baumann and Mangold (10). The slightly yellow oil, 745 mg (95%) had an appropriate mass spectrum (15) having m/e 225 and m/e 224 corresponding to M^+ and $[M\text{-}1]^+$.

3,3-Dideuterio-Pentadecanoic Acid (VI)

The nitrile V, 745 mg, was refluxed in a mixture of 2 ml 90% potassium hydroxide and 2 ml ethanol for 58 hr. Acidification with hydrochloric acid and extraction with chloroform gave, after removal of the solvent, a colorless crystalline residue which was recrystallized from acetone. Yield: 543 mg (74%), mp 53 C [Lit. (16) 52.1 C for pentadecanoic acid]. The mass spectrum revealed peaks at m/e 224 and m/e 199 corresponding to M^+ and $[M-CH_2CD_2CH_3]^+$.

Glyceryl Tri-3,3-Dideuterio Pentadecanoate (VII)

After reacting 228 mg VI with 18.5 mg of glycerol using 15 mg catalyst 134 mg (86%) VII was isolated. Melting point 54 C [Lit. (13) 54 C for glyceryl tripentadecanoate]. Isotopic purity of RCO^+: 94.8% d_2, 5.2% d_1.

4,4-Dideuterio-Hexadecanoic Acid (VIII)

Diethyl malonate, 3 g, was added to a solution of 200 mg sodium in 25 ml anhydrous ethanol. The mixture was refluxed for 1 hr and 1.01 g IV was added, and the solution refluxed for 2 hr and left at room temperature overnight. The pink solution was filtered and most of the solvent removed under vacuum. Water was added and the esters extracted with chloroform. The solvent was distilled under reduced pressure leaving an orange oil which was hydrolized by refluxing for 2 hr in a mixture of 5 ml 90% potassium hydroxide and 20 ml ethanol. The mixture was left at room temperature overnight, acidified with hydrochloric acid and extracted with ether. Removal of the ether left a white crystalline residue which was decarboxylated by heating at 150 C for 2 hr. Traces of acetic acid formed from malonic acid were removed under vacuum. The residue was recrystallized thrice from acetone giving 366 mg (41%) melting at 62.5 C [Lit. (14) 62.67 C for hexadecanoic acid]. The mass spectrum showed m/e 258 M^+ and m/e 213 $[M-CH_2CH_2CD_2H]^+$ as prominent peaks in the high mass region.

Glyceryl Tri-4,4-Dideuterio Hexadecanoate (IX)

From the reaction mixture consisting of 18 mg glycerol, 192 mg VIII and 14 mg catalyst, 39 mg IX were isolated. Melting point 66 C [Lit. (13) 65.6 C for glyceryl trihexadecanoate]. Isotopic purity of RCO^+: 96.8% d_2, 0.6% d_1, 2.5% d_0.

3,3-Dideuterio-Pentadecanol (X)

Unused portions of VI (415 mg), VII (110 mg), mono- and diglycerides from the preparation of VII were pooled and reduced with 400 mg lithium aluminum hydride in 50 ml dry ether to yield X. The mixture was refluxed for 3 hr and worked up as described for III. The crude product, 630 mg, melted at 43.5 C [Lit. (14) 43.84 C for pentadecanol]. The chromatographically homogenous product had an appropriate mass spectrum, m/e 212 corresponding to $[M\text{-}18]^+$.

3,3-Dideuterio-Pentadecyl Mesylate (XI)

From 620 mg X and 1 g methanesulfonyl chloride in 5 ml pyridine, 520 mg (63%) XI was prepared. The melting point was 49-50 C (51 C for pentadecyl mesylate, Baumann, personal communication). The mass spectrum exhibited a peak at m/e 212 corresponding to $[M-CH_3SO_3H]^+$.

5,5-Dideuterio-Heptadecanoic Acid (XII)

Using the same amounts of sodium, diethyl

malonate and solvent, 520 mg XI were used in a malonic ester synthesis as outlined for VIII. The product was crystallized twice from acetone giving 245 mg (53%) XII. The mass spectrum indicated the presence of an impurity, m/e 300. The methyl ester was prepared by reacting the acid overnight with methanol containing 5% hydrochloric acid. The ester was extracted with chloroform and crystallized from petroleum ether. Melting point 28.5 C [Lit. (17) 28.6 C for methyl heptadecanoate]. The ester was hydrolized by refluxing for 2 hr in a mixture of 2 ml 90% potassium hydroxide and 20 ml ethanol. Acidification with dilute HCl, extraction with ether and recrystallization from acetone gave 148 mg XII. Melting point 60.5-61 C [Lit. (14) 61.19 C for heptadecanoic acid]. The mass spectrum revealed prominent peaks at m/e 272 M^+, m/e 229 $[M-CH_2CH_2CH_3]^+$ and no peak at m/e 300.

Glyceryl Tri-5,5-Dideuterio Heptadecanoate (XIII)

Sixty-four milligrams of XII, 5.7 mg glycerol and 6 mg *p*-toluenesulfonic acid gave, after purification, 26 mg XIII which melted at 64 C [Lit. (13) 64 C for glyceryl triheptadecanoate]. Isotopic purity of RCO^+: 94.3% d_2, 3.7% d_1, 1.9% d_0.

5,5-Dideuterio-Heptadecanol (XIV)

Hydride reduction of XII, XIII and mono- and diglycerides which had been recovered from the preparation of XIII yielded 181 mg XIV. Total weight of the starting materials was 202 mg. The initial product which appeared to be chromatographically pure melted at 53-53.5 c [Lit. (14) 53.31 C for heptadecanol]. The mass spectrum exhibited a major peak at m/e 240 and a minor one at m/e 239 corresponding to $[M-H_2O]^+$ and $[M-HDO]^+$.

5,5-Dideuterio-Heptadecyl Mesylate (XV)

From 180 mg XIV and 700 mg methanesulfonyl chloride in 5 ml dry pyridine 234 mg (100%) XV was prepared. The chromatographically homogenous product melted at 58.5 C which corresponds to a value interpolated from melting points of hydrogen homologs (9). No parent peak was found in the mass spectrum, but an intense peak was located at m/e 240 agreeing with the mass calculated for $[M-CH_3SO_3H]^+$.

5,5-Dideuterio-Heptadecyl Cyanide (XVI)

To 234 mg XV in 10 ml dimethyl sulfoxide was added 280 mg potassium cyanide and treated as outlined for V. A slightly yellow oil, 139 mg (74%), was not further purified or

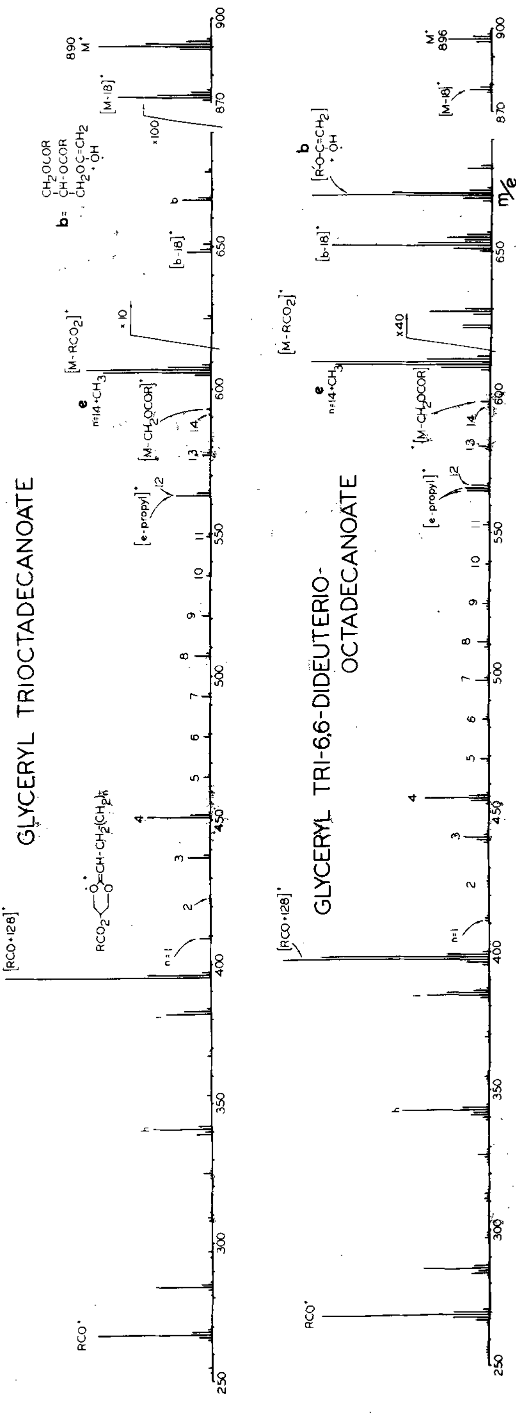

FIG. 1. High mass regions of the mass spectra of a saturated triglyceride and its deuterium-labeled counterpart. Changes in intensity scale refer to total scale magnification.

characterized, but it appeared to be more than 98% pure judging from TLC.

6,6-Dideuterio-Octadecanoic Acid (XVII)

The nitrile XVI, 139 mg, was hydrolyzed as described for VI. Crystallization from acetone gave 102 mg (68%) XVII melting at 68.5 C. Recrystallization gave 48 mg melting at 69.5-70 C [Lit. (14) 69.42 C for octadecanoic acid]. The mass spectrum displayed a strong parent ion at m/e 286 and two ions at m/e 242 and 243 corresponding to $[M-CHDCH_2CH_3]^+$ and $[M-CH_2CH_2CH_3]^+$, respectively, the former being the stronger.

The corresponding methyl ester was prepared by keeping the acid recovered from the mother liquors in 5% hydrochloric acid–methanol at room temperature overnight. The mixture was worked up as described above. The ester crystallized from acetone giving 65 mg melting at 37.5 C [Lit. (18) 37.78 C for methyl octadecanoate]. An intense parent ion at m/e 300 and ions at m/e 269 $[M-OCH_3]^+$, 257 $[M-CH_2CH_2CH_3]^+$ and 256 $[M-CHDCH_2CH_3]^+$ were found in the mass spectrum. The ion at m/e 256 was more intense than that at m/e 257.

Glyceryl Tri-6,6-Dideuterio Octadecanoate (XVIII)

After reacting 55 mg XVII with 5.2 mg glycerol in the presence of 9 mg catalyst, 4.5 mg of pure XVIII was isolated. The crystalline product melted at 72.2 C and at 71.5-72 C when mixed with authentic glyceryl trioctadecanoate. The latter compound melted at 72 C as reported (13). Isotopic purity of RCO^+: 87.7% d_2, 8.7% d_1, 3.6% d_0.

Perdeuterio-Glyceryl Trioctadecanoate (XIX)

In the presence of 8 mg *p*-toluenesulfonic acid 14 mg glycerol-d_8 (ICN, -CD 99 atom %) was reacted with 351 mg octadecanoyl chloride. The product was purified by column chromatography on Florisil, preparative TLC and by three recrystallizations from petroleum ether yielding 10 mg of needles at 71.5 C and 71.5-72 C when admixed with glyceryl trioctadecanoate. The latter melted at 72 C as reported (13).

2-Deuterio-Glyceryl Trioctadecanoate (XX)

After purification, 95 mg (31%) XX were obtained by reacting 988 mg octadecanoic acid with 32 mg 2-deuterioglycerol in the presence of 2 mg catalyst. The melting point, 72 C, was not depressed when mixed with authentic glyceryl trioctadecanoate whose melting point was 72 C as reported (13). Isotopic purity of $[RCO+74]^+$: 96.8% d_1, 3.1% d_0.

2-Deuterio-Glycerol (XXI)

Dihydroxyacetone, 179 mg, was added to a solution of 51 mg sodiumborodeuteride in 2 ml deuterium oxide and the mixture kept at room temperature for 21 hr. Water, 20 ml, was added and the solution filtered through an ion exchange column (1 x 10 cm) consisting of a mixture of anion (IR-45) and cation (IRC-50) exchange material. The resins had been activated with 3% sodium hydroxide and 2 N hydrochloric acid, respectively, and washed to neutrality with distilled water. The resulting aqueous solution was distilled under vacuum leaving a colorless liquid, 154 mg (83%). The mass spectrum revealed no parent peak but the m/e 61 peak of glycerol was shifted to m/e 62 $[CDOH-CH_2OH]^+$. Isotopic purity, 95% d_1, was based on m/e 61, 62.

RESULTS AND DISCUSSION

The reports by Dinh-Nguyen et al. (19,20) on the mass spectra of fatty acid methyl esters were useful in explaining the spectra of triglycerides. Using ^{13}C and deuterium labeling, their studies revealed that four types of reactions contributed to the fragmentations of methyl esters:

(a) Simple cleavage of the alkyl chain giving rise to ions of type a

$$\text{ion a} \quad [CH_3O\text{-}\overset{O}{\overset{\|}{C}}\text{-}(CH_2)_n]^+$$

(b) Expulsion of part of the chain plus one hydrogen atom resulting in ions of type a.

(c) Exchange of hydrogen atoms between position 2 and positions 5, 6 and 7.

(d) McLafferty rearrangement (2,3 cleavage with transfer of one hydrogen from position 4 giving an ion of type b where R is CH_3).

$$\text{ion b} \quad [R\text{-}O\text{-}\overset{OH}{\overset{|}{C}}=CH_2]^+$$

These same reactions have been observed to occur in the fragmentation of triglycerides.

McLafferty Rearrangement (21)

All triglycerides studied displayed ions corresponding to 2,3 cleavage with transfer of one hydrogen, comparable to ion b in which R is the glycerol moiety plus two acyl groups. Only when the 2 or 4 positions were labeled with deuterium (II and IX) did this ion retain deuterium atoms in the rearrangement. This agrees with the report of Dinh-Nguyen et al. (19) who studied methyl esters. Most of the deuteriums were apparently retained when located in the 2 positions (II). This is com-

TABLE I

Ions Related to Loss of Water. Abundances Relative to Ion b, the McLafferty Rearrangement Product

Triglyceride of	M+1	M	M-1	M-17	M-18	M-19	M-20	b+1	b	b-1	b-17	b-18	b-19	b-20
18:0	15	25	7	12	21	3	-	50	100	11	39	86	11	-
2,2-d_2-14:0 (II)	9	25	9	13	34	13	4	43	100	43	73	137	64	37
3,3-d_2-15:0 (VII)	8	16	4	10	25	4	1	35	100	14	42	107	27	8
4,4-d_2-16:0 (IX)	10	22	7	7	17	17	5	45	100	22	18	38	98	50
5,5-d_2-17:0 (XIII)	11	22	5	7	19	5	-	42	100	12	32	69	19	12
6,6-d_2-18:0 (XVIII)	10	25	9	6	13	2	-	56	100	17	42	92	22	6
18:0, 2-d-glycerol (XX)	10	18	5	5	9	2	1	45	100	11	41	91	9	-
18:0, glycerol-d_8 (XIX)	9	17	3	4	13	1	-	60	100	-	52	70	-	-

patible with earlier findings (19,20) indicating that extrusion (reaction type b) or exchange (type c) did not precede or interfere with the McLafferty rearrangement to any significant degree.

$[M-18]^+$, $[b-18]^+$

Barber et al. (3) pointed out that the transition $M^+ \rightarrow [M-18]^+$ was accompanied by a prominent metastable peak indicating ionic fragmentation rather than thermal cracking. Our spectra revealed also a second transition b $\rightarrow [b-18]^+$ with its corresponding metastable peak (Fig. 1). These fragmentations are unique because water is lost from an ester. Re-examination of the spectra of diesters of 1,2-ethane diol and 1,3-propane diol (22) revealed that loss of water also occurs with these esters. Spectra of wax esters (Aasen et al., unpublished data) like octadecyl octadecanoate do not exhibit this ion, $[M-18]^+$, probably because competing modes of fragmentations are favored.

Since the spectra of triglycerides labeled in the glycerol moiety (Table I) showed loss of ordinary water, the hydrogen atoms involved must originate from the acyl moieties. Table I shows that the hydrogen atoms in 2 and 4 positions are involved. The spectrum of IX showed that H_2O, DHO and D_2O were lost in the ratio of about <1:70:30, calculated from the relative populations of $[b-18]^+$, $[b-19]^+$ and $[b-20]^+$. The spectrum of II revealed loss of H_2O, DHO and D_2O in the ratio of about 60:30:10. An enol mechanism with 1,4 elimination similar to that suggested by Williams et al. (23) for the formation of $[\text{cyclohexanone-18}]^+$ is proposed for triglycerides, where R is the glyceryl diacyl moiety:

$[M-19]^+$ ← ion c (R-O-C, HO, D, R′, D) — exchange →

ion d (R-O-C, DO, D, R′, H) → $[M-20]^+$

TABLE II

Relative Abundances of Ions $[RCO+128]^+$ and Neighboring Ions in the Spectra of Some Deuterated Triglycerides

Triglyceride of	RCO+127	RCO+128	RCO+129	RCO+130	RCO+131
18:0	1	100	28	4	1
2,2-d_2-14:0 (II)	4	100	53	11	2
3,3-d_2-15:0 (VII)	1	5	16	100	25
4,4-d_2-16:0 (IX)	9	100	26	6	1
5,5-d_2-17:0 (XIII)	9	100	38	6	1
6,6-d_2-18:0 (XVIII)	11	100	81	20	3
6,7-d_2-18:0[a]	15	100	64	15	2
9,10-d_2-18:0[a]	20	100	29	6	1
18:0, 2-d-glycerol (XX)	1	6	100	27	4
	RCO+132	RCO+133	RCO+134	RCO+135	RCO+136
18:0, glycerol-d_8 (XIX)	7	100	36	5	1

[a]See Reference 1.

Deuterium-hydrogen exchange occurring between the enol-oxygen and C4 might account for the loss of D_2O. The spectrum of II showed that H_2O was lost to a greater extent than DHO and D_2O, indicating competition by reaction c, or extrusion according to reaction b, or both. The loss of water from acyclic alcohols has been found (24,25) to be almost exclusively 1,4-elimination via a hexagonal transition state as shown in ions c and d.

[RCO + 128 + 14n]$^+$

Barber et al. (3) observed that the spectra of all triglycerides displayed a rather intense ion, 128 m/e units heavier than the RCO^+ ion. High resolution measurements (1) indicated that $C_6H_8O_3$ accounted for this increment. Close inspection of the less abundant ions found at higher m/e values revealed that peaks recurred at intervals of 14 m/e units, suggesting the series [RCO + 128 + 14n]$^+$. The ion [M − RCO_2H]$^+$ is represented by [RCO + 128 + 14n + CH_3]$^+$ in which the terminal methyl group has been added to the final member of the series. Several structures might be drawn for this ion, but e appears simplest, is resonance stabilized, and is most consistent with results for the deuterium labeled compounds.

ion e = [M−RCO_2H]

R-C(=O)-O-(ring O, O$^{+\cdot}$)C=CH-CH_2(CH_2)$_n$$CH_3$

ion e′   ion e″

Each member of the series is thought to be formed by simple homolytic cleavage of the alkyl chain of e (reaction a). The high abundance of the first member [RCO + 128]$^+$, might be explained by allylic homolytic cleavage which is energetically favored (26) giving a resonance stabilized ion, f.

ion f   [RCO+128]$^+$

Two other ions, [RCO+170]$^+$ and [RCO+184]$^+$ were usually more intense than others of the series, and the latter was the stronger of the two. This might be rationalized by forming five- or six-membered rings by pairing the electron indicated in e′ or e″ with the unshared electron at C6 or C7. Except for compound II, all the deuterium labeled compounds produced ions agreeing with e (Table II). Deuteriums in all 3 positions of acyl groups effected a shift of two m/e units giving the series [RCO+130+14n]$^+$. Labeled 4 positions resulted in the series [RCO+128+16+14(n-1)]$^+$. Deuteriums farther out in the acyl group, i.e., 5,5; 6,6; 6,7 or 9,10 caused one or two intervals of 16 or 15 m/e units rather than 14. The m/e values of the irregular intervals agreed with postulated ion e having deuteriums in the appropriate positions.

The spectrum of the exception, II, exhibited a stronger [RCO+128]$^+$ ion than the expected [RCO+129]$^+$. The explanation offered for this anomaly is partial loss of deuterium due to exchange with hydrogen (reaction c). It is seen in Table II that the [RCO+129]$^+$ ions of XIII and XVIII and glyceryl tri-6,7-dideuterio-octadecanoate showed increased abundances compared to those of compounds not having deuterium in 5, 6 or 7 position. This suggests that hydrogen in position 2 is replaced by deuterium from position 5, 6 or 7 analogous to what happens in methyl esters (19,20). The spectrum of II shows that most ions are accompanied by intense satellites making it difficult to tell the fate of the deuteriums originally located in the 2 position of the acyl chains. Thus the ions at m/e 384, 398 and 412 representing replacement of one hydrogen with one deuterium in position 5, 6 and 7, respectively, appear to be enriched with deuterium, but so are many other ions. The evidence presented elsewhere (19,20) and above for the interexchange phenomenon suggests that the deuteriums are not transferred by complicated rearrangements to other parts of the molecule, but are largely confined to their original acyl group. Morrison et al. (5) found that little or no scrambling took place between the glyceryl residue and the fatty acid chains in the spectra of deuterated glyceryl 1,3-dioctadecanoates.

The explanation offered for the higher abundance of [M-18]$^+$ than [M-19]$^+$ (Table I) in the case of II was that exchange (reaction c) to a large extent preceded the expulsion of water. The data of Table II indicate that exchange also precedes the formation of ion e, leaving the possibility that exchange occurs already in M^+ and b^+ (see discussion of McLafferty rearrangement above). It was suggested that IX lost some D_2O because exchange had occurred between 4 position and the enol oxygen. This is, however, not reflected in Table

TABLE III

Mass Interval Corresponding to Loss of Propyl Groups From Ions Derived From Deuterium Labeled Methyl Esters and Triglycerides[a]

Position labeled	Propyl group lost: Methyl esters (19,20)	Propyl group lost: Triglycerides
None	43	43
2,2	43,44[b],45	43,44,45[b]
3,3	45	45
4,4	45(44)[c]	45
5,5	43	43
6,6	43,44[b],45	43,44[b],45
6,7	43,44[b]	43,44[b]
9,10	43	43

[a]With methyl esters the loss is from the molecular ion, and with triglycerides loss of propyl is from ion *e*.

[b]Tallest peak in the cluster.

[c]A discrepancy exists between references 19 and 20. Reference 19 agrees with our findings that m/e is lost from VIII and IX.

II by increased intensity of $[RCO+129]^+$, indicating that the enol side of the keto-enol equilibrium is favored.

In the case of maximum interchange, i.e., between 2 and 6 positions, a favorable (27) hexagonal transition state might occur (ion g).

ion g

Absence of detectable metastable peaks makes it difficult to propose an unambigous fragmentation pathway leading to e which is formally obtained by M^+ expelling a neutral carboxylic acid molecule. Because triglycerides labeled in the glycerol moiety retained the deuterium, a mechanism differing from a concerted McLafferty rearrangement must operate. A two-step mode of fragmentation is suggested. Following the loss of an acyloxy radical the enol form of the ester (ion h) may cyclize to ion i which represents the major ion $[M-RCO_2]^+$. Loss of a hydrogen yields e.

ion h → ion i $[M-RCO_2]^+$ → ion e

Albeit formation of e might be envisaged using the keto form of h, the enol form is favored for the following reasons: (a) In view of the mechanism for the loss of water, the enol form is probably present. (b) The enol oxygen would be in closer proximity to the radical site than the keto oxygen. (c) The double bond of e is already present. (d) The enol form of a compound is ionized more easily (28) than is the keto form.

A rather intense ion at m/e 563 in the spectrum of glyceryl trioctadecanoate was originally thought to be a member of the series $[RCO+128+14n]^+$. However, the m/e values of the corresponding ions in spectra of labeled compounds disproved this. The differences in m/e values between e and this ion for various labeled triglycerides coincided (Table III) with the loss of a propyl group. This is comparable to observations (19,20) made on long chain methyl esters labeled in comparable positions. Thus it appears that extrusion (reaction b) of the three methylene groups adjacent to the carbonyl function plus one hydrogen took place in the acyl group of ion e. The clusters found in the spectra of compounds labeled in 2, 6 and 7 positions are consistent with hydrogen-deuterium exchange (reaction c) between these positions. Since major ions such as $[RCO\text{-}propyl+128]^+$, $[RCO\text{-}propyl+170]^+$ and

TABLE IV

Relative Abundances of Ions p and Neighboring Ions Formed From Some Deuterium Labeled Triglycerides

Triglyceride of	RCO+115	RCO+116	RCO+117	RCO+118
18:0	100	59	9	0
2,2-d_2-14:0 (II)	17	47	100	50
3,3-d_2-15:0 (VII)	100	62	9	2
4,4-d_2-16:0 (IX)	100	100	27	6
5,5-d_2-17:0 (XIII)	100	57	14	3
6,6-d_2-18:0 (XVIII)	100	66	25	2
18:0, 2-d-glycerol (XX)	15	100	65	12
	RCO+120	RCO+121	RCO+122	RCO+123
18:0, glycerol-d_8 (XIX)	100	62	20	4

$[RCO\text{-propyl}+184]^+$ are absent, it appears that simple cleavage (reaction a) of the extrusion product does not take place.

In the mechanisms for formation of ions e, m and p six-membered ring structures have been assumed. Ethanediol diesters, if they fragment by mechanisms analogous to triglycerides, should produce ions with five-membered rings. The mass spectra (22) of long chain diesters of ethanediol exhibited a series of m/e 99+14n, of which m/e 99 was intense, suggesting that a five-membered structure is possible (ion j), similar to that postulated for alk-1-enyl ether esters of diols (29). The spectra of long chain diesters of 1,3-propanediol (22) contained a similar series beginning with m/e 113, suggesting a six-membered ring. Unfortunately, with triglycerides the ions $[RCO+128]^+$ can be written with a five-membered ring involving two adjacent ester linkages or a six-membered ring involving the 1,3-ester linkages. These cannot be presently distinguished.

ion j m/e 99 ion k m/e 113

$[RCO + 74]^+$

The proposed structure, m for this characteristic ion is consistent with accurate mass measurements and with retention of deuterium when the glyceryl moiety is labeled (1).

ion l $[M-RCO_2]^+$ ion m $[RCO + 74]^+$

A feasible mechanism for the formation of this ion is loss of a substituted ketene from $[M\text{-}RCO_2]^+$. The origin of the proton attached to the ether-oxygen is unknown. $[RCO+74]^+$ was never altered when the acyl group was labeled in various positions. Exchange of the deuteriums might account for II not displaying $[RCO+74+1]^+$ in its spectrum.

$[RCO + 115]^+$

Two possible structures, p and q, were distinguished by means of labeling. Structure q could arise via a McLafferty rearrangement of M^+ followed by loss of a carboxylic acid molecule, analogous to the reaction h → i → e. Because deuterium atoms in 2 position are retained (18,19) in a McLafferty rearrangement, this sequence applied to II should yield an ion q in which three deuterium atoms are retained, equivalent to RCO+116. However, in structure p, 4 deuterium atoms would be retained, equivalent to $[RCO+117]^+$. The latter was found to be the case as is shown in Table IV.

The following mechanism which is somewhat similar to the proposed pathway leading to e is put forward although there are no metastable peaks to support it.

ion n ion o ion q ion p $[RCO + 117]^+$

Except for IX the intensities of the $[p+1]^+$ ions are about twice the calculated abundance (21-25%) of p's isotopic peak, implying coincidence with a second ion. In the case of IX $[p+1]^+$ is greatly enhanced. This might be due to increase of the second ion, or to retention of some of the dueterium procured in the McLafferty rearrangement. If the latter is the case, the increase of the isotopic peak might come about via the keto-enol equilibrium of b. This would also account for some loss of deuterium in II reflected in the increased $[p-1]^+$ ion.

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Sphingolipid Long Chain Bases

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ABSTRACT

About 60 natural sphingolipid long chain bases have been identified or proposed. A review is given on their nomenclature, distribution, metabolism, biologic properties, chemistry and methods of characterization.

INTRODUCTION

Until a few years ago, sphingolipid long chain bases were thought to be restricted to a few molecular species, namely sphingosine and dihydrosphingosine in animal tissue and phytosphingosine in plant tissue (Fig. 1). At present, however, evidence for at least 60 species of these bases has been presented (Table I), about half of which may exist in a single sphingolipid fraction, e.g., sphingomyelin of bovine milk (1) and bovine kidney (Karlsson et al., unpublished results; 40). The number of bases is therefore similar to the number of sphingolipid fatty acids (2,3), and a great variety of ceramide (fatty acid - base amide) species is possible. The biological meaning of this is unknown. Most sphingolipids, all of which have a ceramide as lipophilic residue, are parts of biological membranes. For a fundamental knowledge of the role sphingolipids may play in membrane function, their complete structures are needed. The purpose of the present paper is to discuss some of our knowledge concerning the long chain bases, the specific parts of the sphingolipids.

NOMENCLATURE

The name of the classical long chain base was invented by Thudichum (4) for a substance "which is of an alcaloidal nature, and to which, in commemoration of the many enigmas which it presented to the inquirer, I have given the name of Sphingosin". The name dihydrosphingosine was first used for the hydrogenated product of sphingosine (6), and phytosphingosine was introduced to indicate an origin of this base in plants (5). In 1967 the IUPAC-IUB Commission on Biochemical Nomenclature (CBN) published proposals for sphingolipid

CH_2OH	CH_2OH	CH_2OH	CH_2OH
$HC-NH_2$	$HC-NH_2$	$HC-NH_2$	$HC-NH_2$
$HC-OH$	$HC-OH$	$HC-OH$	$HC-OH$
CH_2	HC	$HC-OH$	$HC-OH$
CH_2	‖ CH	CH_2	$(CH_2)_3$
CH_2	CH_2	CH_2	HC
CH_2	CH_2	CH_2	‖ CH
$(CH_2)_{10}$	$(CH_2)_{10}$	$(CH_2)_{10}$	$(CH_2)_8$
CH_3	CH_3	CH_3	CH_3
DIHYDROSPHINGOSINE	SPHINGOSINE	PHYTOSPHINGOSINE	DEHYDRO-PHYTOSPHINGOSINE
SPHINGANINE	4-SPHINGENINE	4D-HYDROXYSPHINGANINE	4D-HYDROXY-8-SPHINGENINE
SATURATED DIHYDROXY BASE	UNSATURATED DIHYDROXY BASE	SATURATED TRIHYDROXY BASE	UNSATURATED TRIHYDROXY BASE
d18:0	$d18{:}1^{4trans}$	t18:0	$t18{:}1^{8trans}$

FIG. 1. Fischer projection formulas of the major types of sphingolipid long chain bases. Below the formulas are shown the original trivial names, names according to a recently proposed nomenclature (see text), common word designations and possible shorthand formulas, respectively.

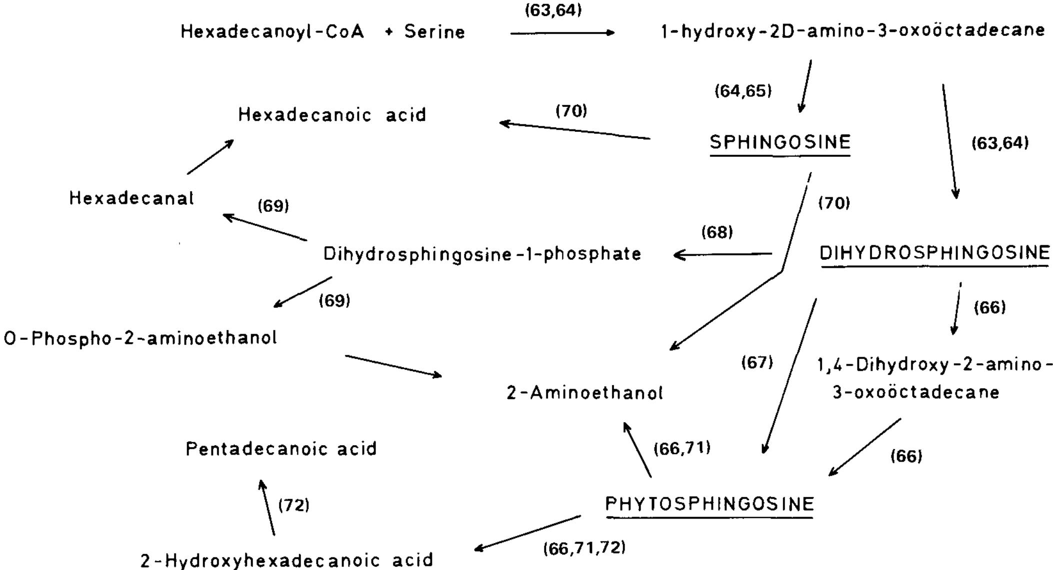

FIG. 2. Summary of long chain base ana- and catabolism. Figures in parentheses are References.

nomenclature, including the long chain bases (7,8). For illustration the bases collected in Table I have been given both systematic names and names according to the suggested nomenclature (Fig. 1). As remarked by CBN, and illustrated in Table I, the new nomenclature is more concise than systematic names only if the terms introduced include both substituents and their configurations. If structural details are unknown, which should be indicated, the new system may even be longer. This may explain why some workers have omitted notations of unknown details. Docosa-4-sphingenine, for example, means D-*erythro*-1,3-dihydroxy-2-amino-*trans*-4-docosene. 1,3-Dihydroxy-2-amino-4-docosene, with unknown configuration and double bond geometry, should however be named 2x,3x-docosa-*x*-4-sphingenine. 4D-hydroxynonadecasphinganine means D-*ribo*-1,3,4-trihydroxy-2-aminononadecane. 1,3,4-Trihydroxy-2-aminononadecane, with unknown configuration, should be translated 4x-hydroxy-2x,3x-nonadecasphinganine.

Several authors have used synonyms of the term sphingolipid long chain base(s), not including specific information on chain length, stereochemistry, etc., for example, sphingosine bases, sphinganines, sphingosines. This does not follow the proposals given, but may indicate a need for a general term in addition to long chain base(s). Sphingosine(s) may be seriously considered, and as a suggestion the term may mean sphingolipid long chain base(s) in a general sense, not implying chain length, substituents or stereochemistry. A deletion of the specific meaning of sphingosine (D-*erythro*-1,3-dihydroxy-2-amino-*trans*-4-octadecene) would be needed. This convention would help indexing, and is analogous to a prostaglandin nomenclature (9), where prostaglandin(s) is a general term, applicable to all substances of the group, and prostanoic acid has a specific chemical meaning, analogous to sphinganine.

For spoken and written communication a simpler system has been added (10), now in use in several laboratories (1,3,11), and not meaning a new nomenclature. It will be used throughout this paper. Groups of bases are named according to major structural characteristics, and this subdivision often follows chromatographic properties, e.g., on thin layers (10,11). A designation refers to number of hydroxyls (e.g., dihydroxy bases), number of double bonds (e.g., dienic bases), or both (e.g., dienic dihydroxy bases). A derived shorthand formula system (1,10,11) may be as given in Figure 1 and Table I where d and t mean dihydroxy and trihydroxy, respectively; the figure before the colon denotes carbon-chain length and that after the colon is for the number of double bonds. The position and geometry of a double bond may be indicated as in Figure 1, and chain branching by the prefixes br or iso and anteiso; n-9 means a double bond in position 9 from the methyl end (7).

NATURALLY OCCURRING BASES

The known naturally occurring bases are listed in Table I. Only 10 of these have been

TABLE I

Natural Spingolipid Long Chain Bases

Number of carbon atoms	Shorthand formula	Systematic name	IUPAC–IUB name	Origin[a]	Isolated[b]	References
Saturated straight chain dihydroxy bases						
12	d12:0	1,3-Dihydroxy-2-aminododecane	2X,3X-dodecasphinganine	A		(1)
14	d14:0	1,3-Dihydroxy-2-aminotetradecane	2X,3X-tetradecasphinganine	A		(1,14,15)
15	d15:0	1,3-Dihydroxy-2-aminopentadecane	2X,3X-pentadecasphinganine	A		(1)
16	d16:0	1,3-Dihydroxy-2-aminohexadecane	2X,3X-hexadecasphinganine	A,P		(1,14,16-20)
17	d17:0	1,3-Dihydroxy-2-aminoheptadecane	2X,3X-heptadecasphinganine	A,P		(1,3,18-20)
18	d18:0	D-*erythro*-1,3-dihydroxy-2-aminooctadecane	Sphinganine	A,B,P	+	(21-23)
19	d19:0	1,3-Dihydroxy-2-aminononadecane	2X,3X-nonadecasphinganine	A,P		(1,20)
20	d20:0	1,3-Dihydroxy-2-aminoeicosane	2X,3X-eicosasphinganine	A,P	+	(20,24-26)
Monounsaturated straight chain dihydroxy bases						
12	d12:1^{4}	1,3-Dihydroxy-2-amino-4-dodecene	2X,3X-dodeca-*x*-4-sphingenine	A		(1)
13	d13:1^{4}	1,3-Dihydroxy-2-amino-4-tridecene	2X,3X-trideca-*x*-4-sphingenine	A		(1)
14	d14:1^{4}	D-*erythro*-1,3-dihydroxy-2-amino-*trans*-4-tetradecene	Tetradeca-4-sphingenine	A	+	(1,14,15,17,28,29,[c])
15	d15:1^{4}	1,3-Dihydroxy-2-amino-4-pentadecene	2X,3X-pentadeca-*x*-4-sphingenine	A		(1,17)
16	d16:1^{4}	D-*erythro*-1,3-dihydroxy-2-amino-*trans*-4-hexadecene	Hexadeca-4-sphingenine	A	+	(1,3,14,15,17,18,29,30-32,[c])
17	d17:1^{4}	1,3-Dihydroxy-2-amino-4-heptadecene	2X,3X-heptadeca-*x*-4-sphingenine	A		(1,3,14,17,18,30,32)
18	d18:1^{4}	D-*erythro*-1,3-dihydroxy-2-amino-*trans*-4-octadecene	4-Sphingenine	A	+	(33-36)
18	d18:1$^{\text{not 4}}$	1,3-Dihydroxy-2-aminooctadecene	2X,3X-sphingenine	A		(14)
18	d18:1^{8}	1,3-Dihydroxy-2-amino-*trans*-8-octadecene	2X,3X-8-sphingenine	P		(37)
19	d19:1^{4}	1,3-Dihydroxy-2-amino-4-nonadecene	2X,3X-nonadeca-*x*-4-sphingenine	A		(1,38)
20	d20:1^{4}	D-*erythro*-1,3-dihydroxy-2-amino-*trans*-4-eicosene	Eicosa-4-sphingenine	A	+	(24-27,39)
20	d20:1^{11}	1,3-Dihydroxy-2-amino-11-eicosene	2X,3X-eicosa-*x*-11-sphingenine	A		(15)
22	d22:1^{4}	1,3-Dihydroxy-2-amino-4-docosene	2X,3X-docosa-*x*-4-sphingenine	A		(27)
22	d22:1^{9}	*Erythro*-1,3-dihydroxy-2-amino-*cis*-9-docosene	*erythro*-docosa-*cis*-9-sphingenine	A	+	(40,[d])
22	d22:1^{13}	*Erythro*-1,3-dihydroxy-2-amino-*cis*-13-docosene	*erythro*-docosa-*cis*-13-sphingenine	A	+	(40,[d])
Diunsaturated straight chain dihydroxy bases						
16	d16:2	1,3-Dihydroxy-2-aminohexadecadiene	2X,3X-hexadecasphingadienine	A		(32,38)
17	d17:2	1,3-Dihydroxy-2-aminoheptadecadiene	2X,3X-heptadecasphingadienine	A		(32,38,41)
18	d18:2^{4,14}	D-*erythro*-1,3-dihydroxy-2-amino-*trans*-4,*cis*-14-octadecadiene	4,*Cis*-14-sphingadienine	A	+	(19,32,40,41)
18	d18:2^{4,8}	D-*erythro*-1,3-dihydroxy-2-amino-*trans*-4,*trans*-8-octadecadiene	4,8-Sphingadienine	A	+	(40,47,[e])
18	d18:2^{4,13}	1,3-Dihydroxy-2-amino-4,13-octadecadiene	2X,3X-*x*-4,*x*-13-sphingadienine	A		(42)
18	d18:2^{4,12}	1,3-Dihydroxy-2-amino-4,12-octadecadiene	2X,3X-*x*-4,*x*-12-sphingadienine	A		(18)
18	d18:2	1,3-Dihydroxy-2-aminooctadecadiene	2X,3X-sphingadienine	A		(14)
20	d20:2^{4,11}	1,3-Dihydroxy-2-amino-4,11-eicosadiene	2X,3X-eicosa-*x*-4,*x*-11-sphingadienine	A		(15)
20	d20:2	1,3-Dihydroxy-2-aminoeicosadiene	2X,3X-eicosasphingadienine	P		(32)
22	d22:2^{4,9}	*Erythro*-1,3-dihydroxy-2-amino-*trans*-4,*cis*-9-docosadiene	*Erythro*-docosa-4,*cis*-9-sphingadienine	A	+	(40,[d])
22	d22:2^{4,13}	*Erythro*-1,3-dihydroxy-2-amino-*trans*-4,*cis*-13-docosadiene	*Erythro*-docosa-4,*cis*-13-sphingadienine	A	+	(40,[d])

Saturated straight chain trihydroxy bases						
16	t16:0	1,3,4-Trihydroxy-2-aminohexadecane	4X-hydroxy-2X,3X-hexadecasphinganine	A,P		(20,40,[d])
17	t17:0	1,3,4-Trihydroxy-2-aminoheptadecane	4X-hydroxy-2X,3X-heptadecasphinganine	A,P		(10,16,20,40,[d])
18	t18:0	D-*ribo*- 1,3,4,-trihydroxy-2-aminooctadecane	4D-hydroxysphinganine	A,P	+	(36,40,43)
19	t19:0	1,3,4-Trihydroxy-2-aminononadecane	4X-hydroxy-2X,3X-nonadecasphinganine	A,P	+	(20,38,40,[d])
20	t20:0	D-*ribo*- 1,3,4-trihydroxy-2-aminoeicosane	4D-hydroxyeicosasphinganine	A,P	+	(20,44-46)
Unsaturated straight chain trihydroxy bases						
18	$t18:1^{8}$	D-*ribo*- 1,3,4-trihydroxy-2-amino-*trans*-8-octadecene	4D-hydroxy-8-sphingenine	P	+	(43)
Saturated branched chain dihydroxy bases						
17	iso d17:0	1,3-Dihydroxy-2-amino-15-methylhexadecane	15-Methyl-2X,3X-hexadecasphinganine	A,B		(1,23,48,[d])
18	iso d18:0	1,3-Dihydroxy-2-amino-16-methylheptadecane	16-Methyl-2X,3X-heptadecasphinganine	A,B		(1,23,48,[d])
19	iso d19:0	1-3-Dihydroxy-2-amino-17-methyloctadecane	17-Methyl-2X,3X-sphinganine	A,B		(1,23,48,[d])
20	iso d20:0	1,3-Dihydroxy-2-amino-18-methylnonadecane	18-Methyl-2X,3X-nonadecasphinganine	A		(1,48)
17	anteiso d17:0	1,3-Dihydroxy-2-amino-14-methylhexadecane	14-Methyl-2X,3X-hexadecasphinganine	A		(1)
19	anteiso d19:0	1,3-Dihydroxy-2-amino-16-methyloctadecane	16-Methyl-2X,3X-sphinganine	A		(1,40,[d])
Unsaturated branched chain dihydroxy bases						
14	iso $d14:1^{4}$	1,3-Dihydroxy-2-amino-12-methyl-4-tridecene	12-Methyl-2X,3X-trideca-*x*-4-sphingenine	A		(1)
15	iso $d15:1^{4}$	1,3-Dihydroxy-2-amino-13-methyl-4-tetradecene	13-Methyl-2X,3X-tetradeca-*x*-4-sphingenine	A		(1)
17	iso $d17:1^{4}$	1,3-Dihydroxy-2-amino-15-methyl-4-hexadecene	15-Methyl-2X,3X-hexadeca-*x*-4-sphingenine	A		(1,48)
18	iso $d18:1^{4}$	1,3-Dihydroxy-2-amino-16-methyl-4-heptadecene	16-Methyl-2X,3X-heptadeca-*x*-4-sphingenine	A		(1,3,10,16,40,48,[d])
19	iso $d19:1^{4}$	1,3-Dihydroxy-2-amino-17-methyl-4-octadecene	17-Methyl-2X,3X-*x*-4-sphingenine	A		(1,40,48,[d])
15	anteiso $d15:1^{4}$	1,3-Dihydroxy-2-amino-12-methyl-4-tetradecene	12-Methyl-2X,3X-tetradeca-*x*-4-sphingenine	A		(1)
17	anteiso $d17:1^{4}$	1,3-Dihydroxy-2-amino-14-methyl-4-hexadecene	14-Methyl-2X,3X-hexadeca-*x*-4-sphingenine	A		(1)
19	anteiso $d19:1^{4}$	1,3-Dihydroxy-2-amino-16-methyl-*trans*-4-octadecene	16-Methyl-2X,3X-4-sphingenine	A	+	(1,3,40,[d])
20	anteiso $d20:1^{4}$	1,3-Dihydroxy-2-amino-17-methyl-4-nonadecene	17-Methyl-2X,3X-nonadeca-*x*-4-sphingenine	A		(40,[d])
Saturated branched chain trihydroxy bases						
17	iso t17:0	1,3,4-Trihydroxy-2-amino-15-methylhexadecane	4X-hydroxy-15-methyl-2X,3X-hexadecasphinganine	A		(40,[d])
18	iso t18:0	1,3,4-Trihydroxy-2-amino-16-methylheptadecane	4X-hydroxy-16-methyl-2X,3X-heptadecasphinganine	A		(40,[d])
19	iso t19:0	1,3,4-Trihydroxy-2-amino-17-methyloctadecane	4X-hydroxy-17-methyl-2X,3X-sphinganine	A		(16,[d])
21	iso t21:0	1,3,4-Trihydroxy-2-amino-19-methyleicosane	4X-hydroxy-19-methyl-2X,3X-eicosasphinganine	A		(49)
19	anteiso t19:0	1,3,4-Trihydroxy-2-amino-16-methyloctadecane	4X-hydroxy-16-methyl-2X,3X-sphinganine	A		(16,40,[d])

[a]A means animal, B bacteria and P plant.

[b]+ means that the base has been isolated.

[c]Karlander et al., in preparation.

[d]Karlsson et al., unpublished results.

[e]Karlsson, unpublished results.

TABLE II

Natural, Covalently Bound, Forms of Long Chain Bases

Base Carbon Atom Involved	Type of Bond	Example of Sphingolipid
1	Glycoside	Glycosphingolipids
1	Phosphoric acid ester	Sphingomyelin, phytoglycolipid
1	Phosphonic acid ester	Ceramide phosphonoethylamine
2	Amide	Ceramide, glycosphingolipid, phosphosphingolipid
3	Ether, vinyl ether	3-0-alkyl or alkenyl cerebrosides (51)

TABLE III

Natural Distribution of Major Types of Long Chain Bases

Type of base	Animals	Plants[a]	Bacteria
dn:0	+	+	+
$dn{:}1^{4}$	+	(52)	
$dn{:}1^{not\ 4}$	+	+	
dn:2	+	(32)	
br dn:0	+		+
br $dn{:}1^{4}$	+		
tn:0	+	+	
tn:1		+	
br tn:0	+		

[a]Figures in parentheses refer to papers where the actual bases have been proposed.

given complete chemical structures, and 17 have been isolated. Most of them have been proposed from studies of oxidation products of mixtures of bases, like aldehydes, alcohols or fatty acids, and in some cases from combined gas liquid chromatography-mass spectrometry (GLC-MS) of derivatives of intact bases (see Methods). The structural characteristics of these bases are summarized as:

Polar end in common: 1,3-dihydroxy-2-amino.
Number of carbon atoms: (12-) 14-22.
Methyl branching of paraffin chain: iso (n-1), anteiso (n-2).
Configurations: 2D, 3D, 4D.
Unsaturation:

(a) Monoenes:	(b) Dienes:
$dn{:}1^{4trans}$	$d18{:}2^{4trans,14cis}$
$d18{:}1^{not\ 4}$	$d18{:}2^{4trans,8trans}$
$d18{:}1^{8?trans}$	$d18{:}2^{4,X}$
$t18{:}1^{8trans}$	$d20{:}2^{4,11}$
$d20{:}1^{11}$	$d22{:}2^{4trans,9cis}$
$d22{:}1^{9cis}$	$d22{:}2^{4trans,13cis}$
$d22{:}1^{13cis}$	

All of them have a 1,3-dihydroxy-2-amino polar residue, and the number of carbon atoms varies from 22 down to 14, or perhaps 12, including odd numbers. Branching of the paraffin chain may be iso or anteiso, with a possible asymmetry in the last case. A third hydroxyl group may be in position 4, and an isolated fourth hydroxy group has been indicated (12,13). The configuration at carbon atoms 2, 3 and 4 is D. Concerning the unsaturation, only one type of trihydroxy base has been found, with an isolated *trans* double bond in position 8 (n-10). Dihydroxy bases may have up to two double bonds. The monoenoic bases possess an allylic *trans* double bond (4 position), or an isolated *cis* (n-9 or n-13) or *trans* (n-10) double bond. In all known dienic bases one double bond is *trans*-4 (allylic), the other an isolated *cis* (n-4, n-9, or n-13) or *trans* (n-10) double bond.

CHEMICALLY BOUND FORMS OF LONG CHAIN BASES

About 3% of the total bases of the yeast *Hansenula ciferrii* (50) have a free amino group (Karlsson et al., unpublished results), and free bases have also been found (Karlsson et al., unpublished results) in human kidney from autopsy. In the majority of cases, however, bases are bound as amides to fatty acids (ceramides), which may exist as such or are the common parts of all other sphingolipids. The types of chemical bonds are given in Table II.

DISTRIBUTION

The natural distribution of major groups of long chain bases is shown in Table III. All types of bases except unsaturated trihydroxy bases, so far specific for plants, have been found in animals. Branched chain bases have not yet been detected in plants, and there is no conclusive evidence for the presence of allylic bases in plants and bacteria. In the following section comments on the patterns of bases in some tissues will be given.

Nerve tissue is the classical object of sphingolipid studies, and the structure of sphingosine (Fig. 1) was obtained mainly from investigations on brain cerebroside (34). Nerve sphingolipids have a relatively simple base composition. Human brain cerebroside and sulfatide contain d18:1, small amounts of d18:0 and traces of lower homologs (40) (Table IV). Brain gangliosides have about equal amounts of 18- and 20-carbon dihydroxy bases (24-27), and d20:1 is found in small amounts in human brain ceramide (40,53), diglycosylceramide

(40) and sphingomyelin (40,53). Human peripheral nerve (*Nervus ischiadicus*) lipids (40) have no 20-carbon bases but higher amounts of lower homologs than brain lipids (40). An indication of species differences is the finding (54) of d20:1 in rabbit brain and spinal cord cerebroside, and the absence of this base in human brain and peripheral nerve cerebroside (40). In the human, d20:1 of ceramide and sphingomyelin is localized almost exclusively in grey matter, combined with stearic acid (53). An increase with age of 20-carbon bases has been noted for ganglioside (59) and ceramide (40). A relatively high amount of d18:0 has been found in human peripheral nerve (40) and rabbit spinal cord (54), which confirms an earlier finding from bovine nerve tissue (62). Trihydroxy bases have not been found in nerve tissue, except for a trace amount in peripheral nerve sulfatide and in brain ganglioside (40). Branched chain bases seem to exist in peripheral nerve, but have not been conclusively detected in the central nervous system (40). This may indicate a blood brain barrier for branched chain precursors of long chain bases, and a suggestion has been made (16) that branched chain bases have their origin in intestinal microorganisms.

The long chain base composition of blood plasma (cell free tissue) ceramide (40,55), sphingomyelin (3,19,32,56) and glycolipids (40,57) has been reported. Ceramide and sphingomyelin are similar and differ from monoglycosyl- and diglycosylceramide, which contain trihydroxy bases (40), and have lower amounts of dienic bases. Table IV presents the composition of human sphingomyelin, based on studies by several workers (3,19,32,40,41,56). Plasma sphingomyelin has the highest content of d18:2 so far reported for mammalian tissues.

Sphingomyelin of atherosclerotic human aorta has a composition similar to but not identical with human plasma sphingomyelin (14). A double bond positional isomer of $d18:1^4$ was proposed to be present, as well as a second dienic base.

In contrast to many other mammalian tissues studied so far (40), kidney has a very complex long chain base pattern and contains most of the known mammalian bases (10,11,16,40). Normal and branched chain di- and trihydroxy bases with 16 to 20 carbon atoms are present, as are dihydroxy bases with two double bonds. Differences have been shown for various regions of human and bovine kidney (10,114) and for different human kidney glycolipids (94), especially concerning relative content of trihydroxy bases. In a recent paper (11) bovine kidney gangliosides were found to be similar to

TABLE IV

Long Chain Base Composition (Relative Amounts) of Human Blood Plasma Sphingomyelin and Brain Cerebroside

Long chain base	Plasma sphingomyelin	Brain cerebroside
d16:0	1	Trace
d17:0	1	Trace
d18:0	2	2
$d16:1^4$	10	Trace
$d17:1^4$	5	Trace
$d18:1^4$	62	98
d17:2	1	—
$d18:2^{4,14}$	14	Trace
iso $d18:1^4$	2	—
anteiso $d19:1^4$	2	—

sphingomyelins (40) but with a higher content of trihydroxy bases. The relative content of branched chain bases is 5 to 10 times higher in bovine than human kidney (10,16), and these bases may be absent in the rat (16). Sphingomyelin of bovine milk (1) is also rich in branched chain compounds. As some protozoa contain branched chain di- and trihydroxy bases (48,49), it was suggested (16) that the origin of such bases in bovine material was an intestinal synthesis by microorganisms. A dietary origin has been discussed for phytosphingosine, mainly present in kidney and intestinal mucosa (58).

Several non-mammalian tissues have been analyzed during the last few years. Some plant sphingolipids have been characterized (13,32,37,43,52) presenting dihydroxy as well as trihydroxy bases, a double bond isomer of $d18:1^4$ (37), and evidence for a tetrahydroxy base (12,13). The presence of allylic bases in plant tissue has been proposed (32,52). A yeast mutant, *Hansenula ciferrii*, with an extracellular deposition of acetylated di- and trihydroxy bases (50), contains predominantly normal saturated 18-carbon bases (20), but no allylic bases. Sphingolipids of bacteria (60) contain saturated normal and iso-branched dihydroxy bases (23). Two protozoans, where branched chain bases were first found, have been investigated (48,49). Of the unusual unsaturated bases listed in Table I and summary above, the 22-carbon bases were isolated from cerebroside of the sea star, *Asterias rubens* (Karlsson et al., unpublished results; 40) $d20:1^{11}$ and $d20:2^{4,11}$ were identified in phosphosphingolipids of a scorpion (15). $d18:2^{4,8}$ was isolated from ceramide phosphonoethylamine of the sea anemone, *Metridium senile* (40), and may be identical with a major base of a shell fish lipid (47,61). Several d20:2 species were reported to be present in yeast (32).

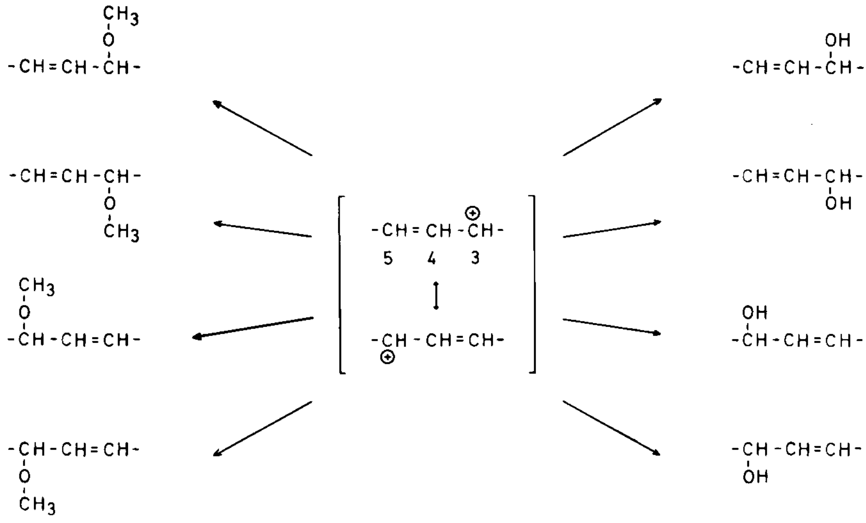

FIG. 3. Illustration of the formation of long chain base by-products due to acid catalyzed rearrangement and substitution at an allylic group. The carbonium ion is a resonance hybrid of carbon atoms 3 to 5 of an allylic long chain base. On the right hand side the possible products are shown of a nucleophilic addition of a hydroxyl ion (from water), and on the left hand side the products of an addition of a methoxyl ion (from methanol). The second product from above on the right is the reformed natural product (3D).

METABOLISM

A detailed discussion of the metabolism of long chain bases is beyond the scope of this review. A summary is given in a scheme (Fig. 2). Synthesis is achieved from acyl CoA and serine to a 3-keto intermediate, which is stereospecifically reduced to dihydrosphingosine. The exact mechanism of formation of the *trans* double bond of sphingosine is not yet known. Phytosphingosine has been shown to originate in dihydrosphingosine, so far only in yeast, but the mechanism is unknown. Nothing is known of the origin of isolated *cis* and *trans* double bonds listed above. Worthy of note is the existence of these unsaturations in both allylic and nonallylic bases: d18:1^{8trans} and d18:$2^{4trans,8trans}$, d20:1^{11} and d20:$2^{4,11}$, d22:1^{9cis} and d22:$2^{4trans,9cis}$, d22:1^{13cis} and d22:$2^{4trans,13cis}$. Also of interest is the parallel presence of *trans*-8 unsaturation in dihydroxy and trihydroxy base: d18:1^{8trans}, d18:$2^{4trans,8trans}$ and t18:1^{8trans}. The fact that d22:1^{4} seems to be absent from the sea star cerebroside (Karlsson et al., unpublished results; 40) may indicate that an isolated double bond is introduced before allylic unsaturation. Branched chain bases of higher animals have been suggested to originate in intestinal microorganisms (16).

All types of bases are catabolized via a cleavage between carbon atom 2 and 3, yielding a fatty acid and ethanolamine, probably through an ATP-consuming 1-phosphorylation of the base with initial formation of ethanolamine-1-phosphate.

BIOLOGIC PROPERTIES

Unbound long chain bases have biological activity. Worthy of mentioning is Thudichum's comment on his use of sphingosine nitrate for the treatment of human disease (73,74). Anticoagulant effects (75,76) and mycostatic and bacteriostatic (20,76) properties, as well as changes in circulation dynamics (76), have been reported. N-acetyl sphingosine and sphingomyelin have effects similar to cortison on tuberculin sensitivity in guinea pigs (77). Immunologic activity of glycosphingolipids has been known for many years (2,78), and recently antibodies have been prepared to sphingomyelin (79) and sphingosylphosphorylcholine chemically bound to protein (80). The finding (81) of lipid specific antibodies to dihydrosphingosine-protein conjugates was the basis of a suggestion for a role of the long chain base in sphingolipid immunology (80,81). Although nothing is known about the influence of long chain base variation on immunological properties of sphingolipids, it is reasonable to assume that di- and trihydroxy bases, free or chemically bound, may have different properties in this respect.

CHEMISTRY

The work leading to the complete chemical structures of the four bases shown in Figure 1, including their synthesis, has been reviewed in a recent monograph (82). The following remarks will be restricted to the chemistry in relation to the isolation and characterization of long chain bases.

It has been known since the beginning of this century (83,84) that sphingosine may produce ethers with methanol or ethanol during the acid degradation of a sphingolipid. The ether production was later found (85) to be related to the presence of an allylic alcohol portion (3-hydroxy-4-ene), known to undergo acid-catalyzed substitution and isomerization reactions (86). The products from model studies on sphingosine derivatives have been isolated and characterized (Karlsson, unpublished results; 40,85,87) and are shown in Table V. Methoxy derivatives appear in acidic methanol and 5-hydroxy derivatives in aqueous acid, due to a nucleophilic substitution with methoxy and hydroxy, respectively, including a carbonium ion (Fig. 3) as an intermediate. The two dehydration products with conjugated double bonds, which appear only when water is present, are probably derived from the two 5-hydroxy bases.

The presence of a small amount of a 3L-hydroxy compound (*threo* sphingosine) after methanolysis (Karlsson, unpublished results) may be explained by the production of water (nucleophilic reagent) when heating methanol-hydrochloric acid (88) or methanol-sulfuric acid. Alternatively, it may be due to a N → O-acyl shift (89) followed by inversion, but this mechanism has not been conclusively shown to operate on allylic long chain bases. The 3L isomer has also been found (Karlsson, unpublished results; 40) after acetolysis (90), and these conditions result in an inversion also of 9,10-dihydroxy stearic acids (Karlsson, unpublished results; 40). The mechanism in this case may therefore be similar to that discussed (91) for carbohydrate as a carbon-to-carbon acetoxyl shift followed by inversion. Trihydroxy bases produce a 3L, 4D isomer, and probably small amounts of 3D, 4L and 3L, 4L isomers (Karlsson, unpublished results; 40). The relative amounts of inversion products following this treatment have been illustrated by thin layer chromatography (TLC) (40).

Trihydroxy bases are to some extent dehydrated in acid to tetrahydrofuran derivatives (Table V), which has been illustrated by GLC (13,38). Several other minor by-products of the acid degradation of sphingolipids have been isolated as dinitrophenyl (DNP) derivatives (Karlsson, unpublished results), but their structures have not yet been determined. Bases stored or chromatographed as free amines are not stable (92), in contrast to hydrochlorides (Karlsson, unpublished results; 92) or DNP derivatives (Karlsson, unpublished results).

The most important by-products are those that are due to the allylic group, as discussed above. This is illustrated in Figure 4, where trimethylsilyl derivatives of long chain bases of bovine brain cerebrosides, degraded in different ways, have been analyzed by GLC. Figure 4A shows the by-product free fraction obtained by a periodate-borohydride-mild acid procedure (93), compared with water free (62) acid degradation (Fig. 4B), methanol free (94) acid degradation in water (Fig. 4C), and a methanol-water acid degradation (31) with a minimum of by-products (Fig. 4D).

METHODS

The classical procedure for long chain base isolation and characterization is acid sphingolipid degradation (62), followed by preparation and isolation of acetylated or benzoylated derivatives (62). The properties of these acylated derivatives allow the interpretation of stereochemistry (27,93). With the finding of complex mixtures of bases, however, which are often present in smaller amounts than in nerve tissue, a need for more sensitive methods of separation and characterization appeared. In the following section, methods used during the last few years for the isolation, structure determination and analysis of long chain bases will be indicated and briefly discussed.

Long Chain Base Analysis Without Degradation of the Sphingolipid. In most cases long chain bases are bound in sphingolipids as shown in Table II. As recently demonstrated (40,56,98-101), it is possible, by direct mass spectrometry of a sphingolipid preferably in silylated form, to gain information on long chain base structure, especially following a separation into sphingolipid species by GLC (56,98,100). By the development of GLC-MS, using different lipid derivatives for analysis and different stationary phases for the separation, detailed information about base structure, including stereochemistry, may be possible without isolation of individual bases. In this connection, *erythro* and *threo* isomers of dihydroxy bases have been separated by GLC (102,103) in a simple ceramide form (O-trimethylsilyl-N-acetyl derivatives). Using conventional methods, however, base species have to be isolated before a detailed characterization, which was the case for all completely

TABLE V

By-Products of Natural Long Chain Bases From the Degradation of the Sphingolipid

Systematic name	Conditions of formation	Probable mechanism	References
Products of dihydrosphingosine (D-*erythro*-1,3-dihydroxy-2-aminooctadecane)			
D-*threo*-1,3-dihydroxy-2-aminooctadecane	Acetolysis	Acyl shift with inversion	(40,[a])
Products of sphingosine (D-*erythro*-1,3-dihydroxy-2-amino-*trans*-4-octadecene)			
D-*threo*-1,3-dihydroxy-2-amino-*trans*-4-octadecene	Acid water, acid methanol	Acyl shift with inversion? Via allylic ion	(89,95)
1-Hydroxy-2D-amino-3D-methoxy-*trans*-4-octadecene	Acid methanol	Via allylic ion	(40,85,87,[a])
1-Hydroxy-2D-amino-3L-methoxy-*trans*-4-octadecene	Acid methanol	Via allylic ion	(40,85,87,[a])
1-Hydroxy-2D-amino-5D-methoxy-*trans*-3-octadecene	Acid methanol	Via allylic ion	(40,85,87,[a])
1-Hydroxy-2D-amino-5L-methoxy-*trans*-3-octadecene	Acid methanol	Via allylic ion	(40,85,87,[a])
1,5D-dihydroxy-2D-amino-*trans*-3-octadecene	Acid water	Via allylic ion	(40,96,[a])
1,5L-dihydroxy-2D-amino-*trans*-3-octadecene	Acid water	Via allylic ion	(40,96,[a])
1-Hydroxy-2D-amino-*trans*-3,*trans*-5-octadecadiene	Acid water	Via allylic ion and dehydration	(40,96,[a])
1-Hydroxy-2D-amino-(*cis,trans*)-3,5-octadecadiene	Acid water	Via allylic ion and dehydration	(40,96,[a])
1-Hydroxy-2D-aminooctadecane	Catalytic hydrogenation	Hydrogenolysis	(33,34)
Products of phytosphingosine (D-*ribo*-1,3,4-trihydroxy-2-aminooctadecane)			
2-Tetradecyl-3-hydroxy-4-aminotetrahydrofurane	Acid	Dehydration	(97)
1,3D,4L-trihydroxy-2D-aminooctadecane	Acetolysis	Acyl shift with inversion	(40,[a])
1,3L,4D-trihydroxy-2D-aminooctadecane	Acetolysis	Acyl shift with inversion	(40,[a])
1,3L,4L-trihydroxy-2D-aminooctadecane	Acetolysis	Acyl shift with inversion	(40,[a])

[a]Karlsson, unpublished results.

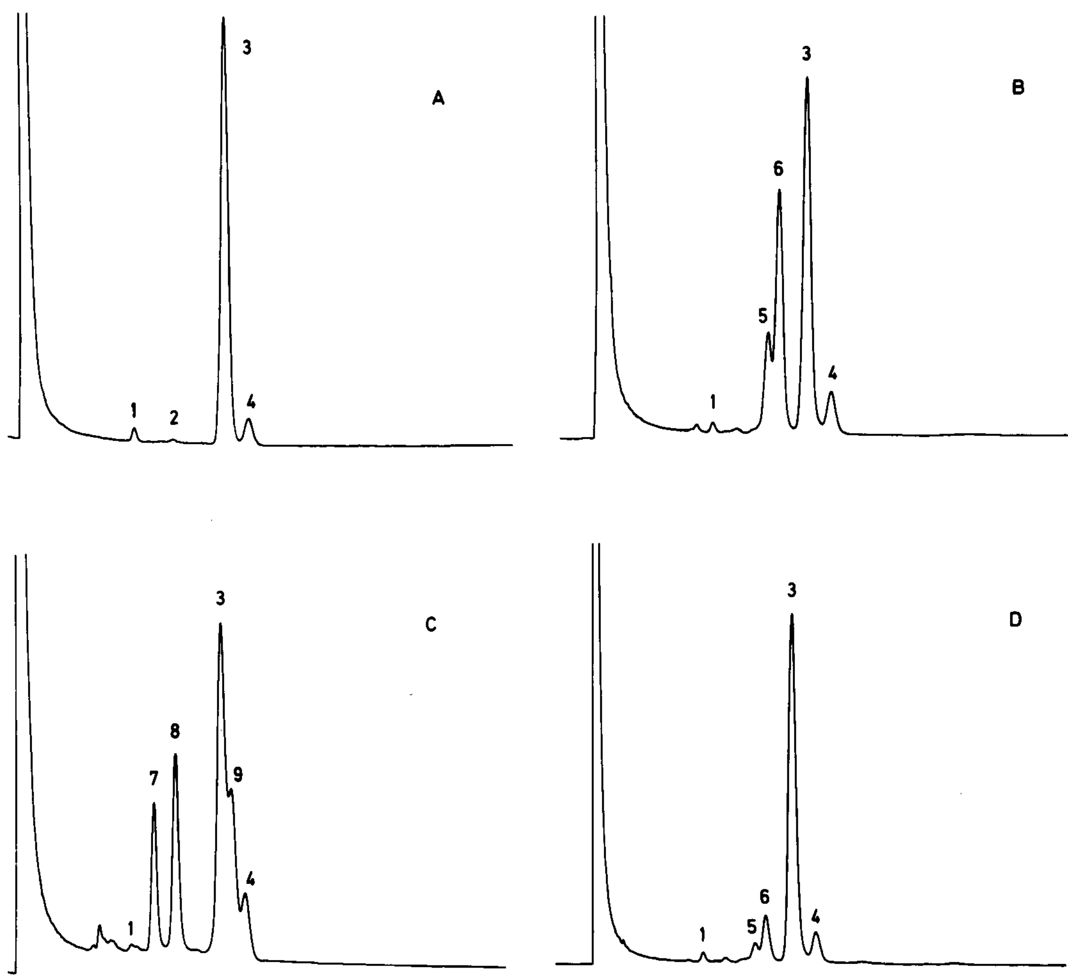

FIG. 4. Gas chromatograms of trimethylsilyl derivatives (110) of long chain bases of bovine brain cerebroside degraded in different ways. A is the by-product free fraction (93). B is the fraction obtained by refluxing for 6 hr in methanol-sulfuric acid 20:1, v/v (62). The base pattern in C was produced by refluxing for 6 hr in 2 M hydrochloric acid in water (94), and in D by a degradation for 18 hr at 70 C in concentrated hydrochloric acid-water-methanol 8.6:9.4:82, v/v/v (31). The peaks indicated are as follows: 1 = d16:1; 2 = d17:1; 3 = d18:1; 4 = d18:0; 5 = 1-hydroxy-2-amino-3-methoxy-*trans*-4-octadecene; 6 = 1-hydroxy-2-amino-5-methoxy-*trans*-3-octadecene; 7 = 1-hydroxy-2-amino-(*cis,trans*)-3,5-octadecadiene; 8 = 1-hydroxy-2-amino-*trans*-3,*trans*-5-octadecadiene; 9 = 1,5-dihydroxy-2-amino-*trans*-3-octadecene. The separation was performed on 3% OV-1 coated on 100-120 mesh Gas-Chrom Q (Applied Science Laboratories), at 235 C and with an argon gas flow of 15 ml/min. The retention time for peak 3 was about 12 min.

known structures (10 species) shown in Table I.

Degradation of the Sphingolipid. The isolation of long chain bases must start with a degradation of the sphingolipid. Concerning the most simple sphingolipid structure, ceramide, an alkaline hydrolysis, gives free long chain bases in quantitative yield and with retained natural structure (93). Alkaline degradation of phosphosphingolipids or glycosphingolipids has not been studied in detail, but a recent suggestion (Morrison, personal communication) of using saturated barium hydroxide in water-dioxane at elevated temperature (120 C) for hydrolysis may prove useful for all types of sphingolipids. An alternative to degrade a glycosphingolipid without changing a dihydroxy base portion (trihydroxy bases are split between carbon atoms 3 and 4) is a periodate oxidation of the glycose, followed by reduction to a polyol and mild acid hydrolysis to ceramide (27,93) (Fig. 4A).

A procedure for the conversion of more complex sphingolipids to ceramide is desirable for two reasons. Ceramides may be easily

hydrolyzed without chemical change of the bases, and ceramides are more easily separated into species (different fatty acid-base combinations) than more complex sphingolipids, preferably by TLC (Karlsson and Pascher, in preparation; 3,56,100,104,105) followed by GLC (56,100). Concerning phosphosphingolipids an enzyme preparation is available (104,105), which converts all lipids tested so far to ceramide: sphingomyelins (56,104,105), ceramide phosphorylethanolamine (106,107), ceramide phosphonoethylamine (40,106,107). Different sphingomyelin species have been hydrolyzed (105), containing both di- and trihydroxy bases and normal and 2-hydroxy fatty acids. Enzymes are known which degrade glucosyl- and galactosylceramide to ceramide (108), but no preparation for practical purposes is yet available.

At present the most frequently used hydrolysis medium for sphingolipids is a hydrochloric acid-water-methanol mixture (31), giving base hydrochlorides with a minimum of by-products (Fig. 4D). Glycosphingolipids are quantitatively degraded, but only half of the sphingomyelin bases are obtained (109). To permit an easier isolation (40) of DNP derivatives of glycosphingolipid bases, a degradation in hydrochloric acid-water has been done (40,94), in spite of a higher proportion of chemically changed bases (Fig. 4C).

Isolation of Individual Long Chain Bases. Several methods have been used in recent years for the isolation of individual long chain bases. Countercurrent distribution was used to isolate O-triacetyl-N-benzoyl derivatives of t18:0 and t18:1 of plant origin (43), and to prepare triacetyl d18:1 and d20:1 from brain gangliosides (27). d18:2 of human blood plasma was enriched by TLC of free bases, followed by preparative GLC of O-trimethylsilyl-N-acetyl derivatives (32). The same base was isolated as a triacetyl derivative by argentation chromatography on thin layer plates (41), and as a DNP derivative by argentation chromatography in combination with reversed-phase paper chromatography (19). The last mentioned set of methods was also used to isolate a number of DNP derivatives of bases listed in Table I (d14:1, d16:1, anteiso d19:1, d20:1, d20:0, d22:1, d22:2, t18:0, t19:0, t20:0).

Structure Analysis of Long Chain Bases. As mentioned above, structure information of long chain bases may be obtained without the isolation of pure species. After hydrolysis, O-trimethylsilyl (31,110) or O-trimethylsilyl-N-acetyl (102,103) derivatives of mixtures of bases may be analyzed by GLC-MS. In this way several bases of human plasma sphingomyelin have been characterized (32,110), and the structure of animal trihydroxy base (human kidney) was established in this way (20). This method was also used to identify a mixture of d20:1^{11} and d20:2^{4,11} and other bases of a scorpion (15), using O-trimethylsilyl-N-acetyl derivatives before and after hydroxylation (111) to get information on double bond positions. Mass spectrometry of derivatives of intact bases is not able to inform about branching of the paraffin chain. This was shown for the silylated DNP derivative of anteiso d19:1 (Karlsson et al., unpublished results; 40), and for an iso branched compound from bacteria (23). In these cases the branching was deduced after oxidation to aldehyde and fatty acid, respectively, and analysis of these by GLC-MS.

Double bond positions have been established after oxidative cleavage and analysis of fragments (14,18,32,41), or by hydroxylation with osmium tetroxide followed by mass spectrometry (40,111). Mass spectrometry may be used to identify an allylic double bond (102), but positions of isolated double bonds cannot be found by this technique without preceding hydroxylation (40,111). The stereochemical configuration of double bonds was shown by IR spectrometry in the cases known and indicated in Table I and summary above. For geometry of d18:2^{4,14} a partial reduction with hydrazine was done, followed by isolation of d18:1^{4trans} and d18:1^{14cis} (41). The position of *cis* and *trans* unsaturation of d22:2 was concluded from *cis* geometry in position 9 and 13 of the two d22:1, present in the same source (40). d18:2^{4,8} has a *trans* double bond absorption with about double the intensity of d18:1^{4}, indicating two *trans* double bonds (40).

Relative stereochemical configuration (*erythro*, *threo*) of dihydroxy bases may be shown by GLC (102,103), which was used to complete the structure of d18:2^{4,14} (41), after reduction to d18:0. There is a good separation by TLC of *erythro* and *threo* isomers of DNP derivatives of saturated dihydroxy bases (40), allowing configurational assignments, and a similar application to trihydroxy bases is possible (40). *Erythro* and *threo* isomers of free d18:1 (112) and of DNP d18:1 (40,94,113) are also separated by TLC or on paper impregnated with silica gel.

The classical way of showing absolute configuration is optical rotation measurement, applied to, e.g., d18:2^{4,14} (41) and animal trihydroxy base (40). It has, however, been shown that ceramides differing in configuration at the fatty acid 2-hydroxy group are well

separated by TLC (Karlsson and Pascher, in preparation; 105,114). As a development of this a separation by GLC has been done of enantiomers of *erythro* and *threo* dihydroxy bases in amide linkage with L-lactic acid, as trimethylsilyl derivatives (unpublished). When developed, this method may allow analysis on a microscale of mixtures of homologs, and should be applicable also to trihydroxy bases.

Analysis of Long Chain Bases. A review of this subject has recently been presented (115). For the detection of bases, TLC of free bases (112) or DNP derivatives (94) have been used, and paper chromatography of free bases (116) or DNP derivatives (113,117). The method in most common use, however, is a periodate oxidation followed by GLC of aldehydes (118) or, more recently, GLC analysis of trimethylsilyl ethers of free bases (31,110) or of O-trimethylsilyl-N-acetyl derivatives (102,103). The GLC methods are also useful for quantitation of individual bases, and for GLC-MS (102,110,111). It has been demonstrated (1,40,119) that different aldehyde or alcohol species are separated better by GLC than their parent bases in different forms, and for a quantitation the oxidation products are therefore preferred. The periodate or tetraacetate (94) oxidation may, however, produce the same aldehyde from a di- and a trihydroxy base, e.g., hexadecanal from d18:0 and t19:0 (38,103). Di- and trihydroxy bases therefore have to be separated before the oxidation (10,11,58), which is easily done by TLC.

As discussed above, acid degradation of a sphingolipid may change the long chain base structure. By-products of major bases may therefore interfere with minor natural bases in the analysis, even in the case of a minimum of by-products (Fig. 4D). To avoid this problem, by-products have been separated before GLC analysis, as is possible by use of DNP derivatives and column chromatography (40,94) or TLC (11). To avoid methyl ethers, which are difficult to separate, the sphingolipid degradation was performed in water (94) (Fig. 4C), in spite of less by-products in water-methanol (31) (Fig. 4D).

Quantitative Determination of Total Bases. The most convenient method for total base determination is the colorimetric method with the methyl orange-complex (120). The incomplete hydrolysis of sphingomyelin, mentioned above, should be borne in mind, and may explain the result from plasma sphingomyelin (121), where only part of the theoretical amount of bases was extractable into the organic phase. DNP derivatives have recently been used for quantitation (1,109), and was also used several years ago in a microanalysis procedure for brain lipids (122).

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The Absorption of Fatty Acids by Functional Bovine Mammary Cells

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ABSTRACT

Freshly dispersed bovine mammary cells rapidly absorbed long chain fatty acids from the culture medium. Differences in the rates of absorption were observed, i.e., palmitic > stearic > oleic > myristic > linoleic acid. The preponderance of the fatty acids absorbed were esterified into triglycerides (>75%) and the remainder were mostly incorporated into phospholipids. The cells secreted triglycerides into the culture medium. Of the phospholipid classes, phosphatidylcholine always contained most of the radioactivity in all experiments with labeled fatty acids. These observations are related to the metabolism of mammary cells in vivo.

INTRODUCTION

The actual contribution of plasma free fatty acids to the synthesis of ruminant milk fat, in vivo, had been equivocal until Annison et al. (1), using a combination of arteriovenous difference measurements and isotope dilution techniques, showed that the lactating goat mammary gland absorbed substantial quantities of plasma free fatty acids. Lauryssens et al. (2) reported that the perfused bovine mammary gland removed labeled stearic acid from the perfusate and Patton and McCarthy (3) and Dimick et al. (4) showed that the goat mammary gland absorbed and metabolized free fatty acids following their intramammary infusion. The lactating mammary gland also absorbs large quantities of free fatty acids (3-4 g/100 ml secreted) which are liberated in the immediate vicinity of the gland by the action of lipoprotein lipase on the triglycerides of the low density lipoproteins (1,5-7).

While it is assumed that the secretory cells absorb and metabolize these free fatty acids in vivo, it has not been actually demonstrated. Hence the present experiments were carried out to study the absorption and metabolism of free fatty acids and to examine the relative rates of absorption of different fatty acids by dispersed secretory cells obtained from lactating bovine.

MATERIALS AND METHODS

The cells were prepared from fresh lactating tissue obtained from Holstein cows immediately post mortem. The method of preparation, washing, and dispersal has been thoroughly described (8,9). The composition of the culture media and the incubation and harvesting methods have been reported (9). Following incubation, the cells (approximately 1×10^8/treatment) were separated from the culture media by centrifugation (500 *g* for 10 min). The lipids were extracted from both the cells and culture media by the procedure of Folch et al. (10). The lipid extracts were fractionated by thin layer chromatography (TLC). The neutral lipid classes were separated on Silica Gel G plates, 250 μ thickness, using a solvent system of petroleum ether-diethyl ether-acetic acid (70:25:1 v/v/v). The phospholipid classes were fractionated by two-dimensional TLC (11). The various fractions were identified by co-chromatographing with standard mixtures of known lipids (Applied Science, State College, Pa.). The various lipid classes were located using iodine vapor. After the lipid spots were marked, the iodine was evaporated by gently heating the plate to 60 C under a stream of nitrogen. The radioactivity in each spot was determined by transferring the silica gel spot and the adsorbed lipids to a scintillation vial, adding the scintillation fluors (2,5-diphenyloxazole and 1,4-bis-(5-phenyloxazolyl-2)-benzene, Nuclear-Chicago, Chicago, Ill.) in toluene, and counting the sample in a (Packard TriCarb) liquid scintillation spectrophotometer. The recovery of radioactivity from these plates ranged from 77% to 103% and appropriate corrections were made for recovery and quenching effects when computing the final data. The efficiency of the scintillation counter (70%) was not considered in the calculation of radioactive data.

The pure 1-^{14}C fatty acids (New England Nuclear, Boston, Mass.) were converted to their respective sodium salts (12) and introduced into the culture flasks at the initiation of each incubation. The TLC equipment and supplies were obtained from Brinkmann Instruments, Westbury, N.Y. and purified solvents (Mallinckrodt, St. Louis, Mo.) were used. Kodak No-Screen medical x-ray film (Kodak, Rochester,

TABLE I

Per Cent Distribution of Radioactivity in Phospholipids Following Incubation of Bovine Mammary Cells With Labeled Fatty Acids (12 hr)

Fatty acids	Quantity of radioactive esters as phospholipids, %	Percentage of total phospholipid radioactivity							
		Sphingomyelin	Phosphatidylcholine	Phosphatidylinositol	Phosphatidylserine	Phosphatidylethanolamine	Lysophosphatidylcholine	Cerebrosides	Cardiolipin
C14:0	9±2	4.8	77.1	7.4	1.3	6.6	2.4	0.4	---
C16:0	10±4	4.5	75.5	10.1	2.9	6.0	1.5	0.5	Trace
C18:0	13±3	5.4	56.3	17.8	3.2	12.9	1.7	0.8	1.9
C18:1	13±5	1.9	70.6	11.3	3.4	9.8	0.7	0.9	1.4
C18:2	20±6	2.0	68.6	17.5	3.9	7.4	Trace	Trace	0.6

[a]Average of three analyses.

N.Y.) was used for autoradiography. The purity of the radioactive fatty acids was checked before and after each experiment by gas liquid radiochromatography (13).

RESULTS

The initial series of experiments showed that the preponderance of the long chain fatty acids absorbed by the cells were esterified. Most of these esters were triglycerides (70-80%) and the remainder were associated with the phospholipids and diglycerides (Table I). Significant quantities of ester lipids, predominantly triglycerides, appeared in the culture medium as the incubations progressed (Fig. 1). The radioactive triglycerides appeared as two and, occasionally three, discrete spots on the autoradiograms. These probably coincide with the triglycerides of different molecular weights which occur in mammary cells and milk (14).

Using the extent of esterification as an index of active absorption, the relative rates of uptake of various long chain fatty acids, normally supplied to the lactating mammary gland, were measured and the results are summarized in Figure 2. The long chain saturated fatty acids were absorbed to a greater extent than the unsaturated acids, particularly linoleic acid.

As demonstrated in the autoradiograms the adsorbed fatty acids were predominantly esterified in triglycerides (70-80%) and most of the remainder was incorporated into the phospholipids (Table I). The phosphatidylcholine fractions contained most of the radioactivity associated with the phospholipids although phosphatidylinositol, phosphatidylethanolamine and phosphatidylserine were significantly labeled by all of the substrate fatty acids studied.

DISCUSSION

These experiments demonstrated that the dispersed secretory cells prepared from lactating bovine mammary tissue actively absorb and metabolize exogenous free fatty acids. The cells showed a preference for saturated fatty acids, which is perhaps logical since the functional ruminant mammary gland normally absorbs large amounts of these, particularly palmitic and stearic acid, from the circulating plasma (1,4-6) even though significant quantities of unsaturated acids are also present. The data confirm the assumption that the secretory cells are responsible for fatty acid absorption and metabolism in the lactating ruminant mammary gland.

The mode of absorption of the fatty acids by mammary cells is unknown but conceivably

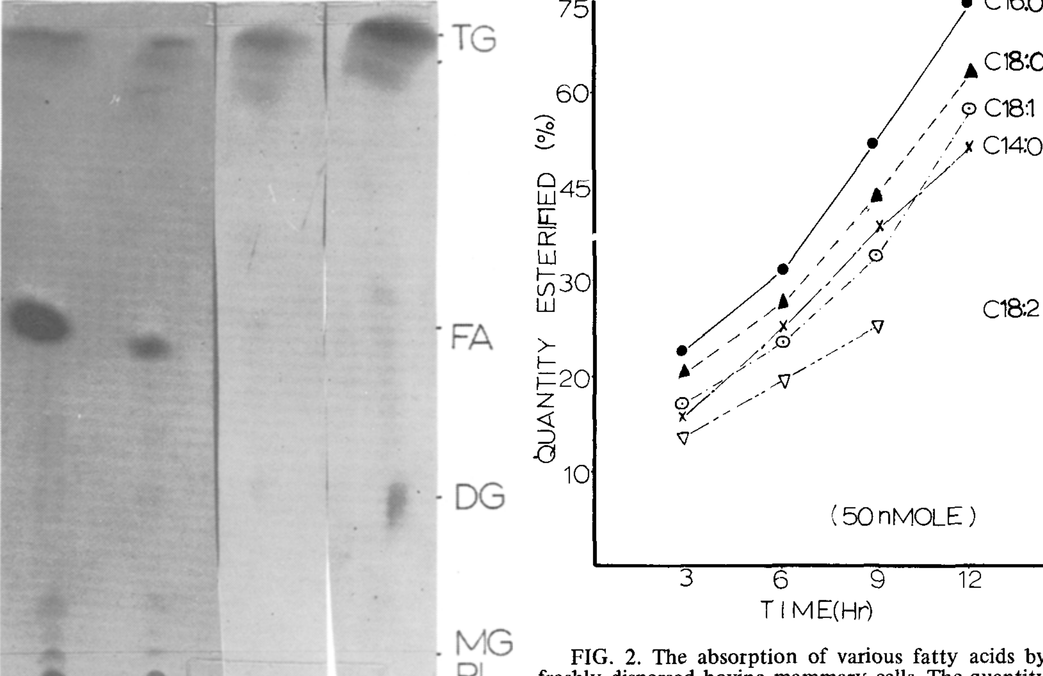

FIG. 1. Thin layer autoradiogram of the radioactive lipids extracted from bovine mammary cells and culture media following incubation with 1-^{14}C-sodium myristate for 4 and 8 hr. PL, phospholipids; MG, monoglycerides; DG, diglycerides; FA, free fatty acids; TG, triglycerides (two spots indicated).

FIG. 2. The absorption of various fatty acids by freshly dispersed bovine mammary cells. The quantity esterified indicates active absorption. C16:0, palmitic; C18:0, stearic; C18:1, oleic; C18:2, linoleic and C14:0, myristic acid.

the mechanism is similar to that proposed by Shapiro (15), i.e., it involves adsorption, activation and absorption of the activated fatty acids. Esterification is the principal mechanism by which free fatty acids are assimilated by tissues (15) and the present experimental observations were consistent with such a mechanism because the preponderance of the fatty acids absorbed by the cells were esterified. Presumably the activation of these exogenous fatty acids occured in the cell membrane since negligible quantities of free acids appeared in the cells and significantly, it has been reported (18) that the cell membrane fraction of the liver cell is the richest locale of long chain fatty acid activating enzymes.

The rapid esterification of absorbed fatty acids has been observed in the mamary gland of the rabbit (16), goat (1), guinea pig (12), mouse (17), and by dispersed mammary cells from the rat (Kinsella, unpublished data) and generally the triglycerides are the major lipid class synthesized. Askew et al. (19) while studying glyceride synthesis by subcellular portions of bovine mammary tissue, observed different rates of esterification for various fatty acids (oleic ~ palmitic > stearic > linoleic). This observation and the experimental data presented above may reflect different activation rates, acyl transferase activities and/or the relative availability of acyl acceptors in the mammary cell.

The bovine cells incorporated the various fatty acids into the cellular phospholipid classes, indicating that the mammary cells can synthesize these molecules at least partially (20). Characteristically, the phosphatidylcholine fraction contained the preponderance of the radioactivity in all of the experiments. Similar labeling patterns have been observed in vivo (3) and in vitro with other substrate precursors (20). These labeling patterns have been rationalized by proposing an active metabolic role for phosphatidylcholine in milk lipid biosynthesis (21). Coincidentally, the pattern of absorption of the fatty acids by the mammary cells is quite similar to that observed by Van den Bosch et al. (22) in experiments with rat liver microsomes and using 2-acyl-3sn-glycerol-phosphorylcholine as the acyl acceptor. If such a mechanism was

operating in the mammary cells it would account for the labeling of the phosphatidylcholine; whether or not it is a route to the synthesis of triglycerides (21) is the subject of further studies.

Finally, this study demonstrates that the bovine mammary cell can absorb and metabolize oleic and linoleic acid when these are presented as free acids. The precise quantitative origin of these fatty acids in milk fat has not been defined. Significant quantities of the oleic acid are derived by desaturation of stearic acid in the mammary gland (1,2,24). However, dietary studies indicate that both oleic and linoleic acids of milk fat can be derived from circulating plasma lipids (23).

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Effects of Diets Rich in Saturated Fatty Acids With or Without Added Cholesterol on Plasma Lipids and Lipoproteins[1]

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ABSTRACT

Semi-synthetic diet I which contained 16% hydrogenated coconut oil and 5% cholesterol, and diet II, identical to I but without cholesterol supplement, were fed to dogs for four months to determine the effects of added cholesterol on lipemia produced by diets high in saturated fatty acids (FA) and lacking essential FA. In addition, diet I was fed to another group of dogs for 12 to 16 months. The initiation of lipemia was very similar in all experimental animals. Plasma from dogs on diets I and II showed significant increases in lipid concentration and changes in FA per cent composition within the first week, as compared to controls, while during the first month there was no difference in lipid concentration or FA distribution in all lipid fractions between I and II. At the 10th and 16th weeks plasma total and free cholesterol and phospholipid were significantly higher in the group on diet I, with the cholesterol supplement, than on diet II with no added cholesterol, but there was no difference in triglyceride concentration between these groups. Dogs on diet I for 12 to 16 months showed a further and substantial increase in plasma FA concentration; these changes were most marked in cholesteryl esters. Little or no lipoprotein with electrophoretic and ultracentrifugal properties of alpha-lipoprotein was present in the plasma. Immunotechniques showed that it was present. The composition of dietary FA had great influence in producing this hyperlipemia. Lipemia produced was not a simple reflection of the FA in these diets as evidenced by the increase in some FA, e.g., $C_{16:1}$, which was absent in the experimental diets and $C_{18:1}$, which contributed only 3.4% of the FA. Large increases in palmitoleate and oleate indicate synthesis or mobilization or both from other tissues. Diets composed predominantly of saturated medium chain length fatty acids, with or without added cholesterol were equally effective in the initiation of hyperlipemia. Data also suggest that added cholesterol is necessary for sustaining hyperlipemia.

INTRODUCTION

Numerous dietary procedures have been used to produce arteriosclerosis but few of them are effective in producing lesions in dogs. One which was effective in causing lesions in this species was described by Malmros and Sternby (1). As part of our search for a suitable model for studying arteriosclerosis, the present studies were made to determine the sequence and type of changes which occur in plasma lipids, fatty acids of lipid fractions, and lipoproteins of dogs fed a semisynthetic, hydrogenated coconut oil containing diet. This diet is high in saturated fat composed predominantly of medium chain length fatty acids deficient in essential fatty acids, and contains a 5% cholesterol supplement. A second group of dogs which received the same diet but without cholesterol supplement was also studied.

METHODS AND EXPERIMENTAL PROCEDURES

Twelve adult mongrel male dogs weighing between 12 and 20 kg were immunized against distemper, examined and found to be in good

TABLE I

Total Fatty Acid Weight Per Cent Composition of Control and Experimental Diets I and II[a]

FA	Control diet	Experimental diets I and II
8:0	---	6.4
10:0	---	4.2
12:0	---	37.0
14:0	2.6	17.0
16:0	23.0	12.0
18:0	13.0	19.0
16:1	3.4	---
18:1	35.0	3.4
18:2	23.0	0.3

[a]Diet I, FA per cent composition same as in diet II but with 5% cholesterol supplement.

[1]Presented in part at the AOCS Annual Meeting, New Orleans, April 1970.

PLASMA PHOSPHOLIPID AND TRIGLYCERIDE (mg/100 ml)

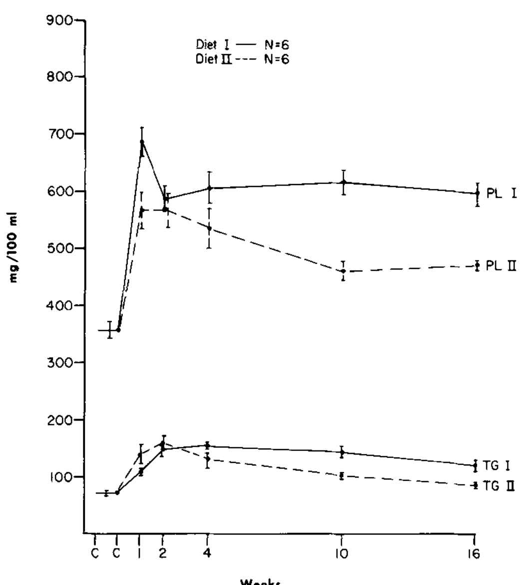

FIG. 1. Plasma total cholesterol (TC) and free cholesterol (FC) concentration in mg/100 ml. Mean concentration with SE are given for control (C) and 1,2,4,10 and 16 weeks on diet with 5% cholesterol (diet I) and without cholesterol (diet II).

PLASMA TOTAL AND FREE CHOLESTEROL (mg/100 ml)

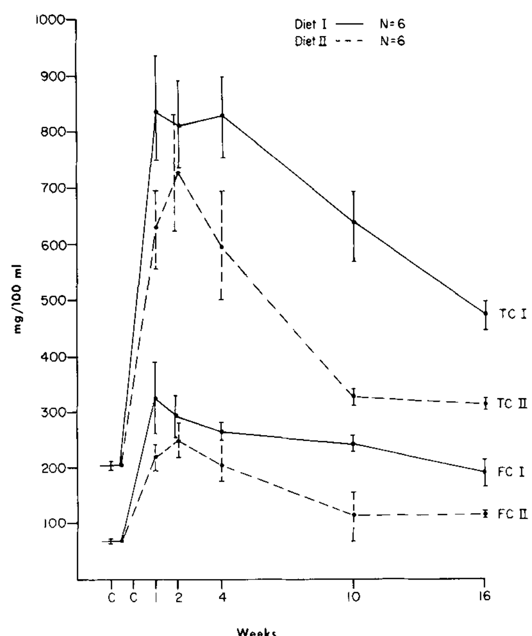

FIG. 2. Plasma phospholipid (PL) and triglyceride (TG) concentration in mg/100 ml. Mean concentration with SE are given for control (C) and 1,2,4,10 and 16 weeks on diet with 5% cholesterol (diet I) and without cholesterol (diet II).

health, free of parasitic infestation and were maintaining a constant body weight on a control ration (Ken L Ration:beef byproduct 5:1). Two control samples of blood were taken at least one week apart from each dog after which they were given one of two experimental diets. Diet I contained 4.77 g cholesterol per 100 g (1), which was replaced in diet II by alphacel. Both contained 16% hydrogenated coconut oil, which was deficient in essential fatty acids and contained a high proportion of saturated fatty acids as shown in Table I. Sucrose (29%), casein (20%), adequate vitamins and salts, and nonnutritive bulk made up the remainder of the diet. [Diets were prepared in pellet form by Nutritional Biochemicals Corp. Cleveland, Ohio.] The salt mixture comprised 2.86% of the diet and the KI level was 0.086% of this mixture. Control diet comprised 25% protein, 8% fat, 55% carbohydrate, 9% ash and 3% crude fiber. The diets were given to dogs in amounts sufficient to maintain body weight, and were available at all times or until the daily ration was consumed. Water was given ad libitum. Dogs were housed in individual cages in a room maintained at 22 ± 1 C. All blood samples were taken from animals fasting at least 17 hr. Blood studies were made after dogs were on diets 1,2,4,10 and 16 weeks. EDTA was the anticoagulant and all samples were immediately chilled. Blood of five other dogs maintained on diet I was also studied periodically for 12 to 16 months.

Groups of dogs on either diet for different time periods are referred to in the text by the diet (I or II) followed by the number of months they were kept on the diet, e.g., the groups in the 4 month short term period are I-4 and II-4 and dogs on diet I for 12 to 16 months are I-14.

Total cholesterol (TC) and free cholesterol (FC), triglyceride (TG), and phospholipids (PL), were determined on plasma. Fatty acid (FA) analyses calculated in terms of weight per cent composition as well as concentration in μg/ml were carried out on isolated cholesteryl esters (CEFA), triglyceride (TGFA), free fatty acid (FFA) and phospholipid (PLFA) fatty acids of plasma by gas liquid chromatography as previously described (2). Serum lipoproteins were determined ultracentrifugally at a density of 1.21 (3) or by paper electrophoresis using albuminated buffer (4). Lipoprotein fractions were separated ultracentrifugally into chylomicron (i.e., d $<$ 1.006 after 30 min spinning at

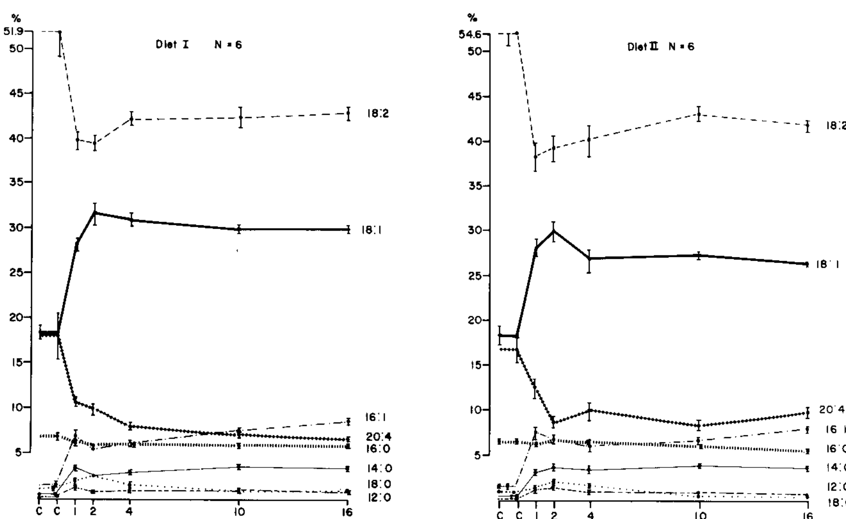

FIG. 3. Plasma cholesterol ester FA distribution. Mean per cent composition with SE are given for control (C) and 1,2,4,10 and 16 weeks on diet with 5% cholesterol (diet I) and without cholesterol (diet II).

30,000 rpm); VLDL fraction (i.e., d < 1.006 after 18 hr spinning), d 1.006-1.063 and d 1.063-1.21 after 18 hr spinning). These fractions separated ultracentrifugally, and the lipoprotein fractions resolved by paper electrophoresis were studied by immunodiffusion or immunoelectrophoretic techniques, using antisera prepared in rabbits against ultracentrifugally prepared d 1.006-1.063 canine beta-lipoprotein repeatedly ultracentrifuged to insure purity, against d 1.063-1.21 alpha-lipoprotein, and against whole canine serum. The lipoproteins and serum used as antigen for antisera preparation were from healthy mongrel dogs on a normal diet. The purity of the antigens used for immunization was checked by thin layer starch-gel electrophoresis (5) and by immunoelectrophoresis (6) using antisera against canine whole serum.

RESULTS

Plasma Lipid Fractions

Control Values. The mean concentration ± SE for each lipid fraction, given in units of mg/100 ml of plasma, was as follows: TC (210 ± 8.9), FC (68 ± 4.4), TG (75 ± 5.5), and PL (351 ± 9.9).

Short-Term Dietary Period. Within one week after starting the special diets, mean TC concentration of plasma was significantly increased from control level (Fig. 1). Since control lipid levels of dogs of group I and group II did not differ significantly the normal values were statistically treated as a single group. Plasma TC of the group on diet I increased from 210 to 835 mg/100 ml and of those on diet II to 625 mg/100 ml at the end of the first week. During the entire period on experimental diets, the dogs' plasma cholesterol concentrations were significantly higher than control levels. While plasma TC levels of dogs on diet I and II did not vary significantly from each other during the first 4 weeks, after 10 and 16 weeks of diet TC levels of the group on diet I were significantly higher than those on diet II which contained no added cholesterol.

The mean FC concentration of plasma of dogs on diet I increased from 68 to 323 mg/100 ml and of dogs on diet II to 218 mg/100 ml during the first week on experimental diets and remained significantly elevated during the entire dietary period (Fig. 1). Like the total, free cholesterol of diet I group was significantly greater than that of diet II after 10 and 16 weeks.

The ratio of free to total cholesterol was 0.32 in control dogs and it varied from 0.32 to 0.40 in experimental dogs with no significant difference between group I and II.

The mean plasma PL concentration of dogs on diet I increased from 351 to 685 mg/100 ml and on diet II to 565 mg/100 ml during the first week (Fig. 2). As was observed for values of TC and FC, significant differences in amount of increase in plasma PL of both experimental groups occurred only after 10 and 16 weeks.

The mean plasma TG concentration of

experimental dogs increased relatively much less than other lipid components (Fig. 2). TG levels were never more than 30% to 50% above control values.

Long-Term Dietary Period. Although some degree of lipemia was sustained in all five dogs of the group maintained on diet I for 12 to 16 months (I-14), variability of lipid levels in the same dog was noticed from time to time and between animals on this diet for identical time periods. The ranges of plasma lipid concentrations at the final bleeding given in units of mg/100 ml were total lipid 1130 to 6320, PL 475 to 1325, TG 160 to 1000, TC 400 to 4000, FC 156 to 700, and esterified cholesterol 244 to 3300.

Plasma Fatty Acids

Cholesteryl Ester FA

Control Values: The sum of all CEFA made up 27.9% of total FA in plasma. It consisted of 48% linoleic acid, 18.5% oleic, 18% arachidonic and 7% palmitic acid. All other FA made up the remaining 8.5% of total control CEFA pattern.

Short-Term Dietary Period: Plasma CEFA pattern, calculated in terms of per cent composition, showed very rapid alterations after dogs were placed on experimental diets (Fig. 3). Changes were maintained with only a slight rebound, for the entire dietary period. Medium chain saturated FA ($C_{12:0}$ and $C_{14:0}$) as well as monounsaturated FA ($C_{16:1}$ and $C_{18:1}$) increased and polyunsaturated FA ($C_{18:2}$ and $C_{20:4}$) decreased in plasma CEFA of both groups. The largest changes were in $C_{18:1}$ which increased from mean control level of 18.5% to 30% and $C_{20:4}$ which decreased from 18% to 8%. Changes in both experimental groups were similar, except for $C_{18:1}$ and $C_{20:4}$ at the 16th week period. Cholesteryl oleate was higher ($p < 0.005$) and arachidonate was lower ($p < 0.025$) in plasma of animals of group I than that of group II. After dogs were on either diet one week, their plasma CEFA concentrations were significantly increased above control levels (Table II) and remained elevated with a few exceptions throughout the entire experimental period. The highest concentrations of some FA esterified with cholesterol were observed after one or two weeks of dietary treatment. Later a decrease from the high early level was noted in most FA in plasma CEFA of both groups of dogs. The decline from the peak concentrations of $C_{18:0}$ and $C_{18:2}$ was especially marked at 10th and 16th week in plasma of dogs on diet II.

While the relative percentage of stearic and linoleic acids was reduced, the absolute concentration of stearic acid was increased for four weeks. Only at the 10th and 16th week periods its concentration was similar to that of controls. Linoleic acid concentration was significantly increased in dogs on diet with cholesterol supplement through the entire experimental period. Its increase in concentration in dogs without cholesterol supplement was significant only for the first four weeks. It remained unchanged from control concentration at the 10th and 16th week periods.

Unlike other plasma CEFA, the concentration of $C_{20:4}$ was increased for one week only in dogs of diet II and for two weeks in group I. The plasma cholesteryl arachidonate concentration of the dogs after 4, 10 and 16 weeks was as low or lower than their normal levels. Medium chain saturated FA, $C_{12:0}$ and $C_{14:0}$, showed the largest increase in relative concentration. Since the amount of these FA in controls was low, their contribution to the total increase in CEFA concentration in experimental dogs was relatively small. Only at the 10th week period total CEFA concentration was lower in dogs without than in dogs with cholesterol supplement.

Long Term Dietary Period: CEFA concentration increased after 4 months and was much higher after the dogs were on diet I for 12 to 16 months. The increase in concentration of cholesteryl oleate was from 159 ± 19 to 7685 ± 2503 μg/ml, $p < 0.01$) and accounted for more than half the net increase in CEFA in plasma of the dogs on diet with cholesterol supplement that were studied for the longer period. While relative percentage of plasma cholesteryl linoleate and arachidonate showed further decrease with prolonged dietary treatment of dogs, being 20% and 4%, respectively after 12 to 16 months compared with 42% and 10% at 4 months of treatment; in terms of concentration however, cholesteryl linoleate increased from 469 ± 36 μg/ml in controls to 1105 ± 176 μg/ml in group I-4 and further increased to 2211 ± 681 μg/ml in group I-14 ($p<0.01$). Arachidonate increased from 154 ± 14 to 198 ± 16 and 381 ± 152 μg/ml, respectively. Because of the larger variability from the mean in group I-14, the latter increase was not significant.

Triglyceride FA

Control Values: The sum of all TGFA made up 9% of the total FA in plasma. Oleic acid (39%) was the most abundant FA component of TGFA pattern of control dogs' plasma. Linoleic acid represented 19%, palmitic 17%, stearic 7%, palmitoleic 5.8% and arachidonic acid 4.5% of TGFA. All other FA made up the remaining 7.7% of total control TGFA pattern.

TABLE II

Plasma Cholesteryl Ester FA Concentration (μg/ml)

FA	Control Mean ± SE	Group	Mean ± SE in experimental groups I and II				
			1st Week	2nd Week	4th Week	10th Week	16th Week
12:0	.08 ± .02	I	29 ± 7	18 ± 4	16 ± 2	21 ± 2[a]	8 ± 2
		II	32 ± 11	25 ± 8	21 ± 9	10 ± 1	7 ± 1
14:0	1.6 ± .3	I	79 ± 14	63 ± 8	69 ± 7	86 ± 11	48 ± 4
		II	83 ± 21	75 ± 18	72 ± 21	49 ± 5	40 ± 5
16:0	59 ± 4	I	145 ± 16	156 ± 25	150 ± 22	158 ± 19[b]	88 ± 9[b]
		II	158 ± 34	149 ± 40	138 ± 37	81 ± 6	62 ± 4
18:0	8 ± 1	I	36 ± 6	63 ± 12	42 ± 11	18 ± 4[b]	10 ± 2
		II	38 ± 10	51 ± 17	38 ± 16	7 ± 2	5 ± 1
16:1	13 ± 1.6	I	175 ± 49	147 ± 26	163 ± 28	198 ± 30[a]	132 ± 16[b]
		II	157 ± 66	119 ± 49	100 ± 48	68 ± 12	73 ± 12
18:1	159 ± 12	I	678 ± 95	870 ± 163	876 ± 180	778 ± 110[a]	476 ± 58[b]
		II	730 ± 151	694 ± 193	643 ± 176	379 ± 21	306 ± 19
18:2	469 ± 36	I	938 ± 77	1055 ± 126	1172 ± 225	1105 ± 176[b]	693 ± 109
		II	900 ± 100	796 ± 156	777 ± 100	579 ± 53	484 ± 25
20:4	154 ± 14	I	238 ± 11	258 ± 40	218 ± 46	174 ± 19	105 ± 18
		II	274 ± 33	176 ± 52	178 ± 36	114 ± 22	115 ± 18
20:3	3 ± .7	I	14 ± 3	29 ± 7	23 ± 7	32 ± 10	21 ± 4
		II	16 ± 4	16 ± 8	25 ± 7	25 ± 8	21 ± 6
Total CEFA	850 ± 75	I	2392 ± 244	2716 ± 375	2780 ± 520	2593 ± 347	1604 ± 213
		II	2487 ± 393	2181 ± 511	2080 ± 402	1324 ± 107	1162 ± 69

[a]Means of group I and II are significantly different ($p < 0.005$-0.001).
[b]Means of group I and II are significantly different ($p < 0.02$-0.01).

Short Term Dietary Period: The per cent composition of plasma TGFA showed significant changes after animals were on special diets for one week; these alterations were sustained throughout the dietary period. Within one week total TGFA concentration was increased and remained elevated throughout the entire experimental period (Table III). Similar increases occurred with both diets except that after 10 weeks, dogs on diet I had higher TGFA levels than those on diet II. The concentration in plasma of saturated fatty acids, $C_{12:0}$, $C_{14:0}$ and $C_{16:0}$ and of unsaturated fatty acids, $C_{16:1}$ and $C_{18:1}$ esterified with triglyceride were significantly increased above normal one week after starting the diets and continued to increase with longer dietary treatment. Higher levels of TG $C_{12:0}$, $C_{14:0}$ and $C_{18:1}$ were observed in plasma of dogs in diet I than those on diet II ($p < 0.05$) after 10 weeks. While the relative percentage of stearic acid was reduced, the absolute concentration was unchanged. Linoleic and arachidonic acids, however, in either diet were reduced in relative percentage as well as in absolute concentration.

Long-Term Dietary Period: After animals were on diet I for 12 to 16 months their plasma total TGFA concentration was six times that observed after four months on that diet. The relative percentage of medium chain saturated FA in group I-14 had increased still further as compared to the short term TGFA pattern; $C_{12:0}$ was increased from 0.1% in controls to 3% in group I-4 at four months, and further to 9% at 12 to 16 months, while $C_{14:0}$ was 1%, 7.5% and 12%, respectively. In contrast, linoleic acid which was 19% in controls, was reduced to 9.2% in this group at four months and further reduced to 2.5% at 12 to 16 months. While the relative percentage of linoleic acid was reduced the absolute concentration was unchanged. Oleic acid esterified with TG showed the highest net gain in concentration. It increased from 110 ± 11 μg/ml in controls to a high of 1200 ± 421 μg/ml in the hyperlipemic dogs of I-14 group. Arachidonic acid also increased, from 12 ± 1.5 μg/ml in control to 29 ± 6.9 μg/ml in I-14 group.

Free FA

Control Values: The FFA made up only 4.4% of total FA present in the control dogs' plasma. As in TGFA, oleic acid comprising 36% of total was the largest FA component. Palmitic acid represented 28%, linoleic 15.5% and stearic 13.5%. All other FA represented only 7% of the total FFA pattern.

Short Term Dietary Period: The per cent distribution of the FA of FFA fraction of dogs' plasma on diets I and II did not change as rapidly as did CE or TGFA. Its concentration also was much less affected by experimental diets than TG or CEFA (Table IV). While the relative percentages of $C_{12:0}$, $C_{14:0}$, $C_{16:0}$ and $C_{16:1}$ were increased, the absolute concentration of only $C_{12:0}$ was increased in both experimental groups. The increase in $C_{14:0}$ concentration was significant only in group II. In both diet groups FFA concentration and changes in their relative per cent distribution were similar except for $C_{18:2}$ which was significantly higher in group I than in group II ($p < 0.025$) after 16 weeks, however, the percent distribution of $C_{18:2}$ aand $C_{20:4}$ was significantly decreased in both groups at one week of dietary treatment. In terms of concentration $C_{18:2}$ and $C_{20:4}$ were reduced significantly only at the 16th week period in both experimental groups ($p < 0.025$ and $p < 0.02$). Other FA and the total FFA concentration was not changed from that of controls.

Long Term Dietary Period: All FA, except $C_{12:0}$, found in FFA fraction were significantly increased in concentration in group I-14 as compared to controls and total FFA concentration increased from 133 ± 20 μg/ml to 433 ± 133 μg/ml. In terms of relative percentage only medium-chain saturated FA and linoleic acid showed further change as a result of long term feeding. The percentage of $C_{12:0}$ increased from 0.1% in controls to 1.5% in I-4 and to 7% in I-14 group. $C_{14:0}$ increased from 1% to 4% and to 7.3%, respectively. In contrast, while $C_{18:2}$ was reduced in percentage from 15.5% in controls to 10.7% in I-4 and to 6% in I-14 groups, the concentration was only reduced from 20.1 ± 3 μg/ml in controls to 10.2 ± 2.9 μg/ml in I-4 group but it was increased to 34 ± 7.7 μg/ml in I-14 group.

Phospholipid FA

Control Values: The PLFA concentration made up 58.8% of total plasma FA. Stearic and arachidonic acids each represented 25% of the PLFA in control dog plasma, while linoleic acid was 16.5%, palmitic 13.5% and oleic 10.5%. Other FA present represented less than 1% each of the total PLFA pattern.

Short Term Dietary Period: PLFA per cent distribution showed changes within a week and total PLFA concentration was significantly increased in plasma of all dogs on experimental diets (Table V). There was great similarity between the two groups in terms of PLFA concentration as well as per cent distribution, except after 10 weeks PLFA of plasma had a higher concentration in group I than in group II. Saturated and monounsaturated FA

TABLE III

Triglyceride Fatty Acid Concentration (μg/ml)

FA	Control Mean ± SE	Group	Mean ± SE in experimental gropps I and II: 1st Week	2nd Week	4th Week	10th Week	16th Week
12:0	.31 ± .07	I	10 ± 5	32 ± 17	18 ± 7	38 ± 13[a]	15 ± 8
		II	4 ± 2	35 ± 14	20 ± 15	6 ± 1	6 ± 1
14:0	2.4 ± .4	I	22 ± 6	41 ± 17	26 ± 7	56 ± 10[a]	41 ± 16
		II	42 ± 26	66 ± 16	42 ± 18	30 ± 3	36 ± 8
16:0	43 ± 4	I	71 ± 8	94 ± 18	96 ± 11	114 ± 13	111 ± 37
		II	92 ± 29	116 ± 18	97 ± 23	84 ± 6	100 ± 26
18:0	20 ± 3	I	29 ± 6	41 ± 12	26 ± 3	24 ± 3	27 ± 11
		II	46 ± 23	44 ± 12	44 ± 22	18 ± 2	20 ± 7
16:1	16 ± 2	I	25 ± 2	33 ± 5	37 ± 5	47 ± 7	53 ± 15
		II	30 ± 9	33 ± 7	33 ± 7	35 ± 4	50 ± 15
18:1	110 ± 11	I	144 ± 16	192 ± 31	207 ± 30	237 ± 28[a]	225 ± 89
		II	185 ± 35	218 ± 35	204 ± 45	169 ± 5	233 ± 79
18:2	51 ± 4	I	39 ± 7	68 ± 19	49 ± 10	31 ± 5	38 ± 11
		II	47 ± 6	37 ± 6	40 ± 7	28 ± 5	28 ± 16
20:4	12 ± 1.5	I	10 ± 3	9 ± 2	5 ± 2	4 ± 1	6 ± 2
		II	11 ± 2	6 ± 1	12 ± 5	6 ± 8	5 ± 1
20:3	3.5 ± .78	I	3 ± .7	6 ± 2	4 ± 1	6 ± 2	9 ± 2
		II	5 ± 2	3 ± 1	5 ± 1	12 ± 4	21 ± 7
Total TGFA	274 ± 24	I	374 ± 45	549 ± 95	485 ± 49	570 ± 69[a]	570 ± 185
		II	526 ± 160	579 ± 90	519 ± 133	404 ± 16	523 ± 161

[a]Means of group I and II are significantly different ($p < 0.05$)

TABLE IV

Free Fatty Acid Concentration (μg/ml)

FA	Control Mean ± SE	Group	Mean ± SE in experimental groups I and II				
			1st Week	2nd Week	4th Week	10th Week	16th Week
12:0	.18 ± .06	I	1 ± 1	1 ± .2	2 ± .9	2 ± .3	8 ± 6
		II	3 ± 1	3 ± .9	2 ± 1	3 ± 1	3 ± .6
14:0	2.4 ± 2	I	7 ± 4	3 ± .7	6 ± .8	6 ± .5	7 ± 1
		II	5 ± 1	6 ± 1	6 ± 2	8 ± 3	9 ± 2
Total FFA	133 ± 20	I	125 ± 18	131 ± 18	174 ± 29	134 ± 20	133 ± 19
		II	162 ± 9	142 ± 28	155 ± 16	146 ± 26	126 ± 18

increased within a week after the initiation of the experimental diets and remained elevated throughout the dietary period, but fluctuated more in diet II than in Diet I group. At the 10th week, $C_{12:0}$, $C_{16:0}$ and $C_{18:1}$ were higher in dogs on diet I than II ($p < 0.05$, 0.05 and 0.01), respectively. Linoleate esterified with PL was also increased in all experimental animals except in the group on diet II after 10 weeks. While the relative percentage of arachidonic acid was reduced from 25 ± 0.07% in controls to 10.6 ± 0.06% in I-4 group and to 11.6 ± 0.9% in II-4 group, its absolute concentration was not changed significantly in either group from that of controls. Concentration of $C_{20:3}$ increased more in the PL than in TG or CE fractions as a result of the experimental diets.

Long Term Dietary Period: PLFA pattern was further affected by long term feeding and there was a threefold increase in total PLFA concentration in dogs of group I-14. The greatest elevation was in $C_{18:1}$ which increased from 182 ± 18.2 μg/ml in controls to a high of 3116 ± 975 μg/ml in group I-14. $C_{18:2}$ was also greatly increased from 308 ± 33 μg/ml to 1131 ± 298 μg/ml, respectively. While the relative percentage of arachidonic acid was reduced from 25% of the PLFA of controls to 13.1% in I-4 and further reduced to 6% in the I-14 group, the absolute concentration was unchanged: $C_{20:4}$ was 430 ± 53 μg/ml in controls, 329 ± 34 μg/ml in I-4 group ($p < 0.2$) and it was 628 ± μg/ml in I-14 group ($p < 0.3$).

The Ratio of Triene to Tetraene Fatty Acids ($C_{20:3}/C_{20:4}$)

It has been suggested by Holman (7) that the ratio of total $C_{20:3}$ to total $C_{20:4}$ greater than 0.4 indicated essential fatty acid deficiency. The ratios for these two acids, calculated from per cent composition for each lipid fraction as well as the total percentage are given in Table VI.

In CEFA the ratio of $C_{20:3}$ acid over $C_{20:4}$ never exceeded 0.4 in any experimental group. In the TG fraction this ratio was 0.4 or more in dogs of groups I-4, starting from the 4th week period and in dogs on diet II-4 from the 10th week. Both dietary groups showed a ratio of 0.4 or more in the PL starting from the 10th week. In contrast, this ratio in the FFA is abnormal from the very first week in dogs of groups I-4 and it is abnormal from the 10th week in dogs on diet II-4. In terms of total concentration, the net increase in $C_{20:3}$ was greatest in PL fraction where the gain in I-4 dogs was 190 μg/ml and 179 μg/ml in the II-4 group. $C_{20:3}$ in CE fraction increased 20.1

TABLE V

Phosphoplipid Fatty Acid Concentration ($\mu g/ml$)

FA	Control Mean ± SE	Group	Mean ± SE in experimental groups I and II				
			1st Week	2nd Week	4th Week	10th Week	16th Week
12:0	.22 ± .12	I	6 ± 2	5 ± 2	3 ± 2	7 ± 1[a]	4 ± 1
		II	10 ± 7	10 ± 3	6 ± 4	3 ± 1	4 ± .6
14:0	2 ± .3	I	28 ± 5	30 ± 8	26 ± 6	38 ± 4	31 ± 8
		II	35 ± 14	42 ± 10	37 ± 13	25 ± 4	28 ± 3
16:0	225 ± 19	I	530 ± 50	503 ± 77	476 ± 24	528 ± 31[a]	451 ± 27
		II	470 ± 70	493 ± 50	467 ± 62	410 ± 18	378 ± 21
18:0	441 ± 54	I	918 ± 85	960 ± 135	859 ± 82	850 ± 77	752 ± 71
		II	697 ± 62	724 ± 55	742 ± 59	716 ± 62	652 ± 29
16:1	15 ± 3	I	43 ± 9	38 ± 10	32 ± 3	41 ± 5	46 ± 4
		II	48 ± 17	42 ± 11	38 ± 13	30 ± 5	33 ± 8
18:1	182 ± 18	I	639 ± 113	566 ± 134	520 ± 51	580 ± 50[b]	503 ± 37
		II	577 ± 127	559 ± 94	513 ± 120	412 ± 14	402 ± 30
18:2	308 ± 33	I	644 ± 77	686 ± 117	596 ± 66	594 ± 56[b]	524 ± 50[b]
		II	544 ± 72	588 ± 43	552 ± 75	417 ± 31	348 ± 26
20:4	430 ± 53	I	590 ± 35	621 ± 34[b]	409 ± 59	355 ± 31	329 ± 34
		II	560 ± 48	459 ± 38	481 ± 53	319 ± 64	307 ± 35
20:3	66 ± 9	I	232 ± 49	258 ± 39	236 ± 38	298 ± 94	246 ± 39
		II	185 ± 27	182 ± 38	238 ± 27	297 ± 60	308 ± 37
Total PLFA	1794 ± 189	II	3912 ± 392	3957 ± 516	3334 ± 301	3422 ± 194[a]	3041 ± 242
			3351 ± 914	3280 ± 299	3319 ± 350	2754 ± 183	2562 ± 142

[a]Means of group I and II are significantly different ($p < 0.05$).

[b]Means of group I and II are significantly different ($p < 0.02$-0.01

μg/ml in the I-4 group and 17.2 μg/ml in the II-4 group. The corresponding values in TG fraction were only 0.7 μg/ml in I-4 and 4.6 μg/ml in II-4. FFA showed the smallest increase, with 0.17 μg/ml and 0.27 μg/ml in I-4 and II-4 groups.

Interrelationships of FA and Their Lipid Classes

There is a strong correlation ($p<0.001$) among most FA to their lipid fractions in plasma. Cholesteryl ester FA, except $C_{20:3}$ and $C_{20:4}$, correlate stronger to total cholesterol concentration than do FA in the TG or PL fraction to triglyceride or phospholipid concentration.

Plasma Lipoproteins

The plasma lipoprotein pattern of normal dogs when studied ultracentrifugally at d 1.21 shows a high concentration of high density -S 0-10 (alpha-) lipoproteins and only about 10% to 20% as much low density, -S 20-40 (beta-) lipoproteins. Electrophoresis, using pH 8.6 albuminated buffer, demonstrated a similar distribution of plasma lipoproteins consisting of a high alpha- and low beta-lipoprotein concentration. Within one to two weeks after initiation of special diets the dogs' plasma showed increased concentration of beta-lipoprotein, and an additional lipid- stained band migrating behind normal alpha-lipoproteins was frequently present. Concentration of the alpha-lipoproteins was unchanged or slightly increased. There was no grossly visible lipemia and no chylomicron fraction was demonstrable by paper electrophoresis at any time. The plasma lipoprotein patterns determined four to six weeks after starting diets, when plasma cholesterol levels were usually similar to or slightly lower than those present after one to two weeks, showed a further increase in concentration of beta-lipoprotein; the fraction migrating between alpha- and beta- lipoprotein was also usually more concentrated, and frequently showed a slower mobility. The alpha-lipoprotein concentration was unchanged or slightly increased from normal. While lipoproteins of dogs on diet I and II had similar mobility characteristics, the increase in concentration of beta-lipoprotein and slow alpha-lipoprotein was greater in dogs on the cholesterol rich diet I.

After long periods of 12 to 16 months on diet I, plasma lipoprotein pattern of the dogs showed no lipoprotein with flotation rate of -S 0-10 when studied ultracentrifugally at d 1.21. Fractions of flotation rate -S 10-20, -S 20-40, and -S 40-70 were present in widely varying concentrations. Electrophoresis showed no lipid-stainable material with mobility of alpha-lipoprotein. All lipid-stainable fractions had mobility of or slightly faster than that of normal beta-lipoprotein. Immunoelectrophoresis and immunodiffusion studies were made using antisera developed in rabbits against high density -S 0-10 alpha-lipoprotein and against low density -S 20-40 beta-lipoproteins prepared from sera of normal dogs. Even when no lipoproteins with electrophoretic or ultracentrifugal characteristics of normal alpha-lipoprotein were present in plasma of dogs on diet I for 10 to 14 months, this plasma by immunoprecipitation techniques showed a precipitin band against anti alpha-lipoprotein. A heavy precipitin band against beta-lipoprotein was always formed when the serum of dogs on diets was allowed to react against anti beta-lipoprotein antisera.

TABLE VI

The Ratios[a] of Triene and Tetraene FA (C 20:3 to C 20:4) in Plasma, Calculated in Terms of Per Cent Composition for CE, TG, FFA and PL Fractions. The Ratio for the Sum of all FA is Calculated From FA Concentration.

Lipid fraction	Control diet	Experimental diets	
		Diet I-4	Diet II-4
Cholesterol ester (CE)	0.02	0.12	0.07
Triglyceride (TG)	0.39	0.82	1.14
Free fatty acids (FFA)	0.34	0.69	0.54
Phospholipid (PL)	0.26	0.56	0.63
The sum of all FA	0.11	0.42	0.45

[a]The ratio of total C20:3 to total C20:4 greater than 0.4 indicates essential FA deficiency (7).

Clinical and Anatomopathological Findings

The general condition of the animals kept on diets for 16 weeks was excellent. Weight was maintained and dogs were even-tempered and alert. In contrast to this some animals on long-term studies became listless and developed difficulty in walking. Their skin became dry and scaled off easily when rubbed. Details concerning pathological changes observed in these dogs will be described in a separate communication. Extensive macroscopic, histological and ultrastructural changes occurred in the aorta and peripheral vessels of dogs on diet I for 12 to 16 months and seemed to be related to the severity of sustained chronic hyperlipemia. No

gross or histological changes were found in arterial tissue of dogs of groups I-4 and II-4. Electron microscopy showed medial changes in the major arteries in all dogs in group I-4 and in one animal in group II-4. These were characterized by appearance of intracellular membrane-bound cytoplasmic vacuoles in smooth muscle cells of the media. Changes of this type were not found in arteries of any of control dogs.

DISCUSSION

Dogs developed hyperlipemia very rapidly after initiation of either diet I or II. The first week after the dogs were on the diets, their plasma lipids and some fatty acids reached the highest levels observed during the four month study. The composition of dietary FA had great influence in producing this hyperlipemia. Medium chain saturated FA which are major components of the diets were increased. For example, laurate in plasma FFA increased 95 times over its concentration in plasma of control animals. The FA composition of hyperlipemic plasma was not, however, a simple reflection of dietary FA. Large increases in palmitoleic and oleic acids indicated increased synthesis or mobilization, or both, from other tissues, since palmitoleic acid was not in the diet and oleic acid represented only 3.4% of total FA of diets I and II, as compared to 35% oleic acid in total FA composition of control diet.

Correlation coefficients derived from linear regression analyses showed that the concentration of each fatty acid within the phospholipid, triglyceride, or cholesteryl ester fraction changed in a highly predictable manner as the total plasma concentration of each lipid fraction increased. The predictability or association in terms of correlation coefficients was greatest in cholesteryl esters.

The diet, high in saturated medium chain FA without added cholesterol appeared to be just as effective in initiation of hyperlipemia in dogs as when combined with 5% cholesterol. Plasma FA per cent compositions were changed from control patterns in the four lipid fractions, CE, PL, TG and FFA, to the same degree within the first week after starting experimental diets I and II and in general were maintained during the entire four-month period. While the relative percentage of some fatty acids, e.g., stearic, linoleic and arachidonic acids were reduced, their absolute concentrations were either increased or remained unchanged from that of control dogs' plasma. It is remarkable that linoleic acid concentration in cholesterol ester and phospholipid fractions of plasma of dogs on diet I and II for four months increased to over twice the control concentration. Its mean concentration was even higher in dogs on cholesterol supplemented diet for 12 to 16 months. The source of linoleic acid does not seem to be the diet, since linoleic acid comprised only 0.3% of experimental as compared to 23% of the control dogs' diet. It was more likely that linoleic acid was mobilized from other tissues. More detailed studies on FA changes in tissues in the same experimental diets will be described in a separate communication.

As with fatty acids, there was also no difference in total and free cholesterol, triglyceride or phospholipid concentrations between the two experimental diet groups within the first four weeks on these diets. After 10 to 16 weeks, however, TC, FC and PL concentrations were significantly higher in dogs on the diet with than in those without cholesterol supplement. It appears that exogenous cholesterol was necessary to sustain the severe hyperlipemia.

Though the $C_{20:3}$ to $C_{20:4}$ ratio, as described by Holman (7) may represent a useful index of essential FA deficiency, care must be exercised in interpreting the results calculated from per cent composition in various lipid fractions. In some lipids the ratio rises primarily due to an actual increase in $C_{20:3}$ and a decrease in $C_{20:4}$ while in others either the triene or the tetraene concentration may be held relatively constant while the other FA is changing. By comparing ratios or per cent distribution values, it appears that triglyceride had the largest increase in $C_{20:3}$ but by measuring the FA concentration, it was shown that the greatest increases in $C_{20:3}$ were observed in the phospholipids and cholesteryl esters.

Dermal symptoms as criteria for essential fatty acid deficiency have been reported in the rat (8,9). The scaliness of the skin in experimental dogs on diet I for 12 to 16 months confirms previous reports (10,11) that in this species skin manifestations may also occur as a result of essential fatty acid deficiencies.

Changes in serum lipoprotein pattern of dogs on experimental diets were rapid. While the initial increase in low density lipoproteins occurred with little change in concentration of high density alpha-lipoprotein, dogs on the diet for 10 to 16 months showed very low levels of high density alpha-lipoprotein, -S 0-10, lipoprotein, with electrophoretic mobility of alpha-lipoprotein. It is significant, however, that immunologic studies showed that alpha-lipoprotein was present in nearly normal amount. In the extremely hyperlipemic situation the alpha-lipoprotein was combined with greater amounts of lipid than in normal, so that

flotation and electrophoretic properties were very different. More detailed studies on the lipoprotein fractions will be described in another report.

The observations by Malmros and Sternby (1) that vascular lesions may be induced in dogs by feeding a cholesterol-supplemented diet lacking essential FA and rich in saturated medium chain FA has been confirmed in the long term study. Their studies also included two dogs on the diet without cholesterol. Data on serum cholesterol and triglycerides, measured at monthly intervals, show variability among the dogs in response to either diet similar to that reported here. Comparisons between groups fed different diets probably would be facilitated by use of pure bred dogs but the degree of response would likely be dependent on the breed chosen.

While it might be argued that the diets used were not physiological insofar as they were devoid of essential fatty acids and in the case of diet I, contained 5% cholesterol, they do afford the opportunity to study the effects of exogenous cholesterol, saturated fat and lack of essential fatty acids on plasma, arterial and other tissue lipids, and on the initiation of arteriosclerosis in a species considered to be resistant to development of spontaneous arterial lesions. There is a possibility that the higher percentage of fat in the experimental diets, 20% in I and 16% in II, as compared to only 8% fat in control, was a factor in the development of the hyperlipemia in the experimental animals. However, this seems unlikely in view of evidence (12-15) that the amount of dietary fat has much less effect than the type of fat on hyperlipemias produced in man and experimental animal. The experimental diets in the present study were abnormal in terms of both degree of saturation and average chain length of fatty acids, the diets being composed predominantly of saturated, medium chain length fatty acids ($C_{12:0}$ and $C_{14:0}$). The possibility of altering the lipemia by supplementing the diet with essential fatty acids is presently being examined.

ACKNOWLEDGMENTS

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Dietary Lipid, Fatty Acid Oxidation and Incorporation of Carbon Into Cholesterol[1]

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ABSTRACT

Female rats (200 g) were fed a nutritionally adequate diet containing 1% by weight of corn oil (low-fat, LF), 21% of corn oil (CO) or 20% of beef tallow plus 1% of corn oil (BT) for two weeks. Food was removed for 8-12 hr, then each rat was refed for 1 hr. Each rat was injected ip with Na-^{3}H-acetate and U-^{14}C-Na-palmitate, (P), -oleate (O) or -linoleate (L). Expired CO_2 was collected for 2 hr. Liver, heart and serum were obtained for analysis of total lipid ^{14}C and ^{3}H and cholesterol ^{14}C and ^{3}H. Oxidation of L was twice as great as O or P when the LF diet was fed. CO and BT diets doubled oxidation of O to equal L, and increased oxidation of P, 50%. In liver and serum P was retained to a greater extent than O or L on BT and CO diets. Incorporation of acetate into total lipid was highest on LF diet and reduced by feeding either CO or BT. Incorporation of acetate into cholesterol was greater when BT or CO was fed than for LF. ^{14}C was incorporated into cholesterol in such small amounts that it was barely detectable and could not be counted accurately. Conclusions are that (a) dietary fat affects rate of oxidation of uniformly labeled palmitate and oleate, but not linoleate, (b) acetate is a more ready precursor to cholesterol than is fatty acid carbon, and (c) the acetate incorporated into cholesterol when polyunsaturated fat is fed is not derived directly from fatty acid carbon. The failure of incorporation of fatty acid carbon into cholesterol within 2 hr of administration opens the question of compartmentation of acetate as to its metabolic source.

INTRODUCTION

Incorporation of acetate into cholesterol by rats, in vivo and in vitro, is greater when 20% fat is included in the diet than when only enough fat for essential fatty acids is included (1-3). Fats containing high concentrations of polyunsaturated fatty acids accelerate acetate incorporation into cholesterol more than those containing primarily saturated and monounsaturated fatty acids (1,4).

One of the differences between saturated and polyunsaturated fatty acids is in their rates of oxidation. 1-^{14}C linoleate is oxidized to $^{14}CO_2$ more rapidly than 1-^{14}C-palmitate or 1-^{14}C-stearate by mice (5) and rats (6) in vivo, by guinea pig heart homogenates and beef heart (7), and rat liver isolated mitochondria (8). U-^{14}C-linoleate is also oxidized more rapidly than U-^{14}C-palmitate, in vivo and in vitro (9). Cholesterol synthesis from acetate, therefore, may be accelerated by rapid formation of acetate from linoleate; the acetate thus produced being incorporated into cholesterol.

Several reports have described studies of incorporation of carboxyl carbon of fatty acids into cholesterol. Mead et al. (5) and De Leo and Foti (10) performed the earliest of such studies and their results were not sufficiently precise to conclude whether linoleate was more active than stearate. Cenedella and Allen (11) recently have reported that 1-^{14}C-linoleate is oxidized more rapidly than 1-^{14}C-palmitate when given to rats intragastrically. They also found incorporation of label into cholesterol proportional to rate of $^{14}CO_2$ formation. Foti et al. (12) reported exactly opposite results when they studied incorporation of 1-^{14}C-stearate and 1-^{14}C-linoleate into cholesterol by cell-free systems from rat liver. Stearate was twice as active a source of ^{14}C for cholesterol as linoleate in nuclei-free homogenates. Systems free of mitochondria incorporated more of either acid into cholesterol, with linoleate exceeding stearate. The authors conclude that incorporation of fatty acid carbon into cholesterol is not via mitochondrial oxidation to acetate.

If rate of oxidation of the fatty acids determines rate of acetate incorporation into cholesterol, then incorporation of labeled acetate would be decreased by dilution of an assumed acetate pool with unlabeled fatty acid carbon. There is evidence that the carboxyl carbon of unsaturated fatty acids is not metabolized via the same pathway as the 9-12 carbons, in muscle (9). Linoleate can be oxidized by gamma cleavage resulting in incorporation of the 9-12 carbons into methylmalonate. The

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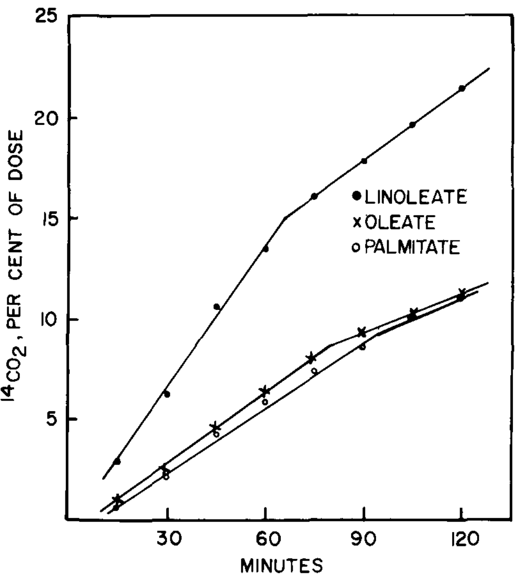

FIG. 1. Accumulative $^{14}CO_2$ expiration following intraperitoneal injection of U-^{14}C-fatty acids in female rats fed low fat diet (1% corn oil).

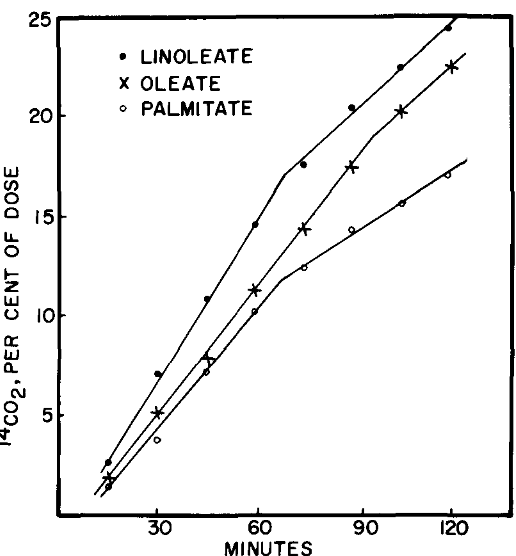

FIG. 2. Accumulative $^{14}CO_2$ expiration following intraperitoneal injection of U-^{14}C-fatty acids in female rats fed high beef tallow diet (20%).

extent of activity of this pathway is not yet known.

With the conflicting results cited, it is not clear how fatty acid oxidation affects cholesterol biosynthesis. The following study was designed to determine the relative rates of incorporation of ^{3}H-acetate and uniformly labeled ^{14}C-fatty acids into cholesterol under dietary conditions known to affect rate of acetate incorporation. The rates of oxidation of fatty acids were studied to determine whether that process regulated incorporation of acetate into cholesterol.

METHODS

Diets, Animals and Experimental Design

Low fat, high corn oil and high beef tallow diets were formulated similar to those used in previous experiments (3). Low fat diet (LF) contained 1% (wt.) of corn oil, high corn oil diet (CO) contained 21.2% of corn oil and high beef tallow diet (BT) contained 1.2% of corn oil and 20% of beef tallow. All diets contained lactalbumin, cornstarch, vitamins, minerals and cellulose (3). The major difference in fatty acid composition of beef tallow and corn oil is 53% linoleate in corn oil vs. 2% in beef tallow and a total of 10% saturated fatty acids in corn oil vs. 37% in beef tallow (13).

Female, Carworth Farms CFE rats weighing approximately 160 g were purchased. Upon arrival they were caged singly and fed Purina Laboratory Chow until they were put on experimental diets. Nine or 12 rats per day, three days a week for three weeks, were weighed and placed on experimental diets. A total of 90 rats were so placed on three diets. At the end of two weeks of ad lib. feeding, the first group of rats was treated as follows: food was removed from the cages; 6-10 hr later it was replaced and rats were injected ip with a U-^{14}C-fatty acid and Na-^{3}H-acetate. Immediately after injection the rat was placed in a metabolic chamber for collection of CO_2. After 2 hr it was removed, anesthetized with Na-pentobarbital, a blood sample was obtained by heart puncture and the liver and heart were excised. Serum, livers and hearts were stored at -20 C for later analyses. Thirty rats were processed each week for three weeks.

Radiohomogeniety of ^{3}H- and ^{14}C-acetate.

Kamen (14) has written a summary of early work (*15,16*) on incorporation of acetate into cholesterol, stating:

> After careful analyses of the specific label content of isolated and purified cholesterol from H^2-labeled and C^{13}, and C^{14}-doubly labeled acetate, it was established that acetate carbon was available as a sufficient carbon source and that it was being incorporated as a unit.

The acetylation of acetate with hydrogens intact (H^2, C^{13}) was determined by Bloch and Rittenberg (15), but the tracer homogeneity of acetate incorporated into cholesterol was not

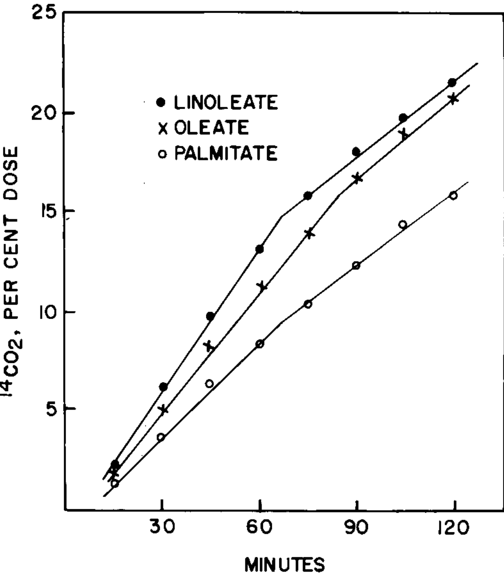

FIG. 3. Accumulative $^{14}CO_2$ expiration following intraperitoneal injection of U-^{14}C-fatty acids in female rats fed high corn oil diet (21%).

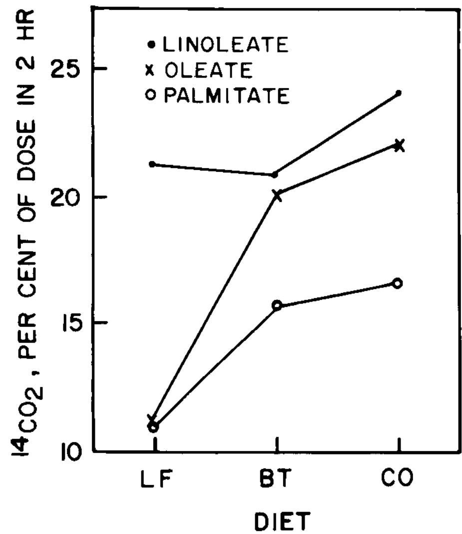

FIG. 4. Interaction between dietary fat and expiration of $^{14}CO_2$ from intraperitoneally injected U-^{14}C-fatty acids. LF, BT, CO explained in text.

determined. If only one hydrogen is lost or exchanged from the methyl group, the result would be incorporation of two thirds of the label into cholesterol. Subsequently an excess of three carboxyl groups in the cholesterol ring would increase the ratio by 10% to a maximum of 0.74. Analysis of the pathway of cholesterol synthesis as described by Cornforth (17) yields an approximate theoretical $^{3}H/^{14}C$ of 0.64.

An experiment was conducted to ascertain the $^{3}H/^{14}C$ ratio of cholesterol synthesized from Na-^{3}H-acetate and Na-1-^{14}C-acetate administered simultaneously to rats. The dose contained 20 μc of ^{3}H and 10 μc of ^{14}C (actual $^{3}H/^{14}C$ = 2.04) so that the theoretical yield ratio should be 0.64 x 2 = 1.28. Two female rats fed the corn oil diet used in the experiment were used. The results for liver cholesterol $^{3}H/^{14}C$ were 1.26 and 1.32. The liver fatty acids would have a theoretical $^{3}H/^{14}C$ of 0.4 x 2 = 0.8. The results were 0.85 and 0.86.

Analytical Methods and Materials

Sodium-^{3}H-acetate (500 mc/mmole, Nuclear Chicago) was injected via 0.5 ml of physiological saline solution containing 18 μc of ^{3}H. U-^{14}C-palmitate (560 mc/mmole), -oleate and -linoleate (630 mc/mmole) were obtained from Applied Science Laboratories in 50 μc containers. Sodium salts of the fatty acids were prepared under N_2 and diluted with physiological saline solution. The dose was 2.5 μc in 0.5 ml.

Carbon dioxide was collected by suction of air through the metabolic chamber via a train which included a NaOH tube prior to the chamber and a tube containing standard 0.5 N NaOH for collection of expired CO_2. Traps were changed every 15 min and the samples stored for ^{14}C analysis and titration with standard HCl to phenolphthalein end point. An aliquot of the collection solution was solubilized with Beckman Biosolv and 15 ml of a solution containing 5 g 2,5-diphenyloxazole (PPO), 100 mg 4-di-2-(5-phenyloxazolyl)-benzene (POPOP), 50 g napthalene, 380 ml dioxane, 380 ml toluene and 250 ml absolute ethanol was added. Counting was performed with a Beckman Scintillation system at an efficiency of 62%.

Lipids of serum, livers and hearts were extracted by the method of Folch et al. (18). Total fat of liver and heart was determined by drying and weighing an aliquot of the chloroform-methanol extract (C–M). Total lipid ^{3}H and ^{14}C were determined by drying an aliquot of C-M and counting in a scintillation solution containing 0.05 g/liter POPOP and 4 g/liter POP in toluene. ^{14}C-toluene and ^{3}H-toluene were used as standards to determine counting efficiencies of the double-labeled samples.

Samples of C-M were saponified, and extracted with petroleum ether for the precipitation of cholesterol as the digitonide (19) for counting and for quantitation of cholesterol using the Zak et al. (20) acid iron color reagent. Cholesterol digitonide was dissolved in 2 ml of

TABLE I

Body and Organ Weights and Lipid Composition of Young Female Rats Fed Diets Varying in Fat For Two Weeks

Diet	Body wt., g	Liver wt., g	Liver Fat, mg	Liver Chol. mg	Heart wt., g	Heart Fat mg	Heart Chol. mg	Serum Chol., mg/nl
LF[a]	202 ± 3.0[b]	6.08 ±0.18	265 ±10.6	15.5 ±0.50	0.72 ±0.01	20.7 ±1.39	0.97 ±0.03	60 ± 2.4
BT[a]	208 ± 3.6	5.87 ±0.15	288 ± 8.8	15.1 ±0.37	0.71 ±0.01	21.9 ±1.91	0.94 ±0.03	60 ± 3.3
CO[a]	209 ± 2.6	6.05 ±0.13	290 b 11.4	16.6 ±0.45	0.71 0.01	21.5 1.15	0.95 0.03	62 ± 2.4

[a]LF, low fat diet, 1% corn oil; BT, 20% beef tallow + 1.2% corn oil diet; CO, 21.2% corn oil diet.

[b]Mean ± standard error of the mean of 30 rats.

methanol then counted using the same scintillation solution as used for total lipid.

Mean, standard error of the mean, analysis of variance, t test for differences between means and interaction of variables were determined using a CDC 6400 computer.

RESULTS

Fatty Acid Oxidation

Table I shows the general characteristics of the rats after two weeks on low fat, high corn oil or high beef tallow diets. There were no differences in body weight, liver weight, fat or cholesterol; heart weight, fat or cholesterol; or serum cholesterol. Carworth CFE rats are quite uniform in physiological parameters and young female rats are able to adapt to diet very well. A two-week period was not sufficient for metabolic alterations to affect tissue concentrations of lipids.

Expiration of $^{14}CO_2$ following injection of uniformly labeled fatty acids is described by Figures 1-4. Figure 1 indicates accumulative expiration of $^{14}CO_2$ by rats fed the low fat diet. The total CO_2 (unlabeled) expired was not affected by diet. Thus per cent of dose expired is indicative of contribution of the label to fuel of respiration. Palmitate (P) and oleate (O) were oxidized at the same rate with a total of 11.1% and 11.3%, respectively, of the injected dose expired in 2 hr. Linoleate (L) was expired at a significantly more rapid rate than P or O, with a total of 21.4% expired in 2 hr.

Figure 2 illustrates the effect of 20% beef tallow in the diet upon oxidation of fatty acids. P oxidation was accelerated 50% by feeding beef tallow in place of cornstarch ($P < .05$); O oxidation was increased almost 100% ($P < .01$). The total $^{14}CO_2$ expired in 2 hr from P was 15.8% and from O was 20.6% of the dose. L oxidation was not affected by feeding beef tallow compared to cornstarch (oxidation in 2 hr was 21.0% of dose). Oxidation of O was enhanced to equal the rate of oxidation of L.

The effect of feeding 20% corn oil in place of cornstarch is illustrated in Figure 3. P and O were oxidized at rates similar to those seen when beef tallow was fed (16.9% and 22.4% of dose expired in 2 hr, respectively). L was oxidized at a slightly more rapid rate (24.1% of dose expired in 2 hr) than when LF or BT was fed, but neither difference was statistically significant.

The interaction between diet and fatty acid oxidation is shown in Figure 4. The statistical evaluation of interaction was significant ($P < .005$). The interpretation of statistical interaction is that different diets affected different fatty acids in dissimilar manners.

Fatty Acid Retention

The amount of ^{14}C left in the liver after 2 hr of exposure to U-^{14}C-fatty acids was inversely related to the rates of oxidation of palmitate and linoleate when CO was fed (Table II and Figure 5). Although L was oxidized more rapidly than P on the LF and BT diets it was also conserved in the liver. When BT or CO was fed, however, neither O nor L was conserved in the liver as much as P. This phenomenon is illustrated by the interaction plot in Figure 5. The significant interaction ($P < .005$) is due to the corn oil diet. Corn oil contains 53% linoleate (13) so that the CO diet contained 11.2% (wt) of linoleate (21.2% of dietary calories).

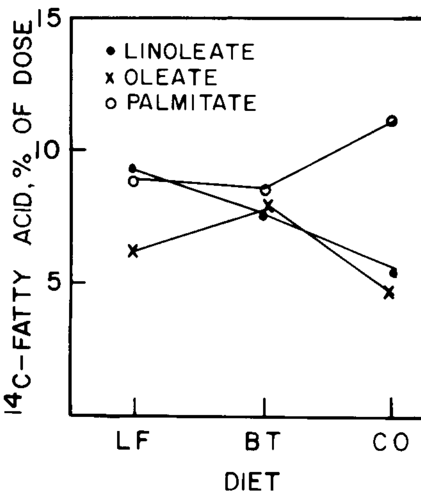

FIG. 5. Interaction between dietary fat and retention of ^{14}C-lipid in liver, 2 hr after intraperitoneal injection of U-^{14}C-fatty acids. LF, BT, CO explained in text.

TABLE II

^{14}C Left in Organ Lipids 2 hr After Intraperitoneal Injection of U-^{14}C-Fatty Acids

Diet	Linoleate, %	Oleate, %	Palmitate, %
Liver			
LF[a]	9.1 ± 1.0[b]	6.1 ± 0.8	8.9 ± 1.2
BT	7.2 ± 0.8	7.3 ± 0.9	8.7 ± 1.1
CO	5.1 ± 0.7	4.4 ± 0.7	10.9 ± 1.1
Heart X 10^2			
LF[a]	4.4 ± 0.8	4.0 ± 0.6	5.8 ± 1.3
BT	5.9 ± 2.0	5.0 ± 0.8	4.2 ± 0.5
CO	5.6 ± 1.1	4.0 ± 0.7	6.6 ± 1.0
Serum X 10^2, per ml			
LF[a]	5.4 ± 1.0	4.9 ± 1.1	4.1 ± 0.5
BT	3.7 ± 0.2	3.5 ± 0.7	4.8 ± 0.8
CO	3.0 ± 0.2	2.8 ± 0.2	4.7 ± 0.6

[a]LF, low fat diet, 1% corn oil; BT, 20% beef tallow + 1.2% corn oil diet; CO, 21.2% corn oil diet.

[b]Mean ± standard error of the mean of 7-10 rats.

Beef tallow is composed primarily of biosynthesized fatty acids, therefore, beef tallow would be an energy source similar to body fat.

Labeled L, O and P remaining in serum were not statistically different on LF diet. P was retained to a greater extent than L or O when BT was fed, although the difference was not significant and differences between BT and LF effect were not significant. CO resulted in significantly less L in serum than LF ($P < .05$). P was retained to a greater extent than L or O on CO. The serum data for retention of fatty acids generally parallel the liver data.

There were no differences in ^{14}C left in total lipids of the heart. Neither diet nor source of fatty acid resulted in selective retention by the heart.

Acetate Incorporation Into Lipids

Table III contains data describing incorporation of ^{3}H-acetate into total lipid and cholesterol of liver, serum and heart. High fat diets, whether beef tallow or corn oil, depressed acetate incorporation into total lipid of the liver. Serum reflected the liver data quite closely. Heart total activity (per cent of injected dose) of total lipid was less on BT and CO than LF, but not as much less as serum and liver TA.

Incorporation of acetate into cholesterol was enhanced by feeding fat. Liver cholesterol TA and SA were more than twice as great when BT or CO was fed than when LF was fed. Serum cholesterol TA and SA magnified the differences due to diet. BT caused a 60% greater serum cholesterol TA and CO 400% greater. Heart cholesterol contained too little radioactivity to detect by the methods used.

Incorporation of U-^{14}C-Fatty Acids Into Cholesterol

Carbon-14 in cholesterol was extremely low. The counting errors for such low levels prohibit use of the data for computations magnifying differences which are probably due to counting error. To obtain an estimate of the relative rates of acetate and fatty acids as carbon source for cholesterol a second experiment was conducted. The object was to inject a larger dose of linoleate into smaller rats to obtain samples with a higher counting rate. The results are shown in Table IV.

Experiment 2 verifies that linoleate carbon is incorporated into liver cholesterol, but at a very low rate. The younger rats (average wt. 161 g) used in Experiment 2 probably were synthesizing cholesterol at a higher rate than those used in Experiment 1 (4).

DISCUSSION

Effect of Dietary Fat on Fatty Acid Oxidation

The results reported here indicate that feeding either 20% beef tallow or 20% corn oil enhanced oxidation of U-^{14}C-palmitate and -oleate when compared to their oxidation on a 1% fat diet. Linoleate oxidation was not affected by diet. Earlier results (6) using carboxyl labeled fatty acids showed no effect of diet on 1-^{14}C-stearate, -oleate or -linoleate.

TABLE III

Incorporation of Na-^{3}H-Acetate Into Organ Lipids of Female Rats in 2 hr After Intraperitoneal Injection (Actual, not adjusted for theoretical loss of ^{3}H)

	Total lipid			Cholesterol	
Diet	TA[b]	SA[c]	TA X 10^2	TA X 10^3	SA
Liver					
LF[a]	5.9 ± 0.7[d]	22.1 ± 2.4		38 ± 6	2.4 ± 0.3
BT	2.7 ± 0.2	9.3 ± 0.7		81 ± 20	5.2 ± 1.1
CO	2.3 ± 0.1	8.2 ± 0.4		94 ± 12	5.6 ± 0.7
Heart					
LF			4.5 ± 0.6		
BT			3.3 ± 0.4		
CO			3.5 ± 0.3		
Serum (per ml)					
LF			2.7 ± 0.4	0.7 ± 0.2	1.2 ± 0.2
BT			1.3 ± 0.1	1.2 ± 0.1	2.2 ± 0.3
CO			1.5 ± 0.1	3.1 ∓ 0.4	4.7 ± 0.6

[a]LF, low fat diet, 1% corn oil; BT, 20% beef tallow + 1.2% corn oil diet; CO, 21.2% corn oil diet.

[b]TA, per cent of injected dose per tissue.

[c]SA, TA/g lipid.

[d]Mean ± standard error of the mean for 24-30 rats.

The two studies were performed on different strains of rats, Carworth CFE and Holtzman; otherwise they were conducted in almost identical manner. Thus, either the two rat strains differ in their metabolism of fatty acids, carboxyl carbon is metabolized differently from other carbons of fatty acids, or the two studies were not done under sufficiently similar conditions. It seems most likely that feeding fat should cause adaptation to oxidation of fatty acids for energy. In that case, linoleate is oxidized at a maximum rate regardless of amount consumed, but oleate and palmitate oxidation are amenable to adaptation. There is reason to believe that unsaturated fatty acids are oxidized by both beta and gamma mechanisms and gamma oxidation could accelerate metabolism of acetate derived from beta oxidation (9). On that basis the carboxyl carbon may not be representative of all of the carbons of the fatty acid. To resolve the issue, a study must be done in which 1-^{14}C and U-^{14}C (or specifically labeled) fatty acids are compared under different dietary conditions. Such an experiment is planned in this laboratory.

Incorporation of Fatty Acid-^{14}C Into Cholesterol

Fatty acid carbon was not as active a precursor to cholesterol as was acetate in this study. Obviously the degree of labeling of the immediate precursors to hydroxy-methyl-glutaryl-CoA was greater from externally

TABLE IV

Comparison of Incorporation of ^{3}H-Acetate and U-^{14}C-Linoleate Into Liver Cholesterol

				Cholesterol per cent of dose liver	
Expt.	Label	Dose, dpm	Liver, dpm/liver	Actual	Corrected[a]
1	^{3}H-acetate[b]	3 x 10^7	2.2 x 10^4	0.094	0.147
2	U-^{14}C-L[c]	1.3 x 10^7	1426	0.011	0.011

[a]Experimental ^{3}H/^{14}C of acetate incorporation into cholesterol is 0.64

[b]30 rats.

[c]3 rats.

administered acetate than from externally administered fatty acids. Fatty acids, however, were converted to acetyl CoA at rapid rates as indicated by production of $^{14}CO_2$. The failure of this acetate to be incorporated into liver cholesterol in amounts comparable to exogenous acetate may be caused by intracellular compartmentalization or by inter tissue metabolism. Exogenous acetate can be incorporated into cholesterol by the cytosol and microsomes or can be oxidized by the mitochondria (21). The fatty acid could enter the liver and be oxidized by the mitochondrion; the resulting acetate would have to be transported out of the mitochondrion to be a precursor to cholesterol. Perhaps this intracellular separation of acetyl CoA accounts for the observed differences.

The conflicting reports of Cenedella and Allen (11) and Foti et al. (12) cannot be resolved by this study. Neither of those studies compared fatty acid carbon with acetate as a precursor to cholesterol.

The more likely explanation for relatively less carbon from fatty acids than acetate being incorporated into cholesterol is tissue compartmentation. The fatty acid could be secreted from the liver as lipoprotein and transported to extrahepatic tissue. Muscle is more active in oxidation of fatty acids than is liver (11,22). Liver is the most active site of cholesterol biosynthesis (23). This organ specificity could easily result in separation of fatty acid carbon from sites of cholesterol synthesis.

The following conclusions may be drawn from this experiment. Dietary fat affects rates of oxidation of uniformly labeled palmitate and oleate, but not linoleate. In comparison to data on carboxyl labeled fatty acids, this suggests that there may be a difference between oxidation of carboxyl and other carbons of fatty acids. Exogenous acetate is a more ready precursor to cholesterol than exogenous fatty acid carbon, in vivo. The acceleration of acetate incorporation into cholesterol by fat feeding is not the result of formation of acetyl CoA from fatty acids and incorporation of that acetate into cholesterol.

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The Fatty Acids of *Entomophthora coronata*

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ABSTRACT

The total lipids and fatty acid composition of *Entomophthora coronata* were determined. The fungus was grown on a chemically defined medium and a chemically nondefined medium (Sabouraud dextrose yeast extract) for a period of 26 days. The organism contained from 16.2% to 44.6% total lipids depending upon the days of growth. The major fatty acids were 12:0 (5.5-9.0%), 13:0 (1.2-8.2%), 14:0 (33.5-43.5%), 16:0 (9.7-13.9%), $18:1_9$ (20.4-22.4%), and $18:2_{9,12}$ (3.5-10.5%). Lesser amounts of 15:0, 16:1, 16:2, 17:0, 18:0, two other 18:2 (both having conjugated double bonds), $18:3_{6,9,12}$, another 18:3 (conjugated double bonds), $20:3_{8,11,14}$, $20:4_{5,8,11,14}$, another 20:4 (conjugated double bonds), and 24:1 acids were found. Trace amounts of 20:0, 20:1, 20:2, 22:0 and 24:0 were also present. The relative percentage of most of the fatty acids did not vary appreciably with growth. However, $18:2_{9,12}$ and $20:4_{5,8,11,14}$ increased with age of the chemically defined culture. Peak E (18:2, conjugated double bonds) increased and 13:0 and $18:3_{6,9,12}$ decreased with age of the chemically nondefined culture. The fatty acids were predominately saturated (56.9-69.1%) and contained a high percentage of shorter chain fatty acids (C 12 to C 15). The fatty acids of the chemically defined culture were more unsaturated than the Sabouraud culture and the unsaturation increased with age of the culture.

INTRODUCTION

The genus *Entomophthora* of the class Phycomycetes, consists of at least 104 different species with varying degrees of pathogenicity and host specificity to insects (1-3). This genus is of considerable interest because of the increase in emphasis on biological control of insect pest populations. The entomogenous fungus *Entomophthora coronata*, whose phylogenetic position is not completely settled (4-6), is a well characterized species; it is easily grown, highly pathogenic to certain insects, and is host nonspecific (2-3). Its economic importance has been the subject of a number of investigations (7). If entomogenous fungi are to be used as insect control agents it is important to thoroughly understand the host, the pathogen and the host-pathogen relationship.

This paper reports the fatty acid composition of one isolate of *E. coronata* grown on two different liquid media for a period of 26 days. The numerous long chain fatty acids were identified by mass spectrometry.

EXPERIMENTAL PROCEDURES

Stock cultures of *Entomophthora coronata*, isolated by Yendol and Paschke from a termite (7), were grown in petri dishes on Sabouraud dextrose yeast extract agar for 10 days. A 4 cm disc was removed from the petri dishes, placed in 100 ml of sterile distilled water and blended for approximately 20 sec at low speed. A 1 ml aliquot of the homogenate was added to 100 ml of sterile medium in a 250 ml Erlenmeyer flask. The fungus was grown on two media: a chemically defined medium and a chemically nondefined medium (Sabouraud dextrose yeast extract) (SAB+GY). The chemically defined medium consisted of 20 g glucose, 2 g L-asparagine, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.6 g KH_2PO_4 and 2.4 g K_2HPO_4 per liter. The chemically nondefined medium (SAB+GY) contained 10 g Neopeptone (Difco), 40 g glucose, and 2 g yeast extract per liter. The organism was grown in stationary

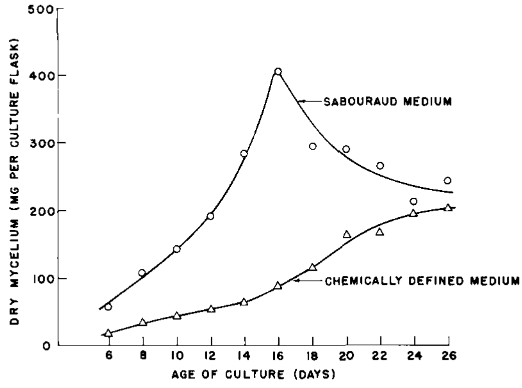

FIG. 1. Growth curves of *E. coronata* grown in a chemically defined medium and in a chemically nondefined medium (Sabouraud dextrose yeast extract).

TABLE I

Percentage Lipids in *E. coronata* Grown on Chemically Defined Medium and on Sabouraud Dextrose Yeast Extract Medium

	SABY+GY medium		Defined medium	
Days	Mg lipid per flask	Percentage[a] lipids	Mg lipids per flask	Percentage[a] lipids
6	13.3	16.2	7.3	24.7
8	36.3	21.2	12.8	27.1
10	77.5	31.4	18.8	28.8
12	146.0	40.7	31.4	35.5
14	297.7	44.8	39.4	37.9
16	421.2	39.2	61.1	38.5
18	263.1	38.0	85.6	38.9
20	300.7	31.9	117.6	40.0
22	317.9	39.6	147.8	44.6
24	304.0	41.0	163.6	43.1
26	387.1	38.5	179.3	44.4

[a]Based on total dry weight of the organism.

cultures at 25 C under constant light for 26 days. The content of four to five culture flasks from each medium was analyzed every other day. The mycelium was harvested and extracted essentially in the manner previously reported (8).

Methyl esters were prepared in two ways: (a) by transesterification with 12.5% methanolic-boron trifluoride for 45 min at 110 C (9), and (b) by transesterification with 0.5 M sodium methoxide in methanol. The methyl esters were analyzed by gas chromatography (Micro Tek Model 220, flame ionization detector) and peak areas were determined by an electronic digital integrator (Aerograph Model 471). Routine analyses were performed with a 6 ft 15% diethylene glycol succinate (DEGS) column at 180 C and a 6 ft 3% OV-1 column at 180 C (4 mm in diameter). A 15% DEGS (92 g) column, 9 ft in length and 9 mm in diameter, was used (Barber-Colman Model 5000) for preparative gas chromatography. The fatty acids were identified by comparison of retention times with methyl ester standards (Supelco, Bellefonte, Pa. and Northern Regional Laboratory, Peoria, Ill.), by log of retention time plots versus carbon number on both DEGS and OV-1, by bromination and by hydrogenation. These peaks were then analyzed by UV spectroscopy and by direct gas chromatography-mass spectrometry (LKB Model 9000). Peaks A through I were also oxidized with osmium tetroxide; the resulting polyols were methylated and analyzed by direct gas chromatography-mass spectrometry (10).

RESULTS AND DISCUSSION

Entomophthora coronata was grown in stationary cultures at 25 C under constant light on a chemically defined medium and on a chemically nondefined medium (SAB+GY). The growth curves, based on dry extracted mycelium weight, are given in Figure 1. The SAB+GY culture reached a maximum growth at 16 days, while the chemically defined medium culture continued to increase in weight throughout the duration of the experiment. The SAB+GY culture growth curve suggests degradation has taken place after 16 days of growth as is typical of most microorganisms following their maximum growth (11). Table I shows the milligrams of lipid per flask and the relative per cent of lipids. The cultures on SAB+GY contained a greater amount of lipid (13.3-421.2 mg) than those on the defined medium culture (7.3-179.3 mg). When comparisons are made on a relative percentage lipid basis after the 10th day, there is very little difference between the two cultures and very little change with culture age. The SAB+GY culture reached its maximum per cent of lipid (44.8%) on the 16th day while the defined medium culture continually increased in per cent of lipid with age (maximum 44.4%).

A typical gas chromatographic analysis of the methyl esters of the fatty acids of *E. coronata* is shown in Figure 2. Methyl esters having retention times equal to or greater than methyl γ linolenate (peak C) are shown in twice their normal size because of their occurrence in low relative percentage and because of the presence of several unusual fatty acids. The use of a digital electronic integrator eased the identification and quantification of the fatty acids and especially the identification of the long retention time fatty acids. Peaks A through K (Fig. 2) of the 26 day SAB+GY culture were isolated by preparative GLC, analyzed indi-

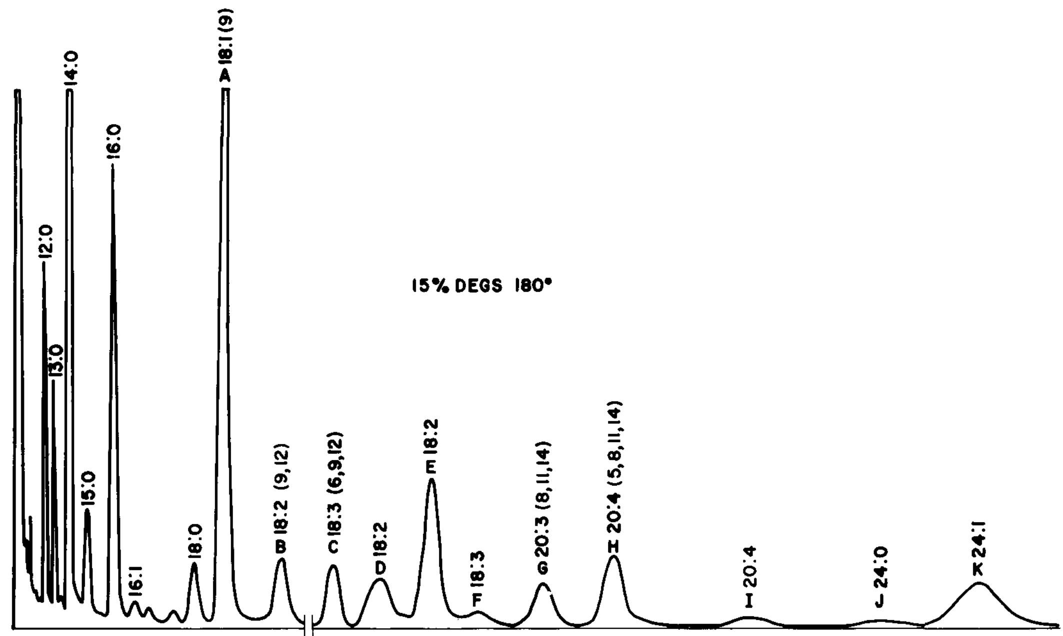

FIG. 2. Gas chromatograph of methyl esters of fatty acids from 26 day old *E. coronata* grown on SAB+GY medium. Peaks C through K are represented in twice their normal intensity.

vidually by GLC-mass spectroscopy, and by hydrogenation followed by GLC. Table II summarizes these results. The equivalent carbon lengths (ECL) were determined for each peak and for the hydrogenation products of the trapped peaks on both DEGS and OV-1 (12-14). The position of the double bonds in 18:1 and the methylene-interrupted polyunsaturated fatty acids were determined by the method of Niehaus and Ryhage (10). Peaks A and B were $18{:}1_{9}$ and $18{:}2_{9,12}$. Peak C proved to be mainly γ linolenic ($18{:}3_{6,9,12}$) with a trace of 20:0 (2-3%). Peak D consisted of approximately 75% 18:2 and 25% 20:1 and peak E consisted of another 18:2 and a trace of 20:2. The 18:2 fatty acids in peaks D and E absorb strongly in the ultraviolet at 231.5 nm, which suggest conjugated double bonds and these methyl esters have ECL values similar to that reported for *cis-trans* or *trans-trans* isomers (15); their complete structure awaits further analyses. Peak F consists mainly of an 18:3, possessing conjugated bonds, and a trace of 20:2. Peak G was identified as $20{:}3_{8,11,14}$ and a trace of 22:0. Peak H consists entirely of $20{:}4_{5,8,11,14}$. Peak I also consists of another 20:4 which contains conjugated double bonds.

Since unsaturated fatty acids possessing conjugated double bonds were observed, it seemed important to investigate whether the methanolic-boron trifluoride method of forming methyl esters could be producing these unusual fatty acids. The methylations were repeated using basic conditions, methanolic-sodium methoxide. The fatty acid composition was the same as in that observed with the methanolic-boron trifluoride method.

The fatty acid composition of *E. coronata* grown on SAB+GY and on chemically defined media was analyzed every other day for a growth period of 6 to 26 days. Table III summarizes these data for the 6th, 14th, 20th and 26th day of growth. The relative percentage of most of the fatty acids did not vary greatly with the age of the culture. However, $18{:}2_{9,12}$ and $20{:}4_{5,8,11,14}$ increased with age of the defined medium culture. Peak E (18:2) increased and 13:0, $18{:}2_{9,12}$ and $18{:}3_{6,9,12}$ decreased with age of the SAB+GY culture. In comparison the relative percentages of fatty acids of the two cultures were very similar except for the odd carbon length fatty acids and the fatty acids possessing conjugated double bonds, which were more common in the SAB+GY culture. *E. coronata* possessed a large percentage (ca. 50%) of shorter chain fatty acids (C 12 to C 15) and this value decreased with the culture age. The fatty acids of the defined medium culture were more unsaturated (30.1-43.1%, unsaturation index of 0.40-0.74) than the fungus grown on the nondefined medium (34.4-38.4%, unsaturation index of

TABLE II

Summary of Information on Gas Chromatography Peaks

Peaks	Equivalent carbon Length(ECL)[a]		ECL of hydro-genated peaks[a,b]	
	15% DEGS	3% OV-1	15% DEGS	Identification[c]
A	18.45	17.65	18.0	$18{:}1_9$
B	19.15	17.5	18.0	$18{:}2_{9,12}$
C	20.0	17.4 (98%) 20.0 (2%)	18.0(96%) 19.0(1%) 20.0(3%)	$18{:}3_{6,9,12}$ 20:0 trace
D	20.6	18.15(70%) 19.7 (30%)	18.0(75%) 20.0(25%)	18:2, conjugated[d](75%) 20:1 (25%)
E	21.0	18.4 (93%) 17.8 (6%) 19.0 (1%)	18.0(98%) 20.0(2%)	18:2, conjugated[d] 20:2 trace
F	21.4	18.1 (90%) 19.0 (5%) 19.5 (5%)	18.0(95%) 20.0(5%)	18:3, conjugated[d] 20:2 trace
G	21.9	19.3	20.0	$20{:}3_{8,11,14}$ 22:0 trace[e]
H	22.4	19.1	20.0	$20{:}4_{5,8,11,14}$
I	23.4			20:4, conjugated[d]

[a]When more than one methyl ester was detected the approximate composition is given.

[b]Samples were obtained by trapping methyl ester peaks from preparative GLC on 15% DEGS and subsequently hydrogenated.

[c]All the methyl esters and the polymethoxy methyl ester derivatives (10) of peaks A, B, C, G and H were analyzed by direct gas chromatography-mass spectroscopy. This technique identified the molecular weight of the fatty acid and the position of the double bonds. The position of the double bonds in peaks D, E, F and I were not determined because these methyl esters were not oxidized with osmium tetroxide under our experimental conditions.

[d]Absorbs strongly in the ultraviolet at 231.5 nm.

[e]Based on mass spectral data.

0.51-0.65) (Table III).

The large percentage of 14:0 and 18:1 acids, the high degree of saturation, and the significant quantity of 12:0, 13:0 and 15:0 acids are consistent with the data reported by Tyrrell (16) for other *E. coronata* strains. No appreciable amount of 14:1 was found as has been reported (17,18).

The presence of significant levels of 13:0 and 15:0 acids in the SAB+GY culture may reflect the composition of the medium. The odd chain fatty acids are biosynthesized initially from propionic acid which is primarily derived from amino acids. The SAB+GY medium contains significant amounts of peptone and yeast extract which provides the amino acid pool for the formation of propionic acid and subsequent odd carbon chain fatty acids. The composition of the haemolymph of the insects, in which the *E. coronata* grows, is rich in amino acids and is more like that of the SAB+GY medium than the defined medium.

The rigorous identification of the GLC peaks was conducted on the 26 day old SAB+GY culture and these data may not necessarily apply to all cultures and ages. The identification of $18{:}3_{6,9,12}$, $20{:}3_{8,11,14}$ and $20{:}4_{5,8,11,14}$ is consistent with the fatty acids found in other Phycomycetes (16,18,19). The unusual fatty acids possessing a strong UV absorption are only present in significant quantities in the SAB+GY culture after the 16th day. Perhaps these fatty acids are associated with the degradation processes. The presence of

TABLE III

Percentage Fatty Acids and Unsaturation in *E. coronata*

		SAB+GY medium, days				Defined medium, days			
Peaks	FA	6	14	20	26	6	14	20	26
	12:0	5.5	7.4	6.6	7.3	8.2	9.0	8.2	7.7
	13:0	8.2	6.1	6.3	5.4	1.6	1.2	1.3	1.4
	14:0	36.1	36.6	33.6	34.9	43.5	36.9	33.5	33.5
	15:0	2.6	2.0	3.3	2.1	0.6	0.3	0.5	0.6
	16:0	10.6	10.8	10.4	9.7	13.9	12.0	12.1	12.4
	16:1	1.0	0.5	1.1	0.3	0.7	0.9	0.6	0.7
	16:2	0.3	0.3	0.6	0.2	Trace	Trace	0.1	0.1
	17:0	0.2	0.4	0.8	0.1	0.0	0.0	Trace	Trace
	18:0	1.8	2.3	2.2	1.9	2.0	1.3	1.4	1.2
A	18:1 (9)	21.5	21.0	20.4	21.3	21.4	22.4	21.3	21.9
B	18:2 (9,12)	4.8	4.9	4.6	3.5	5.2	8.0	10.5	10.4
D	18:2	0.2	0.4	0.8	0.4	0.1	0.2	0.2	0.2
E	18:2	0.2	1.1	2.4	4.8	Trace	0.2	0.6	0.8
C	18:3 (6,9,12)	2.4	1.2	1.4	1.1	0.9	2.1	2.2	2.2
F	18:3	0.0	0.0	0.3	0.3	0.0	0.0	Trace	0.1
G	20:3 (8,11,14)	1.2	1.0	1.0	1.1	0.2	0.6	0.6	0.5
H	20:4 (5,8,11,14)	2.9	2.3	2.8	2.9	0.9	3.4	4.8	4.6
I	20:4	0.0	0.0	0.5	1.3	0.0	0.0	0.0	0.0
J	24:0	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace
K	24:1	0.3	1.7	1.4	1.2	0.7	1.3	3.0	1.6
Per cent unsaturated		34.8	34.4	37.3	38.4	30.1	39.1	42.9	43.1
Unsaturation index		0.56	0.51	0.60	0.65	0.40	0.63	0.72	0.74

a small percentage of unusual fatty acids may not impart any noticeable properties, however, if these fatty acids were located in certain lipid classes it could be highly significant. The mycelium grows well in both light and dark conditions. The fungus produces more conidia, the infectous stage, under constant light conditions and therefore, is more pathogenic when grown in light. The relationship of lipid composition to culture conditions will be investigated further.

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The Variation of Triglyceride Structure With Fatty Acid Composition in Pig Adipose Tissue

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ABSTRACT

Forty-five triglyceride samples with a wide range of fatty acid compositions were selected from a large number of pig adipose tissue samples (inner and outer back fats and perenephric fat) available from nutritional experiments. These samples were subjected to stereospecific analysis to determine the changes occurring in the positional distribution of the component fatty acids. The oleic acid content of the triglycerides was taken as the standard of comparison and as this increased, the proportions of the other unsaturated fatty acids also increased in a linear manner and the concentrations of the saturated components decreased proportionately. In position 1, the palmitic acid concentration remained constant while the stearic acid concentration decreased linearly and the concentrations of the unsaturated fatty acids increased. In position 2 the stearic acid concentration remained almost constant while the palmitic acid concentration decreased linearly in response to increases in the concentrations of the unsaturated acids. The least change occurred in position 3 where there were slight decreases in the concentrations of saturated acids as the concentrations of unsaturated acids increased. The precise quantitative relationships depended on the tissue examined. Constant proportions of the available myristic and palmitoleic acids were found in all three positions and constant proportions of the available stearic and oleic acids were found in position 1. These results are discussed in relation to possible pathways of triglyceride biosynthesis in pig adipose tissues.

INTRODUCTION

Pig adipose tissue triglycerides have an unusual fatty acid distribution in that position 2 is occupied largely by palmitic acid with the bulk of the remaining saturated fatty acids in position 1. Position 3 contains largely oleic acid with most of the linoleic acid (1-3). Triglycerides of this type are rare in nature but have been found in the adipose tissue of several species of the pig family (4), in the elephant (5) and in human milk (6). The fatty acid composition of pig adipose tissue triglycerides may readily be altered by dietary manipulation and it is known that quite small changes in fatty acid composition can have apparently disproportionate effects on the structure and physical properties of the triglycerides (2). In the course of several nutritional experiments (7; also, Mitchell et al., in preparation) in which the effect of the inclusion of copper in the diet of pigs on fat composition has been studied, we have accumulated large numbers of pig adipose tissue samples differing widely in fatty acid composition. It was decided to perform stereospecific analyses on a wide range of these to ascertain what changes occurred in the positional distribution of fatty acids as the overall fatty acid composition of the triglycerides was altered.

EXPERIMENTAL PROCEDURES

Fat Samples

In a series of nutritional experiments described in greater detail elsewhere (7; also, Mitchell et al., in preparation), pigs (Large whites) were given a control diet or the control diet supplemented with various levels of copper from weaning until they reached 90 Kg live weight when they were slaughtered. A sample of perenephric fat, and strips of back fat about 2 cm in width were taken from each animal. The inner and outer layers of back fat were separated along the line of connective tissue and each sample was extracted with chloroform-methanol (2:1 v/v). Portions of the lipid extracts (which were >99% triglyceride by TLC) were transmethylated and examined by gas liquid chromatography (GLC). Oleic acid was the major component and tended to show the greatest variation in concentration so a series of 20 inner back fat samples, 15 outer back fat samples and 10 perenephric fat samples were selected which offered the widest possible range of oleic acid contents for each tissue.

Gas Liquid Chromatography

The procedure was as described previously (3,8).

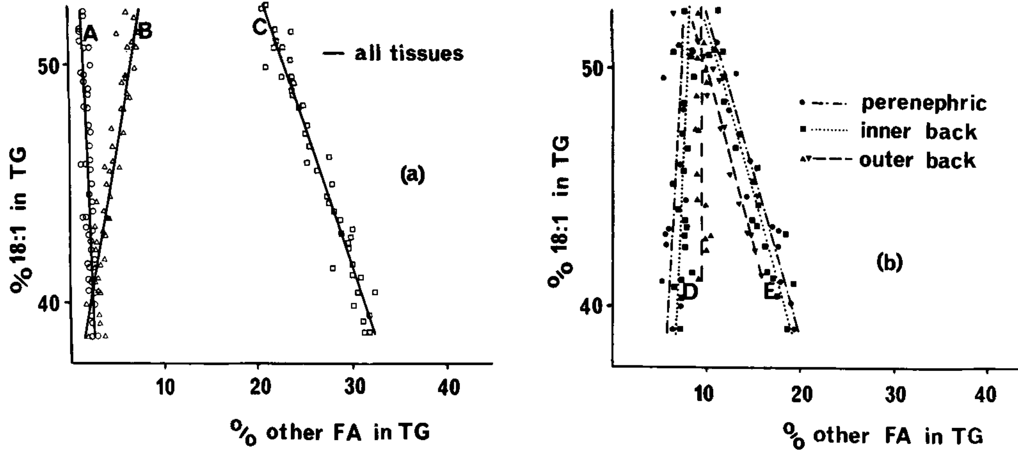

FIG. 1. Variation of the content of each fatty acid (FA) in the triglycerides (TG) with the 18:1 content of the triglycerides. A = 14:0; B = 16:1; C = 16:0; D = 18:2; E = 18:0.

Stereospecific Analysis

The procedure for stereospecific analysis of triglycerides has been described earlier (3,8). Results for position 1 were obtained by analysis of the lysophosphatide produced in the final stage of the procedure, those for position 2 were obtained by pancreatic lipase hydrolysis and those for position 3 were calculated by difference from the known triglyceride composition. Checks were possible on the results for positions 2 and 3 and only those analyses which conformed to the standards of accuracy described earlier (8) were accepted.

RESULTS

Only six fatty acids, myristic, palmitic, stearic, palmitoleic, oleic and linoleic acids were found in the pig adipose tissue triglycerides in appreciable concentrations (other components together constituted <1% of the total). The relationships which existed between the concentrations of these component acids in the triglycerides of the three tissues examined (inner and outer back fats and perenephric fats) are illustrated graphically in Figure 1 in which the concentrations of each fatty acid have been plotted against the oleic acid contents of the triglycerides. Oleic acid was chosen as the standard of comparison as the concentration of this acid (38-54%) varied more than that of any other component and as it occurred in appreciable concentrations in all three positions of the triglycerides.

Over the range of values examined, linear relationships with highly significant correlation coefficients ($P < 0.001$) were found between the concentrations of each component acid and the oleic acid contents of the triglycerides of the three tissues. The linear correlation coefficients for all the comparisons made in this study are listed in Table I. Where poor correlation coefficients were obtained, it invariably implied that the value of the dependent variable in the comparison was constant over the measured range of values of the independent variable and the standard error of the mean (SEM) of the dependent variable has been quoted in confirmation. The precise point scatter obtained was illustrated in Figure 1 only because of the technical difficulties in displaying very large numbers of separate points.

In general, as the concentration of oleic acid in the triglycerides of each tissue increased, the proportions of the saturated fatty acids decreased but the proportions of the other unsaturated acids increased. The precise relationship between components depended, however, on the tissue concerned although only with the stearic and linoleic acids were the differences between tissues appreciable. For example, as the oleic acid content of the triglycerides of the perenephric fat increased from 38% to 52%, the linoleic acid content increased from 5% to 8%, but in the outer back fat over a similar range of oleic acid values, the linoleic acid content remained constant at 9.6% (SEM = ± 0.17).

Stereospecific analyses were performed on each of the triglyceride samples by a procedure described elsewhere (3,8) based on the method devised by Brockerhoff (9). The results are illustrated graphically in Figure 2 in which, for purposes of comparison, the concentrations of each fatty acid in each position have again been plotted against the concentrations of oleic acid

TABLE I

Correlation Coefficiencies for the Various Comparisons Between the Concentrations of Fatty Acids in the Various Positions of Pig Adipose Tissue Triglycerides

Comparison	Correlation coefficients (r)					
	14:0	16:0	18:0	16:1	18:1	18:2
FA in TG v. 18:1 in TG	-0.488	-0.969	-0.868	0.813	---	0.740[a]
FA in position 1 v. 18:1 in TG	-0.138	0.103[b]	-0.896	0.754	0.893	0.686[c]
FA in position 2 v 18:1 in TG	-0.585	-0.939	0.373	0.859	0.938	0.763
FA in position 3 v. 18:1 in TG	---	-0.635	-0.731	0.604	0.694	0.643[d]
FA in position 1 v. same acid in TG	0.488	-0.040[c]	0.975	0.968	0.893	0.944
FA in position 2 v. same acid in TG	0.868	0.975	0.020[f]	0.980	0.938	0.774
FA in position 3 v. same acid in TG	---	0.564	0.877	0.874	0.694	0.944

[a]Except for outer back fat where r = 0.017 but standard error of mean (SEM) = ± 0.17.
[b]SEM = ± 0.26.
[c]Outer back fat, r = 0.529, SEM = ± 0.21.
[d]Outer back fat r = - 0.176, SEM = ± 0.40.
[e]SEM = ± 0.26.
[f]SEM = ± 0.11.

in the original triglycerides. Linear relationships were found over the range of fatty acid compositions examined (see Table I for correlation coefficients).

As the concentration of oleic acid in the original triglycerides increased, there were marked increases in the concentrations of oleic and palmitoleic acids in position 1; a less pronounced increase occurred in the concentration of linoleic acid in position 1 except in the outer back fat where the level of this acid remained constant. To compensate for the increase in the concentrations of the unsaturated acids, there was a marked decrease in the proportion of stearic acid in position 1 together with a slight decrease in the myristic acid concentration. The palmitic acid concentration, however, remained constant (11.8%, SEM = ± 0.26) over the entire range of oleic acid values.

When the oleic acid content of the original triglyceride increased there were pronounced increases in the concentrations of oleic and palmitoleic acids in position 2; the linoleic acid concentration also increased but only slightly. To compensate for this, there was a large decrease in the proportion of palmitic acid in position 2 together with small decreases in the concentrations of myristic and stearic acids. The differences in fatty acid composition between the three tissues were least in position 2 but this was probably because the two fatty acids that showed the major tissue differences, stearic and linoleic acids, were only minor components in this position.

The fatty acid composition of position 3, largely oleic acid (about 70%) and linoleic acid (12-20%), was affected least by alterations in the composition of the original triglycerides. The oleic, palmitoleic and linoleic acid concentrations in this position again increased somewhat as the oleic acid concentration of the original triglycerides increased; these increases were compensated for largely by decreases in the stearic acid content and to a lesser extent in the palmitic acid content. There was no detectable myristic acid in position 3.

There were many ways in which the results of the 45 stereospecific analyses could be correlated with each other and displayed graphically. It was particularly instructive to compare the variation in the concentration of each individual fatty acid, in each of the three positions, with the variation in the concentration of that acid in the entire triglycerides. At the same time, the proportions of each fatty acid (expressed as a percentage of the total available) in all positions were also correlated with the total amount of the same acid in the triglycerides. In doing this, it was found that all tissue specific effects were eliminated. The results for the saturated fatty acid components are illustrated in Figure 3 and those for the unsaturated components in Figure 4. Linear relationships were always found when the concentration of each fatty acid in each position was plotted against the concentration of the same acid in the triglycerides (correlation coefficients are listed in Table I) over the ranges of the fatty acid composition examined. However, both linear and curvilinear relationships were found when the proportions of each fatty acid in the three positions were correlated with the amount available.

Myristic acid was found only in positions 1

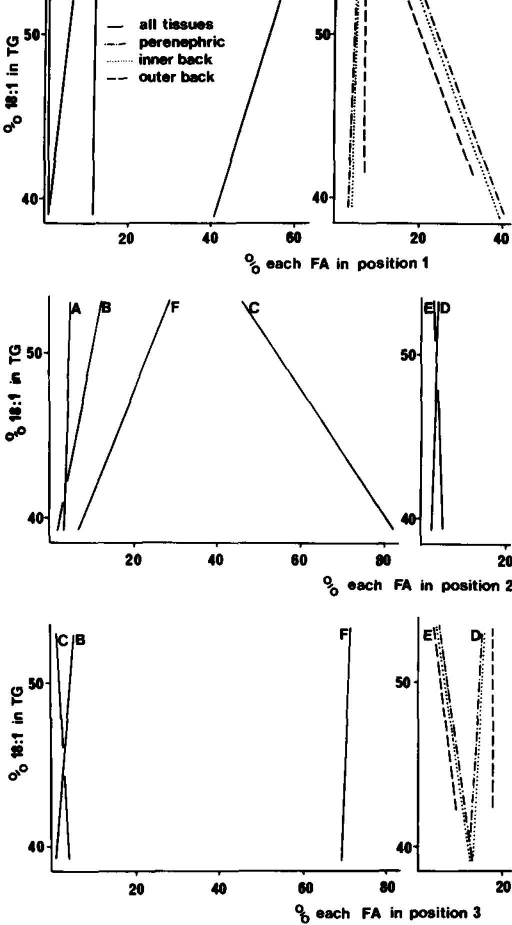

FIG. 2. Variation of the content of each fatty acid in each position of the triglycerides with the 18:1 content of the triglycerides. A = 14:0; B = 16:1; C = 16:0; D = 18:2; E = 18:0; F = 18:1.

and 2 and the concentrations of this acid increased in both positions with increasing myristic acid content of the triglycerides, although the most pronounced increase was observed in position 2. When expressed as a percentage of the total myristic acid in the triglycerides, the proportion of myristic acid in each position remained constant, however, at 17.5% (SEM = ± 0.79) in position 1 and 82.5% (SEM = ± 0.86) in position 2.

The concentration of palmitic acid found in position 1 remained constant at 11.8% over the entire range of concentrations (19-32%) of palmitic acid in the original triglycerides. Most of the change in palmitic acid concentration was observed in position 2 and only to a small extent in position 3. The proportions of palmitic acid in each position bore curvilinear relationships to the concentrations in the original triglycerides; the more palmitic acid available, the greater the proportion that occupied position 2.

As the concentration of stearic acid in the triglycerides increased, the concentration of this fatty acid in position 2 remained constant (3.4 ± 0.11%) but there were linear increases in the concentrations of stearic acid in positions 1 and 3; in position 1, the increase was more pronounced than in position 3. On the other hand, when expressed as a percentage of the total stearic acid in the triglycerides the proportion of stearic acid entering position 1 remained constant (70.5% ± 0.59%) at all concentrations of this acid in the triglycerides; the proportions in positions 2 and 3 varied in a curvilinear manner, a higher proportion entering position 3, the greater the concentration of stearic acid in the triglycerides.

As the concentration of palmitoleic acid in the triglycerides increased from 2% to 10%, the concentration of palmitoleic acid increased linearly in all three positions, but the most marked increase was observed in position 2. The proportion of the total palmitoleic acid in the triglycerides found in each position remained constant, however; 26.8 ± 0.39% was found in position 1, 50.8 ± 0.76% in position 2 and 22.4 ± 0.90% in position 3 at all concentrations of palmitoleic acid in the triglycerides.

When the concentration of oleic acid in the triglycerides increased from 38% to 54%, linear increases in the concentration of this acid were observed in all three positions; the smallest change occurred in position 3. The proportion of the total oleic acid in the triglycerides which was found in position 1 was constant throughout, however, at 35.9% ± 0.22% although the proportions found in positions 2 and 3 changed in a curvilinear manner, a higher proportion of oleic acid entering position 2, the more that was available in the triglycerides.

Linear increases in the concentrations of linoleic acid were also found in all three positions as the total concentration in the triglycerides increased, the most pronounced increase occurring in position 3. On the other hand, when expressed as a percentage of the total linoleic acid in the triglycerides, the proportions of linoleic acid found in each position varied in a curvilinear manner, and greater proportions of linoleic acid were found in positions 1 and 2 at higher concentrations of linoleic acid in the triglycerides.

DISCUSSION

Although there have been a number of investigations of how the fatty acid compo-

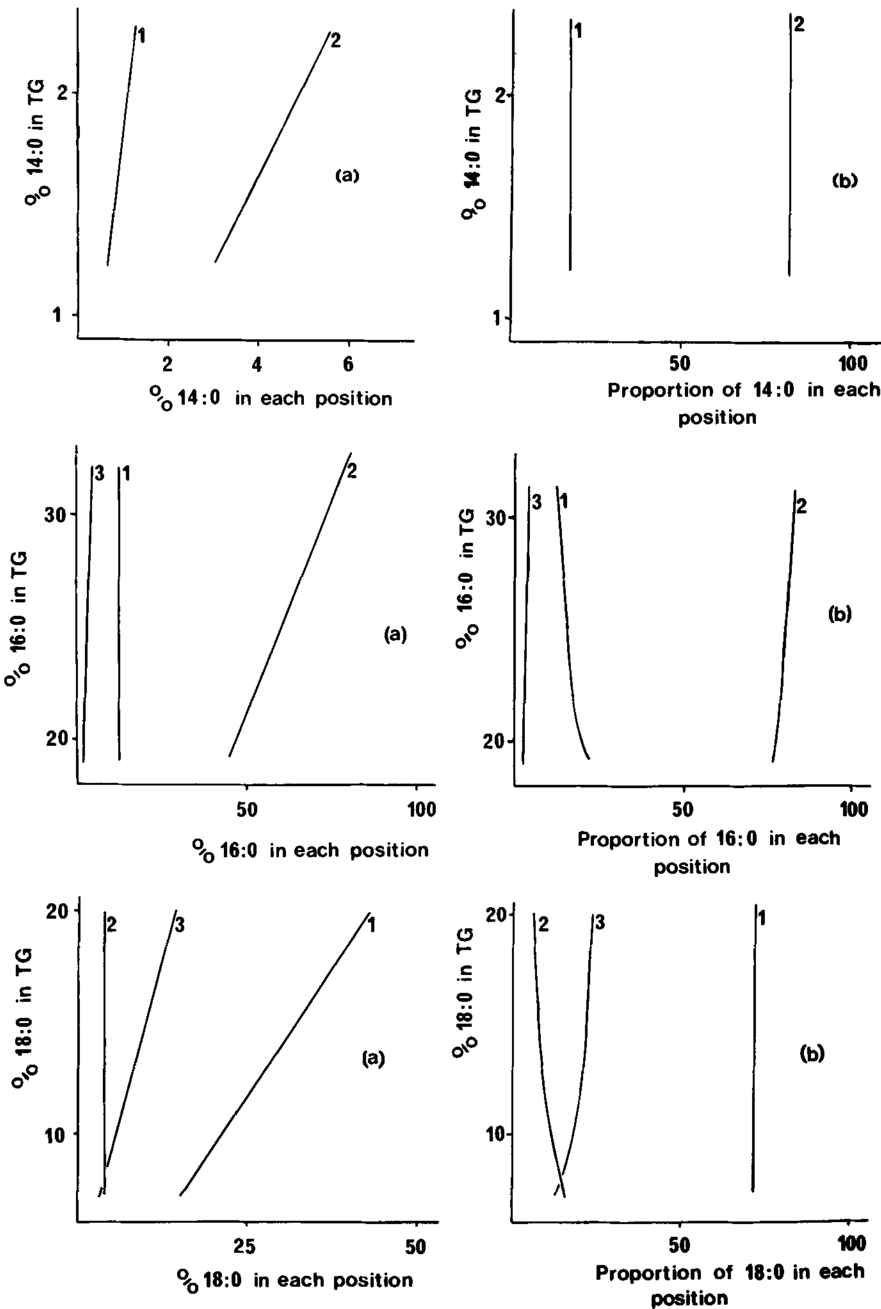

FIG. 3. Variation in the concentration and proportionate concentration of each of the saturated fatty acids with the concentration of the same fatty acid in the triglycerides.

sition of position 2 may change with alteration of the overall fatty acid composition of triglycerides, in particular in mouse adipose tissue (10) and in rabbit liver (11), this is apparently the first study in which similar variations in positions 1, 2 and 3 of triglycerides have been simultaneously determined.

The changes in fatty acid composition of the pig adipose tissue triglycerides in this investigation were obtained by adding copper at various levels (from zero to 250 ppm) to the diet of the animals. This is known to produce adipose tis-

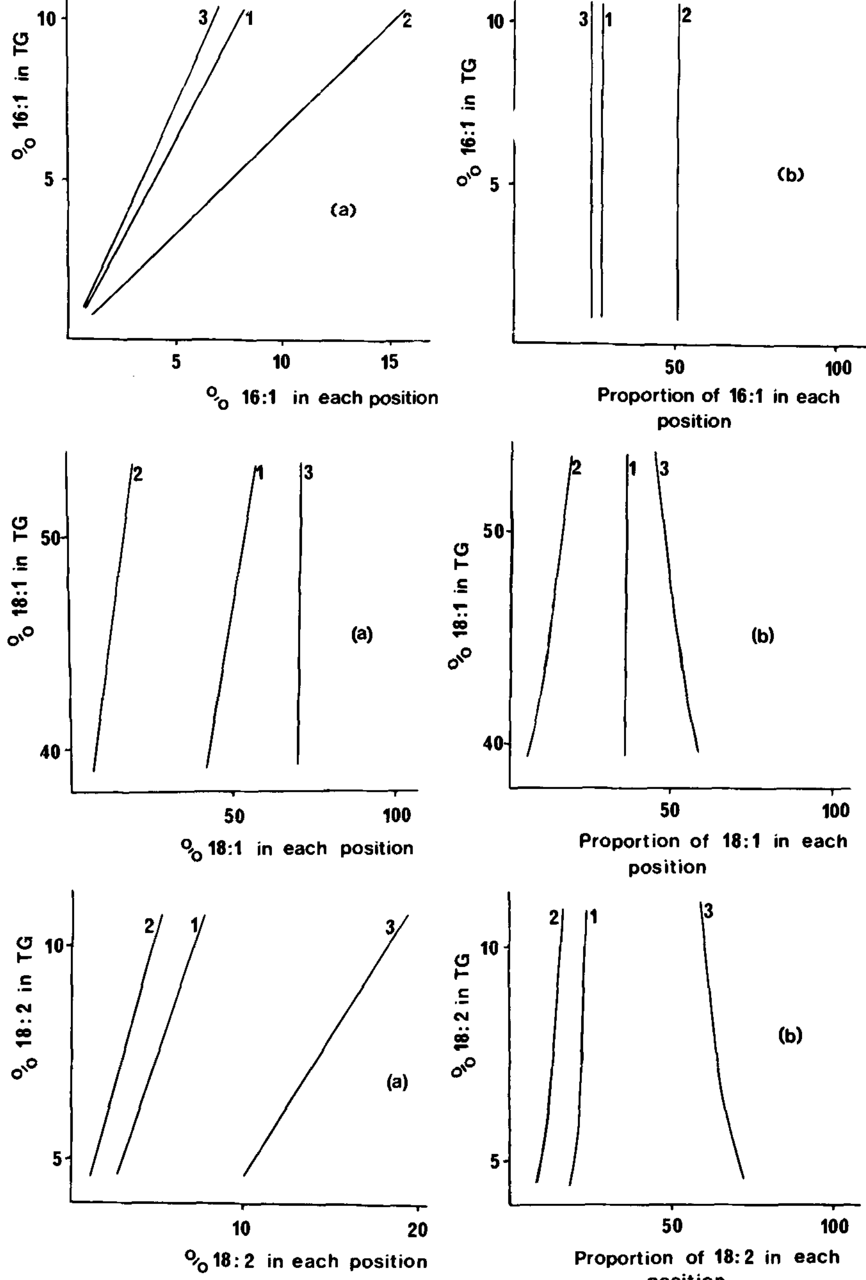

FIG. 4. Variation in the concentration and proportionate concentration of each of the unsaturated fatty acids with the concentration of the same fatty acid in the triglycerides.

sue fats with a higher degree of unsaturation than in control animals (7,12,13; also, Mitchell et al., in preparation) although the precise mechanism by which the effect is produced is unknown. It is therefore uncertain whether the variations in triglyceride structure produced in this way are qualitatively similar to those obtained by other dietary or physiological means. The triglyceride samples chosen for stereospecific analysis were selected solely on their oleic acid contents, without regard to the contents of the other component fatty acids, so

that triglycerides with as wide and continuous a range of concentrations of oleic acid as possible were analyzed. Such triglycerides obviously had quite different structures as their melting points varied between 5 C and 48 C (7; also, Mitchell et al., in preparation).

The results obtained provided useful information on how the supply of fatty acids for acylating the three positions of the glycerol moiety during triglyceride synthesis in pig adipose tissue was controlled, although they cannot be used to define the precise mechanism of biosynthesis. Strictly speaking the results apply only to the range of fatty acid compositions examined, but some extrapolation is possible.

A constant concentration of the available palmitic acid was always found in position 1. Constant proportions of the available myristic, stearic, palmitoleic and oleic acids were also found in this position which would imply that the plots of the concentrations of each of these acids in position 1 against the concentrations of each in the triglycerides are linear at all concentrations and pass through the origin. The concentration of the only other component in position 1, linoleic acid, bore a more complex relationship to the amount available. A small but constant concentration of stearic acid was always found in position 2 together with constant proportions of the available myristic and palmitoleic acids again implying that for these two acids, the concentrations in position 2 would be linear down to zero concentration when plotted against the concentration in the triglycerides. With the other components both the concentration and proportion of the total of each found in position 2 bore more complex relationships to the amounts available in the triglycerides. In position 3, only the palmitoleic acid concentration bore a simple relationship to the concentration in the triglycerides, as a constant proportion of the total was always found (no myristic acid was detected in this position).

Triglycerides are probably synthesized in pig adipose tissue by the glycerophosphate pathway (14,15), i.e., L-α-glycerophosphate is esterified in positions 1 and 2 with fatty acids to form phosphatidic acid. This is dephosphorylated and in turn acylated in position 3 to triglyceride. It has yet to be established whether position 1 or 2 of L-α-glycerophosphate is esterified first or indeed whether both positions are esterified simultaneously. The unique manner in which position 1 is esterified in pig adipose tissue would suggest that the fatty acid requirements of this position must be satisfied first during triglyceride biosynthesis and that there is sequential acylation of positions 1, 2 and 3.

It would appear that adipose tissue is the major site for the de novo synthesis of fatty acids in the pig (16) but it is not known how such fatty acid synthesis is coupled to triglyceride biosynthesis. It is possible, for example, that fatty acids synthesized de novo in the tissue constitute a separate pool from those fatty acids taken up from the plasma and are utilized separately for triglyceride synthesis in the adipose tissues. It may be significant, with regard to this problem, that constant proportions of the two fatty acids which must be synthesized almost entirely endogenously, viz., myristic and palmitoleic acids, are found in all three positions. In consequence it could be suggested that position 1 in pig adipose tissue triglycerides is occupied largely by fatty acids synthesized within the adipose tissues and that in the other positions, fatty acids derived from the blood complicate the pattern. The only fatty acid which must be entirely of exogenous origin, linoleic acid, exhibits a complex distribution pattern in all three positions of the triglycerides. In vitro, biosynthetic studies will be necessary, however, to test this hypothesis.

The graphs in Figures 3 and 4 may be used to roughly predict the distribution of fatty acids in triglycerides of pig adipose tissue if the total fatty acid composition is known. Further, as it has been established that there is an approximately 1 random, 2 random, 3 random arrangement of fatty acids in pig adipose tissue (3), they can also be used to roughly predict the proportions of the various molecular species of triglycerides.

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Aliphatic Hydrocarbon Contents of Various Members of the Family Micrococcaceae

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ABSTRACT

The composition and nature of the hydrocarbons of various members of the family Micrococcaceae were studied by gas chromatography. The hydrocarbons found in *Sarcina lutea* strains, *Sarcina flava, Sarcina subflava, Micrococcus lysodeikticus* strains and their transformed hybrids consisted of monounsaturated hydrocarbons showing either iso or anteiso methyl branching. The hydrocarbons of the various organisms differed in the range of hydrocarbon distribution, the relative proportions of the major hydrocarbon fractions and the individual components within each fraction. *Staphylococcus aureus* strains and *Gaffkya tetragena* contained no appreciable quantities of hydrocarbons. The fatty acid compositions of the *Sarcina species, Micrococcus lysodeikticus* strains and the strains derived by transformation were qualitatively identical. *Staphylococcus aureus* and *Gaffkya tetragena* showed similar fatty acid patterns that were different from those of *Sarcina* and *Micrococcus*. A taxonomic relationship between these organisms was established on the basis of the hydrocarbon compositions.

INTRODUCTION

The hydrocarbons of *Sarcina lutea* ATCC 533 have been identified as a complex mixture of branched unsaturated chains composed of 16 to 29 carbon atoms, the major fractions being C_{25}, C_{26} and C_{27} hydrocarbons (1). Albro and Dittmer (2) confirmed this hydrocarbon composition for *S. lutea* strain ATCC 533, but found that *S. lutea* strain FD 533 contained C_{27}, C_{28} and C_{29} hydrocarbons as major fractions. Albro and Dittmer (2) attributed these differences to the fact that *S. lutea* strain ATCC 533, distributed by American Type Culture Collection and studied by Tornabene et al. (1), was no longer a *S. lutea* but probably another bacterium in the family *Micrococcaceae*.

In an attempt to clarify the identity of *S. lutea* ATCC 533, in addition to an extension of our previous work done on microbial hydrocarbons (1,3-7), this paper reports the results of an investigation of the aliphatic hydrocarbon composition of a variety of bacteria classified in the family Micrococcaceae. Such a study may also help clarify the taxonomic confusion that is associated with this group of organisms.

MATERIALS AND METHODS

Organisms

The organisms studied were *Sarcina lutea* strains ATCC 533 (1), FD 533 (2), ATCC 272, ATCC 381 and ATCC 382 S.I.; *Sarcina subflava* ATCC 7468; *Sarcina flava* ATCC 540; *Staphylococcus aureus* 83, 655 and a laboratory strain; and *Gaffkya tetragena* ATCC 10875. The strains of *Micrococcus lysodeikticus* tested were ISU, PU, UM, WRU and ATCC 15801. The remaining organisms studied were transformed hybrids prepared with *S. lutea* ATCC 272 as the donor and *M. lysodeikticus* ISU as the recipient. These organisms are presented in Table I. Most of the above hybrids are double transformants for both pigment and nutritional markers, incorporated with an appreciable amount of *S. lutea* genetic material.

For sources of organisms listed above and for a description of general characteristics, see Kloos et al. (8-10).

Culturing Conditions

All the organisms reported in this paper were grown to the stationary phase of growth in 1200 ml Trypticase Soy Broth (BBL) on a rotary shaker at 27 C, except for *S. aureus* and *G. tetragena* which were grown at 37 C to maintain optimal growing conditions. In an additional experiment, *S. aureus* was grown in 100 ml Trypticase Soy Broth containing 50 μCi 1-^{14}C-sodium acetate according to previously described procedures (7). All cells were harvested by centrifugation and washed twice with 0.9% saline solution. The lipid contents of the Trypticase Soy medium and the effect of different media on the biosynthesis of hydrocarbons have been reported elsewhere (1,5,11).

TABLE I

Transformation of Pigment and Nutritional Markers in *Micrococcus Lysodeikticus*

Hybrid No.	Donor	Recipient	Hybrid genotype
1	*S. lutea* ATCC 272 (pig-p, ade$^+$, hisD$^+$, trpE$^+$)[a]	*M. lysodeikticus* ISU (pig-y, ade$^+$-1, trpE$^+$, hisD20)	pig-p hisD$^+$
2	*S. lutea* ATCC 272 (pig-p, ade$^+$ hisD$^+$, trpE$^+$)	*M. lysodeikticus* ISU (pig-y, ade$^+$-1, hisD$^+$, trpE16)	pig-p trpE$^+$
3	*S. lutea* ATCC 272 (pig-p, ade$^+$, hisD$^+$, trpE$^+$)	*M. lysodeikticus* ISU (pig-y, ade, hisD$^+$, trpE$^+$)	pig-p ade$^+$
4	*S. lutea* ATCC 272 (pig-o, ade$^+$, hisD$^+$, trpE$^+$)	*M. lysodeikticus* ISU (pig-y, ade$^+$-1, trpE$^+$, hisD20)	pig-o hisD$^+$
5	*S. lutea* ATCC 272 (pig-w, ade$^+$, hisD$^+$, trpE$^+$)	*M. lysodeikticus* ISU (pig-y, ade$^+$-1, hisD$^+$, trpE16)	pig-w trpE$^+$
6	*S. lutea* ATCC 272 (pig-w, ade$^+$, hisD$^+$, trpE$^+$)	*M. lysodeikticus* ISU (pig-y, ade, hisD$^+$, trpE$^+$)	pig-w ade$^+$

[a]Symbols: pig-w = white; pig-p = pink; pig-o = orange; pig-y = yellow; ade = adenine requirement; ade$^+$ = adenine independent; trpE = L-tryptophan requirement; trpE$^+$ = L-tryptophan independent; hisD = L-histidine requirement; hisD$^+$ = L-histidine independent (followed by arabic numeral indicates order of isolation).

Extraction and Column Fractionation

Cell suspensions were extracted by the method of Bligh and Dyer (12) modified as previously described (13). The chloroform soluble materials were fractionated on silicic acid columns as described by Tornabene et al. (6). The hydrocarbons were eluted from the columns with *n*-hexane (nanograde). All solvents used in this study were purchased from Mallinckrodt Chemical Products and were redistilled before use. The procedure for handling the samples and their preparation for analyses has been described (1,3,6). A comparison of the total lipid composition of the organisms studied will be presented elsewhere.

Analytical Methods

The dry weights of the extracted cellular materials were obtained by drying the samples to a constant weight in vacuo. The total hydrocarbon content on a weight basis was determined by calibration of the gas chromatographic peak areas with known amounts of hydrocarbon standards.

The fatty acids were liberated from the lipid components and methylated by refluxing the lipid samples in a mixture of methanol and HCl according to the method described by Kates(14).

The aliphatic hydrocarbons and fatty acid methyl esters were analyzed on a F & M 5750 Gas Chromatograph equipped with dual-flame ionization detectors. The chromatographic analyses were carried out in 31 m x 0.05 cm and 93 m x 0.075 cm stainless steel columns coated with OV-17 (methyl phenyl silicone) and Igepal CO 990 (nonyl phenoxy polyoxyethylene ethyl alcohol), respectively. The identification of the hydrocarbons and fatty acid methyl esters of *S. lutea* ATCC 533 by combined gas chromatography-mass spectrometry has been previously presented (1). The identities of all components separated by gas chromatography were determined by comparing their retention times with those of established standards. The identities assigned to the hydrocarbon components of *S. lutea* FD 533 and *S. flava* ATCC 540 were confirmed by mass spectral analyses using a combination of a Barber-Coleman gas chromatograph and an AEI MS-12 mass spectrometer. The mass spectra were taken as each component emerged from the capillary column.

The lipids were chromatographed on thin layer plates and silicic acid impregnated papers and labeled components were detected by autoradiography as previously described (6,7,13).

TABLE II

Relative Mole Per Cent Compositions of the Hydrocarbons of *Sarcina*[a]

Peak No.	Peak identities	*S. lutea* ATCC 533	*S. lutea* FD 533	*S. lutea* ATCC 272	*S. lutea* ATCC 381	*S. lutea* ATCC 382 S.I.	*S. flava* ATCC 540	*S. subflava* ATCC 7468
1	i-23:1[b]	2.60	---	Trace[c]	---	---	---	0.18
2	a-23:1	1.80	---	0.28	---	0.30	---	0.56
3	a-23:1	0.67	---	0.41	---	0.61	---	1.28
4	i-23:1	0.33	---	Trace	---	---	---	0.08
5	i-24:1	5.51	---	0.06	---	0.12	Trace	0.20
6	a-24:1	5.15	---	0.53	---	0.23	Trace	0.83
7	i-24:1	0.70	---	Trace	---	---	Trace	---
8	a-24:1	1.60	---	Trace	---	---	Trace	---
9	i-25:1	6.01	0.46	0.39	0.32	---	0.13	0.71
10	a-25:1	20.75	1.31	2.54	1.81	2.45	0.15	1.63
11	a-25:1	12.63	2.39	5.03	3.20	6.40	0.59	6.98
12	i-25:1	1.54	0.23	0.17	0.49	---	0.18	Trace
13	i-26:1	2.21	0.11	0.61	1.38	0.55	0.46	0.68
14	a-26:1	7.15	0.71	3.81	3.91	1.69	1.00	4.61
15	i-26:1	1.00	0.13	0.19	Trace	0.06	0.43	---
16	a-26:1	4.48	0.15	0.11	---	0.07	1.00	---
17	i-27:1	1.14	2.04	2.51	4.90	2.68	0.83	1.74
18	a-27:1	8.12	5.41	7.24	4.95	11.73	16.48	11.68
19	a-27:1	16.61	7.31	15.17	10.43	25.25	5.89	21.80
20	i-27:1	---	1.14	0.47	2.47	Trace	3.50	Trace
a	br-olefin	---	0.60	---	---	---	8.89	---
21	i-28:1	---	2.20	1.82	6.30	1.19	2.78	1.48
22	a-28:1	---	3.30	7.68	13.16	2.49	4.98	8.76
23	i-28:1	---	5.49	1.44	1.91	0.78	5.36	---
24	a-28:1	---	3.50	0.72	1.72	0.65	13.35	---
25	i-29:1	---	12.62	6.88	5.29	5.61	0.41	4.51
26	a-29:1	---	24.33	17.35	16.58	18.71	4.99	6.28
27	a-29:1	---	23.02	23.90	19.33	18.43	18.35	26.01
28	i-29:1	---	1.37	0.69	1.85	Trace	2.05	Trace
b	br-olefin	---	---	---	---	---	2.76	---
29	i-30:1	---	---	---	---	---	0.82	---
30	a-30:1	---	---	---	---	---	0.37	---
31	i-30:1	---	1.58	---	---	---	0.99	---
32	a-30:1	---	0.63	---	---	---	3.29	---

[a]The mole per cent compositions of the hydrocarbons were calculated on the basis of their gas chromatographic areas, which were obtained by multiplying the peak heights by the widths at half peak heights. The first number in the identities indicates the number of carbon atoms; the second number indicates the number of double bonds.

[b]Symbols: i = iso; a = anteiso; br = branching.

[c]Trace amount (detected only on analysis of concentrated samples).

TABLE III

Relative Mole Per Cent Compositions of the Hydrocarbons of *Micrococcus Lysodeikticus* Strains

Peak No.	Peak identities	ISU	WRU	PU	UM	ATCC 15801
1	i-23:1[a]	---	---	---	---	Trace
2	a-23:1	---	---	---	---	Trace
3	a-23:1	---	---	---	---	Trace
4	i-23:1	---	---	---	---	Trace
5	i-24:1	---	---	---	---	---
6	a-24:1	---	---	---	---	0.44
7	i-24:1	---	0.02	---	---	---
8	a-24:1	---	---	---	---	---
9	i-25:1	0.38	0.02	---	---	0.44
10	a-25:1	0.28	0.12	0.20	---	0.49
11	a-25:1	1.31	0.28	0.72	---	1.23
12	i-25:1	Trace	Trace	---	---	Trace
13	i-26:1	---	0.02	---	0.16	0.10
14	a-26:1	1.69	0.18	0.30	0.68	2.66
15	i-26:1	1.02	0.01	---	---	Trace
16	a-26:1	---	0.04	Trace	---	0.34
17	i-27:1	11.40	0.25	0.27	6.73	11.23
18	a-27:1	2.37	5.32	1.59	1.60	3.65
19	a-27:1	6.95	10.61	6.53	2.85	5.07
20	i-27:1	0.60	0.02	---	0.68	1.40
21	i-28:1	0.83	0.24	0.41	0.97	0.99
22	a-28:1	12.01	7.77	5.13	10.34	14.48
23	i-28:1	1.11	0.21	0.70	2.10	1.77
24	a-28:1	0.35	0.51	1.65	2.87	5.27
25	i-29:1	3.35	2.54	1.68	4.64	2.02
26	a-29:1	12.89	19.70	21.87	24.70	12.27
27	a-29:1	42.08	50.40	58.97	41.68	34.93
28	i-29:1	1.39	1.75	Trace	---	1.23

[a]Abbreviations as in Table II.

RESULTS

Hydrocarbon Composition

The cells of the *Sarcina spp.*, *M. lysodeikticus* strains, and of the transformed hybrids studied were found to contain aliphatic hydrocarbons in the range of 17.24-22.07% of the total lipids. The hexane fractions of all three *Staphylococcus* strains studied, as well as *G. tetragena*, were found to contain detectable hydrocarbons on the order of 0.08-0.24% of the total lipids; however, these hydrocarbon fractions obtained were not considered as having been produced by the bacteria for the reasons discussed below.

Hydrocarbon Compositions of Sarcina, Micrococcus and Transformed Hybrids

Gas chromatographic patterns for the aliphatic hydrocarbons of representative *Sarcina spp.* and *M. lysodeikticus* strains are presented in Figures 1 and 2, respectively. The peak identities and quantitative data are given in Tables II and III. The range of hydrocarbons in *S. lutea* ATCC 533 is from C_{23} to C_{27}. The major fractions consist of 25, 26 and 27 carbon chains. The analysis shows the presence of tetrads of unsaturated components all containing methyl branching. Each tetrad is composed of four isomers identified as two iso-olefins and two anteiso-olefins. The iso-olefins emerge ahead of the anteiso-olefins except for the last two components of the odd carbon-numbered tetrads. In the C_{27} fraction the fourth component is relatively minor and is not visible in this scan [See, however, Fig. 4 in Ref. (1)]. The nature of the hydrocarbons of *S. lutea* FD 533 (Fig. 1) is the same as the hydrocarbons of *S. lutea* ATCC 533 but with major fractions of 27, 28 and 29 carbons, exactly two carbon units higher. The hydrocarbon compositions are in general agreement with those reported by Albro and Dittmer (2,15). However, Albro and Dittmer (2) have tentatively identified one of the C_{29} hydrocarbon components as having iso and anteiso branchings on opposite ends of the molecule. A similar claim was also made for one of the hydrocarbons of *S. lutea* ATCC 533 (15). Our previous (1) and present mass spectral data show the presence only of symmetrical branched structures for these hydrocarbons. Current studies indicate, however, that some of the resolved components in each tetrad are

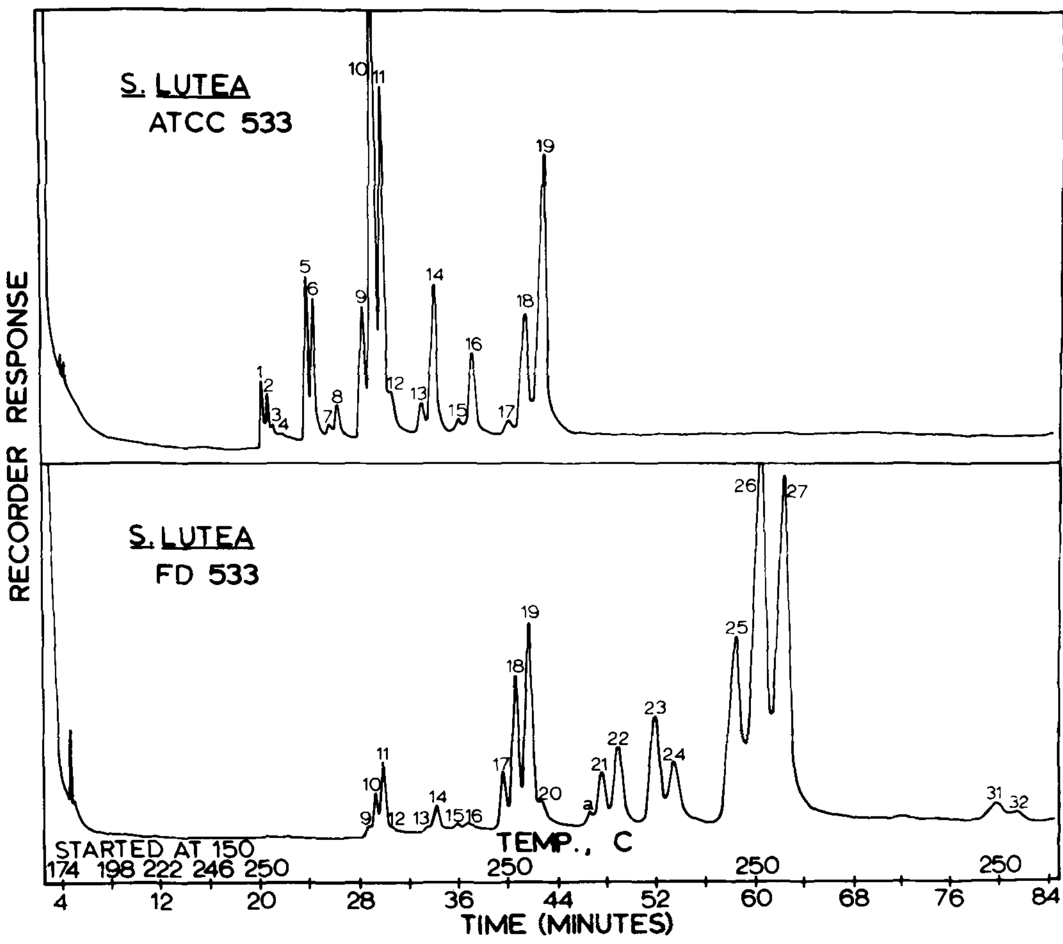

FIG. 1. Gas chromatographic separations of the hydrocarbons of *S. lutea* ATCC 533 and *S. lutea* FD 533 on a stainless steel column (31 m x 0.05 cm, i.d.) coated with 10% OV-17. Approximately equal quantities of the samples were injected. Nitrogen flow rate at 12 ml/min; no split. F & M 5750 apparatus equipped with dual-flame ionization detectors. Range, 10^2; attenuation, 1. Temperature programmed at approximately 6 °/min from 150 to 250 C and held isothermally. Chart speed, 0.25 in/min. Peak identities are given in Table II.

themselves mixtures of two components with different double bond positions, both near the center of the molecule (paper in preparation). Other components, designated a and b, have not yet been identified but are presumed to be branched-chain olefins.

The hydrocarbon pattern of *S. lutea* FD 533 varied somewhat with age. In older preparations, in addition to the three C_{29} components (Fig. 1), a fourth component was detected; also the second anteiso $C_{29:1}$ (peak 27, Fig. 1) was the major component. Such variations in the hydrocarbons of *S. lutea* ATCC 533 have not been observed.

M. lysodeikticus ISU and WRU show hydrocarbon patterns (Fig. 2) that are qualitatively and quantitatively similar to *S. lutea* FD 533 (Fig. 1). Distinguishable differences between the patterns are seen, however, in the relative proportions of the hydrocarbon components in the C_{27} fractions and in the even carbon-numbered fractions.

Quantitative analyses of the aliphatic hydrocarbons in the *Sarcina spp.* and *M. lysodeikticus* strains are presented in Tables II and III, respectively. The results of the analyses of the transformed hybrids are presented in Table IV. The trace quantities indicated in the Tables represent only a qualitative expression of those components detectable when more concentrated samples were analyzed. The general chemical nature of the hydrocarbons of these

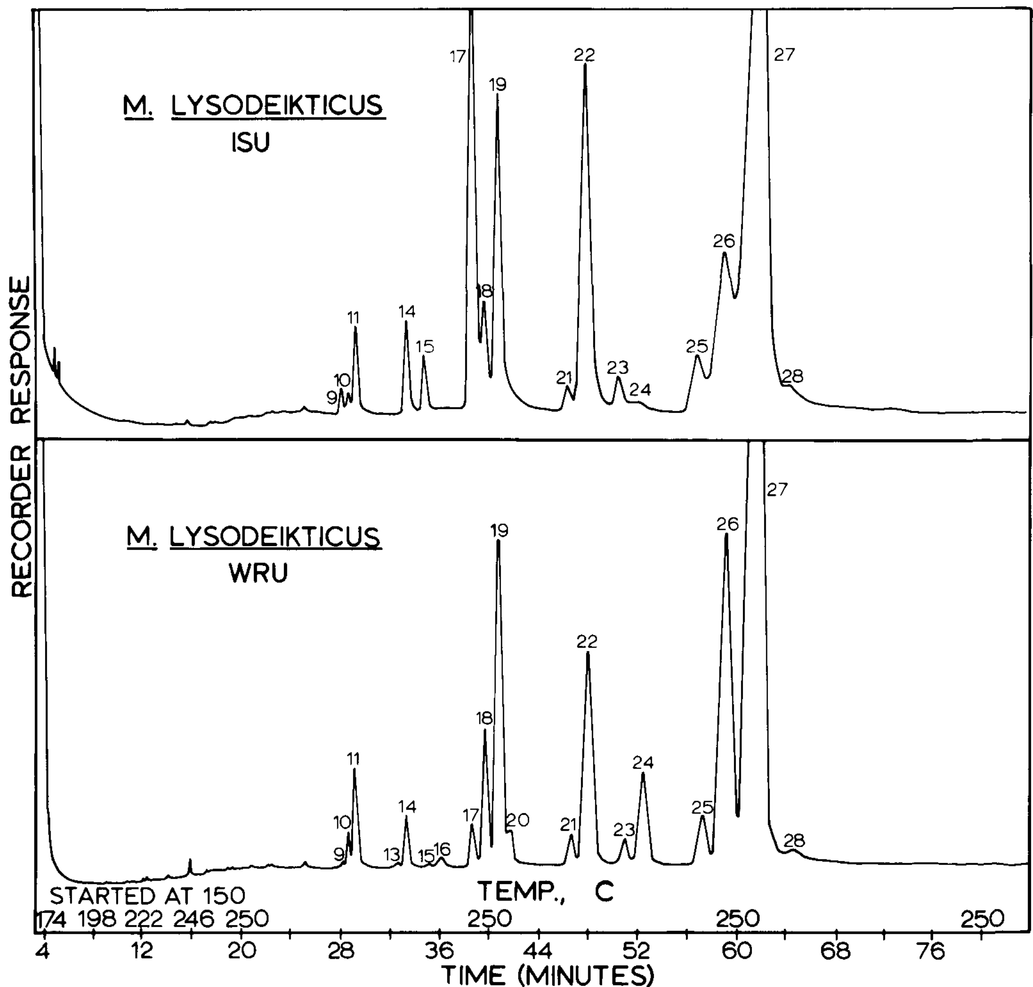

FIG. 2. Gas chromatographic separations of the hydrocarbons of *Micrococcus lysodeikticus* ISU and *Micrococcus lysodeikticus* WRU. Conditions as described in Figure 1. Peak identities are given in Table III.

organisms was the same as described above (Fig 1,2). All the hydrocarbons were identified as branched-olefins. Most of the organisms analyzed contained hydrocarbons in the range C_{23} to C_{29} inclusive. Hydrocarbons containing 30 carbons were also detectable in *S. lutea* FD 533 (Fig. 1), *S. flava* ATCC 540 (Table II), and in hybrid strains 2, 5 and 6 (Table IV), while hydrocarbons lower than C_{23} were seen in *S. lutea* ATCC 533 (1).

The relative proportions of the components in the odd carbon-numbered tetrads of all the *Sarcina spp.* except *S. lutea* FD 533 (Fig. 1, Table II) were similar. There were more pronounced variations, however, in the relative proportions of the components in the even carbon-numbered fractions. In many cases only doublets instead of tetrads were detectable. This situation was most pronounced in *S. subflava* which showed only a doublet of iso and anteiso hydrocarbons in the even carbon-numbered chains (Table II). The hydrocarbon pattern of *S. flava* showed a characteristically high content of branched hydrocarbons designated as a and b. These components were also detected in *S. lutea* FD 533 (Fig. 1) but were only occasionally found in *S. lutea* ATCC 533 (1).

The differences between the patterns of several of the *M. lysodeikticus* strains (Fig. 2, Table III) were also more pronounced in the even carbon-numbered chains. *Micrococcus lysodeikticus* strains UM, ISU and ATCC 15801 can be distinguished from strains PU and WRU

TABLE IV

Relative Mole Per Cent Compositions of the Hydrocarbons of Transformed Hybrids

Peak No.	Peak identities	1	2	3	4	5	6
9	i-25:1[a]	---	0.43	Trace	0.01	0.13	0.24
10	a-25:1	0.25	0.89	0.55	0.30	0.39	0.53
11	a-25:1	0.79	0.54	5.68	1.01	0.20	0.56
12	i-25:1	---	0.04	---	---	---	0.09
13	i-26:1	0.13	---	---	---	---	---
14	a-26:1	---	0.10	0.78	0.35	---	---
15	i-26:1	---	0.18	---	---	Trace	---
16	a-26:1	---	0.11	---	---	---	---
17	i-27:1	0.14	3.15	0.15	0.35	2.29	1.56
18	a-27:1	20.38	4.71	3.19	1.87	4.22	3.20
19	a-27:1	10.87	3.13	19.74	7.45	3.40	3.61
20	i-27:1	---	0.55	---	---	0.46	0.47
a	br-olefin	---	0.16	---	---	0.59	0.61
21	i-28:1	0.41	1.22	Trace	0.30	0.89	0.60
22	a-28:1	3.20	1.17	3.90	2.19	1.03	1.55
23	i-28:1	---	6.12	Trace	Trace	8.85	4.98
24	a-28:1	---	2.68	Trace	Trace	2.66	1.98
25	i-29:1	1.79	23.60	0.57	1.17	18.64	11.81
26	a-29:1	16.48	31.95	10.75	18.62	27.70	25.61
27	a-29:1	45.54	16.09	54.66	66.36	20.86	35.43
28	i-29:1	---	1.35	---	---	2.96	3.98
b	br-olefin	---	---	---	---	Trace	Trace
29	i-30:1	---	---	---	---	Trace	Trace
30	a-30:1	---	1.39	---	---	Trace	Trace
31	i-30:1	---	0.44	---	---	3.71	2.73
32	a-30:1	---	---	---	---	1.00	0.48

[a]Abbreviations as in Table II. See Table I for explanation of hybrid numbers.

by the relative proportions of the isomers in the C_{27} fraction. The UM, ISU and ATCC 15801 strains showed the first anteiso-olefin of the tetrad as the predominant component (Fig. 2, Table III), while the predominant component in strains PU and WRU was the second anteiso-olefin component of the C_{27} tetrad (Fig. 2, Table III). The relative proportions of the hydrocarbon components of *Micrococcus* strains PU and WRU more closely resembled those of the distribution patterns of the *Sarcina spp.* (Fig. 1, Table II).

The relative proportions of the hydrocarbon components of the transformed hybrids (Table IV) varied considerably. Pronounced differences existed among the components comprising the major C_{29} fraction. The hydrocarbon patterns of the transformed hybrids (Table IV) were neither identical to the donor (*S. lutea* ATCC 272, Table II) nor the recipient (*M. lysodeikticus* ISU, Fig. 2, Table III). Components a and b, undetected in both the ISU and recipients, were detected in hybrids 2, 5 and 6 (Table IV). By considering the overall relative proportions of the individual hydrocarbons, hybrids 1, 3, 4 and 6 had slight similarities to the donor (Table II), while hybrids 2 and 5 resembled neither the donor nor the recipient.

In addition to the variations in the relative proportions of the individual hydrocarbon components within a fraction, the predominant hydrocarbon tetrads varied among the organisms studied. In *S. lutea* ATCC 533 the predominant tetrads were the C_{25}, C_{26} and C_{27} hydrocarbons. The major tetrads in *S. lutea* FD 533 were C_{27}, C_{28} and C_{29}, while the major hydrocarbons of *S. lutea* ATCC 382 S.I., *S. flava* and *S. subflava* were C_{27} and C_{29}. In the remaining organisms studied, the fractions containing 29 carbons were more predominant. Most of the *Micrococcus* strains and the hybrids studied showed that the C_{29} fraction accounted for more than 50% of the hydrocarbons (Table IV). In this respect, all the hybrids resembled the recipient organism.

Hydrocarbon Composition of Gaffkya and Staphylococcus

The quantities of hydrocarbons found in the hexane fractions of *Gaffkya tetragena* and the *Staphylococcus* strains amounted only to 0.08-0.24% of the total lipids. The gas chromatographic analyses of the hydrocarbon samples showed a complex mixture of components in the range C_{12}-C_{30} with major peaks at 17, 18 and 19 carbons. The hydrocarbon

fractions obtained were not considered as having been produced by the bacteria for the following reasons: (a) The quantities detected were very small; (b) the gas chromatographic patterns were identical to the controls run on concentrated non-redistilled organic solvents indicating possible contamination; and (c) the hydrocarbon fraction obtained from staphylococcal cells treated with 1-^{14}C-acetate contained no measurable amount of incorporated ^{14}C atoms.

Gas Chromatography of Fatty Acid Methyl Esters

The fatty acid composition patterns for *Sarcina, Micrococcus* and hybrid strains studied were qualitatively similar. All the patterns showed fatty acids in the range C_{12}-C_{18}, consisting of iso and anteiso structures for odd carbon-numbered chains and iso and normal structures for even carbon-numbered chains. In all bacteria studied, iso- and anteiso-$C_{15:0}$ were the predominant fatty acids. A representative fatty acid composition has been previously presented in detail for *S. lutea* ATCC 533 (1,4).

The fatty acids of the three *S. aureus* strains studied had compositions similar to those previously reported (16,17), with iso- and anteiso-$C_{15:0}$ as the major components. Although the general composition of the fatty acids of *Staphylococcus* was similar to the *Sarcina spp.* and *M. lysodeikticus* strains, the fatty acid patterns of *Staphylococcus* differed significantly by the presence of considerable quantities of $C_{16:0}$, $C_{18:0}$ and $C_{20:0}$ acids.

The fatty acid pattern of *G. tetragena* was similar in general to that of the staphylococcal fatty acids with anteiso-$C_{15:0}$ (30.9%) as the major component and considerable quantities of $C_{16:0}$ (5.5%), $C_{18:0}$ (15.4%) and $C_{20:0}$ (8.2%). Other fatty acids were iso-$C_{15:0}$ (10.4%), iso-$C_{17:0}$ (6.6%) and anteiso-$C_{17:0}$ (12.5%). Minor components were iso-$C_{14:0}$ (0.9%), $C_{14:0}$ (1%), iso-$C_{16:0}$ (0.5%), $C_{18:1}$ (1%), iso-$C_{19:0}$ (3%) and anteiso-$C_{19:0}$ (3.7%).

DISCUSSION

It was shown by gas chromatographic analyses that all *Sarcina spp.*, *M. lysodeikticus* strains, and strains derived by transformation of *M. lysodeikticus* studied have qualitatively similar aliphatic hydrocarbon compositions. The hydrocarbons consisted of families of iso- and anteiso-branched monoolefins with even and odd carbon-numbered chains. The hydrocarbon pattern was common to both the ATCC 533 and FD 533 strains of *S. lutea*. However, the hydrocarbons of the ATCC 533 strain differed strikingly from those of the FD 533 strain with respect to carbon chain length: the major hydrocarbons in strain ATCC 533 were C_{25}, C_{26} and C_{27}, while those in FD 533 were C_{27}, C_{28} and C_{29} (Fig. 1, Table II). The *G. tetragena* and *Staphylococcus* strains were clearly distinguished from the *Sarcina spp.* and *Micrococcus* strains analyzed by the absence of hydrocarbons and their different fatty acid patterns.

The hydrocarbon patterns found for the *Sarcina* and *Micrococcus* strains and species studied here have not been reported for any other organisms (3,4,18-28). It may therefore be suggested that these hydrocarbon patterns are characteristic of the *Sarcina* and *Micrococcus* genera in the Micrococcaceae family.

Comparisons of the hydrocarbon patterns have shown variations in the relative proportions of the major fractions as well as the individual components within each fraction and in the range of the hydrocarbon distribution. The differences between the hydrocarbon patterns may have been influenced by the age of the cells. Some evidence for this was indicated for *S. lutea* FD 533 but not for *S. lutea* ATCC 533 (5). It was previously shown that variations in the nutrients resulted in changes in the relative proportions of aliphatic hydrocarbon components without affecting the overall hydrocarbon composition of the cells (5). Since all organisms studied in this paper were grown in the same medium, this suggests the possibility that some of the variations seen in the relative proportions of the hydrocarbons were a demonstration of the specific nutrient requirements of the different organism. The hydrocarbon patterns reported here were readily reproduced from 2 to 5 independent samples with virtually no variations in the patterns.

The *Sarcina* and *Micrococcus* strains and species that have related hydrocarbon patterns also have similar DNA base ratios (G-C) and can participate in genetic exchange reactions (8-10). *S. lutea* ATCC 533 and *S. aureus*, which failed to transform *M. lysodeikticus* (9), have different hydrocarbon compositions. [*S. lutea* FD 533 serving as a donor in transformation with *M. lysodeikticus* was inadvertently presented as *S. lutea* ATCC 533 in previous reports (8,9)]. Transformation studies involving *G. tetragena* have not been reported. The ability to transform *M. lysodeikticus* may serve as one of several criteria to define one species of *Micrococcus*; however, such a criterion is limited in its scope to define other *Micrococcus* species or subgroups. Many *Sarcina* and *Micrococcus spp.*, in addition to those described above, are genetically incompatible. Work is in progress to determine the hydrocarbon composition of

these organisms and those that appear to bridge the various genera in the family *Micrococcaceae*. Perhaps such a study will aid the taxonomists in their attempts to combine these organisms into their respective *Micrococcus* subgroups.

ACKNOWLEDGMENT

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Metabolic Fate of Gossypol: the Metabolism of ^{14}C-Gossypol in Rats

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ABSTRACT

Balance studies designed to obtain information concerning the metabolic fate of gossypol in rats were carried out utilizing two groups of animals. One was fed a basal diet and the other the same diet plus 500 ppm of iron as $FeSO_4$. Single doses of 5 mg each of ^{14}C-labeled gossypol (spec. act. 19.8 μCi/mmole) were administered. The animals were maintained in metabolic cages and killed after various periods of time. The data indicate that gossypol was poorly absorbed from the gastrointestinal tract and rapidly eliminated from the animal body. Although the main route for gossypol elimination from the animal was by fecal excretion in both treatments, the percentage of the total activity eliminated via the feces varied and depended on the level of iron supplied in the diet. The results demonstrate that gossypol was least excreted via urine and that urinary excretion of radioactivity was diminished by iron supplementation to the diet. Most of the radioactivity retained was found in the contents of different parts of the gastrointestinal tract. Tissues, the liver, muscle, kidney and blood had the highest radioactivity, with the liver having the highest specific activity. The data also demonstrate that addition of iron to the ration diminishes ^{14}C radioactivity in the animal body. This effect might be attributed to the formation of chelates that could not be absorbed through the small intestine. Catalysis of the decarbonylation of gossypol by iron also appears to be a factor.

INTRODUCTION

The use of cottonseed flour in feeding has been limited by the presence of gossypol [Chemical name, (2,2'-Binaphthalene)-8,8'-dicarboxaldehyde-1,1',6,6',7,7',-hexahydroxy-5,5'-diisopropyl-3,3'-dimethyl] which is toxic to nonruminant animals (1). Gossypol is reported to cause death to nonruminant animals by reducing the oxygen-carrying capacity of blood and causing hemolytic effect on erythrocytes (2). Iron supplementation, however (3), resulted in the hemoglobin, hematocrit and iron-binding capacity approaching those of the control group, but the levels of glutamic-oxaloacetic transaminase were significantly elevated, indicating some liver damage. The possibility that a chemical reaction between gossypol and protein may take place resulting in a reduction in the availability of lysine and hence a reduction in protein quality has been proposed (4). The condition of pigs fed various cottonseed meals were improved by addition of lysine (5). In a study to determine the effect of diet

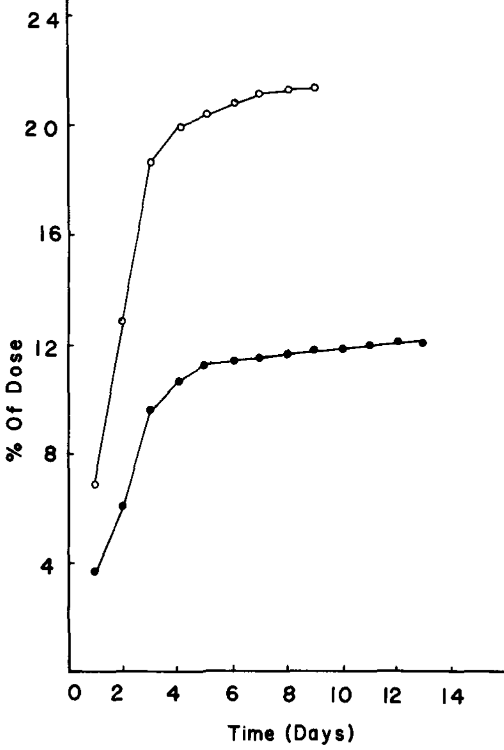

FIG. 1. Accumulated ^{14}C radioactivity in the expired air by rats fed basal (•) and ferrous sulfate supplemented diets (o) following the administration of a single oral dose of formyl labeled gossypol.

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on the accumulation of gossypol in the organs of growing pigs, highly significant reductions in the level of both free and bound gossypol were reported in certain organ tissues from the pigs receiving dietary iron (6). In dogs fed cottonseed meal, it was suggested that certain compounds of gossypol were hydrolyzed in the intestinal tract, therefore resulting in higher values of free gossypol in the feces. The highest concentration of gossypol fed to rainbow trout was found in the liver and lowest in the muscle tissue (8).

The present investigations were designed to determine the pattern of absorption, distribution and elimination of dietary gossypol in rats fed ^{14}C-formyl labeled gossypol. The effect of the iron supplemented diet on the fate of gossypol in rats was also investigated.

EXPERIMENTAL PROCEDURES

^{14}C-Formyl Labeled Gossypol. ^{14}C-formyl labeled gossypol (spec. act. 19.8 μc/mM) was prepared as described (9).

Care and Treatment of Animals. White male rats (about three months old and weighing about 200 g), selected for uniformity of weight, were placed in individual Cary animal cages, (Glass Instruments, Inc., Pasadena, Calif.). The Cary animal cage, which is designed primarily for expired $^{14}CO_2$ studies, contained course and fine mesh screens to separate fecal material from the urine which was collected in a separate flask. The expired air was drawn out through this flask to eliminate the possibility of high specific activity air being trapped and slowly leaking back into the cages to distort expiration rate records. Water and solid food containers were provided. The cage inlet and outlet were protected to prevent the animal from interrupting the normal air flow into the container.

The animals were allowed to adjust to their environment for one week. A single 5 mg oral dose of radioactive gossypol was given in a gelatin capsule to ensure that the complete sample had entered the stomach. The animals were returned to their cages and given free access to food and water. The rats were killed after varying intervals of time, i.e., 1, 2, 4, 9 or 13 days. For each time period two groups of three animals were used. One group was given a basal diet and the other the same diet plus 500 ppm of iron as $FeSO_4$. At the end of the experiment, the animals were anesthetized with chloroform and decapitated. The blood was collected and the individual organs were removed and weighed.

Determination of ^{14}C Radioactivity. ^{14}C radioactivity was determined by the use of a Beckman model LS 250 liquid scintillation spectrometer after preparation of the samples

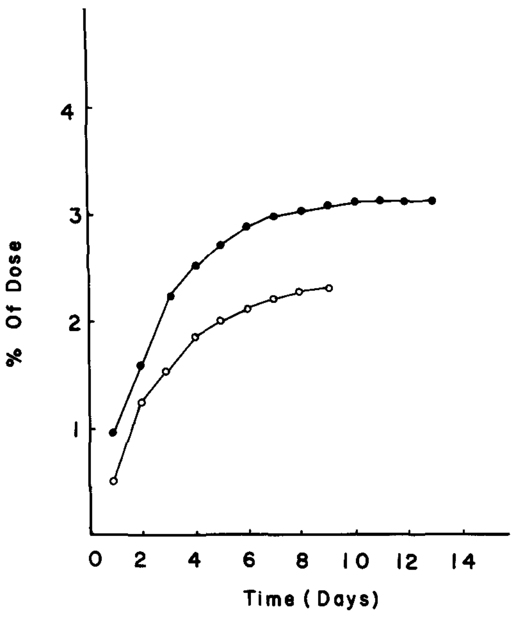

FIG. 2. Accumulated total urinary excretion of ^{14}C radioactivity by rats fed basal (•) and ferrous sulfate supplemented diets (○) following the administration of a single oral dose of formyl labeled gossypol.

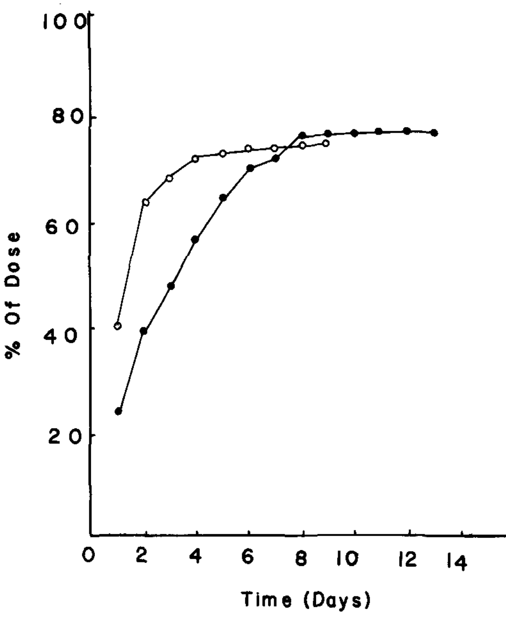

FIG. 3. Accumulated total fecal excretion of ^{14}C radioactivity by rats fed basal (•) and ferrous sulfate supplemented diets (○) following the administration of a single oral dose of formyl labeled gossypol.

TABLE I

Percentage Recovery of Radioactivity From Rats[a] Fed Basal[b] and Ferrous Sulfate[c] Supplemented Diets and Given a Single 5 mg Oral Dose (0.18 μCi) of ^{14}C-Labeled Gossypol

Days	Diet	Expired air	Urine	Feces	Tissues	Gastrointestinal tract contents	Total
1	Basal	3.7 ±0.57	1.0 ±0.11	24.0 ±2.01	12.5 ±1.41	57.5 ±5.71	98,7 ±9.81
	$FeSO_4$	6.8 ±0.85	0.50 ±0.01	40.2 ±3.62	6.2 ±0.81	43.7 ±4.32	97.5 ±9.61
2	Basal	6.0 ±0.87	1.6 ±0.10	39.1 ±3.23	9.7 ±1.12	41.0 ±4.40	97.4 ±9.72
	$FeSO_4$	12.7 ±1.30	1.3 ±0.12	63.1 ±5.15	5.3 ±0.81	17.0 ±1.30	99.4 ±8.68
4	Basal	10.6 ±1.12	2.5 ±0.15	56.6 ±5.10	6.3 ±0.79	19.6 ±1.41	95.6 ±8.67
	$FeSO_4$	19.7 ±1.51	1.9 ±0.13	71.8 ±6.31	1.1 ±0.01	4.0 ±0.62	98.5 ±8.58
9	Basal	11.8 ±1.26	3.1 ±0.41	76.5 ±6.79	0.63 ±0.01	4.0 ±0.65	96.0 ±9.12
	$FeSO_4$	20.9 ±1.80	2.30 ±0.39	74.4 ±6.92	0.06 ±0.00	0.85 ±0.11	98.5 ±9.22
13	Basal	12.1 ±1.20	3.1 ±0.51	77.4 ±7.21	0.28 ±0.02	0.66 ±0.07	93.5 ±9.01

[a]Each value is an average of six determinations from three animals ± standard error.

[b]Experiment period was 13 days.

[c]Experiment period was nine days.

as indicated below. The scintillation medium was toluene-ethylene glycol monomethyl ether (2:1 v/v) containing 5 g of 2,5-diphenyl oxazole (PPO) per liter. Quench corrections were made from a quenching curve prepared utilizing standard ^{14}C-benzoic acid (New England Nuclear).

^{14}C-Carbon dioxide was trapped in a solution of ethanolamine-ethylene glycol monomethyl ether (1:2 v/v) (purified reagents, Fisher Scientific Company, Houston, Tex.). The ^{14}C radioactivity was measured directly in urine by adding a 1 ml aliquot or urine and 3-4 ml of ethylene glycol monomethyl ether to 10 ml of the scintillation solution.

Both tissue and feces samples were prepared for liquid scintillation counting by flask oxygen combustion as described by Buyske et al. (10) and by Davidson and Oliverio (11). The feces were freeze-dried, weighed ground, and 50 to 100 mg samples placed in cellophane combustion envelopes (Ivers Lee Company, Newark, N.J.). Two-tenths ml of a 10% sucrose solution and enough water to wet the entire sample were added. The envelopes were then air dried. The contents of the gastrointestinal tract parts were separated and the different parts were washed with ethanol-ether (5:2 v/v). The wash of each part was added to its corresponding content, dried and analyzed as described for the feces. Fresh tissues (200-300 mg) were placed directly into combustion envelopes. Two-tenths ml of a 10% sucrose solution was added and the envelopes dried as above.

A bag containing the dried sample was wrapped in black paper and placed in a platinum basket carried by a glass rod on the glass stopper which was firmly positioned in a 2-liter heavy-walled Erlenmyer flask that had previously been purged with oxygen for 10 sec. The samples were ignited with a Thomas-Ogg IR igniter (A.H. Thomas Company, Philadelphia) and the evolved $^{14}CO_2$ was trapped in a solution of ethanolamine-ethylene glycol monomethyl ether (1:2 v/v) as described before (12).

Samples of tissue and excreta from animals fed ^{14}C-gossypol were extracted with ethanol: ethyl ether (5:2 v/v) according to the method of Smith (13). The radioactivity of the extract (E) and the residue (R) were determined as described above after the solvent was removed by evaporation. It should be noted that gossypol is extracted from tissues under these conditions but that certain conjugates of gossypol with other molecules such as protein are not (14).

Determination of Distribution Ratios. A

solution of 1 ml of the gossypol in chloroform (0.5 mg/ml) was placed in a 250 ml Erlenmeyer flask. The chloroform was evaporated before the addition of 25 ml ethyl acetate, followed by 25 ml 0.1 M-phosphate-citrate buffers with pH ranged from 2.2 to 8.9. A similar experiment was conducted with chloroform substituted for the ethyl acetate. The flasks were then shaken mechanically for 1 hr at room temperature. The organic solvents were separated from the aqueous solutions. The gossypol concentration in the organic solvent layer was measured directly by reading the optical density with a Beckman D U spectrophotometer at wave length 360 mμ utilizing a 1 cm cell. The aqueous layers were acidified, extracted with chloroform and the optical density was determined as above. The gossypol was determined from standard curves, which were prepared in both solvents. All determinations were done in duplicate.

TABLE II

Ratio (E/R) of Extractable to Nonextractable[a] Radioactivity in the Feces of Rats[b] Fed a Single 5 mg Oral Dose (0.18 μCi) of ^{14}C-Labeled Gossypol

Days	Basal diet	$FeSO_4$ diet
1	0.91 ± 0.12	0.72 ± 0.09
2	1.52 ± 0.10	1.08 ± 0.10
3	2.09 ± 0.25	1.86 ± 0.17
4	2.19 ± 0.23	1.95 ± 0.19
5	2.32 ± 0.24	2.01 ± 0.18
6	2.47 ± 0.25	2.13 ± 0.20
7	2.59 ± 0.27	2.14 ± 0.23
8	2.69 ± 0.29	2.31 ± 0.21
9	2.74 ± 0.31	2.37 ± 0.22
10	2.85 ± 0.28	
11	2.91 ± 0.26	
12	3.01 ± 0.30	
13	3.15 ± 0.32	

[a]Solvent system is ethanol:ethyl ether (5:2 v/v).

[b]Each value is an average of six determinations from three animals ± standard error.

RESULTS

^{14}C Radioactivity in Expired Air. Figure 1 shows the total ^{14}C radioactivity in the expired air from rats fed the basal diet and the same diet supplemented with iron salts. During the period of the experiments there was a steady increase in the total ^{14}C radioactivity in the expired air. The addition of iron to the ration resulted in a marked increase in the total ^{14}C radioactivity in the expired air. Iron also enhanced the elimination of gossypol from the animal body, so that no radioactivity was detected in the expired air from rats fed an iron supplemented diet nine days after administering a 5 mg dose of labeled gossypol. The total $^{14}CO_2$ recovered in the expired air after nine days of administration was 11.8% and 20.9% of the administered dose to rats fed basal and iron supplemented diets, respectively (Table I).

Excretion of ^{14}C Radioactivity in the Urine. The total excretion of ^{14}C radioactivity in the urine by the rats is presented in Figure 2. It shows a rapid increase in accumulated total ^{14}C in the urine of rats fed a single 5 mg dose. It also shows that the addition of iron salts to the ration decreased the urinary excretion of ^{14}C.

Table I shows that nine days after a 5 mg oral dose of gossypol was administered, the urinary excretion of ^{14}C radioactivity by the rats fed the basal and iron supplemented diets was 3.1% and 2.3%, respectively.

Fecal Excretion of ^{14}C Radioactivity. The data on fecal excretion of ^{14}C radioactivity are presented in Figure 3 and in Tables I and II. Figure 3 shows that when 5 mg of gossypol was given, a sharp increase in the total fecal excretion of ^{14}C radioactivity through the fourth day and a slow increase thereafter occurred. It also shows that iron supplementation increased the rate of excretion of ^{14}C radioactivity. At the end of the ninth day, the total fecal excretion of ^{14}C radioactivity by rats fed a basal diet and $FeSO_4$ supplemented diet was 76.5% and 74.4% of the administered dose, respectively.

Table II lists the ratio of extractable radioactivity (E) to nonextractable radioactivity (R) in the feces of rats fed a single oral dose of ^{14}C labeled gossypol. The amount of R in the feces was increased when supplemented with iron salts. Also in animals fed the basal or the iron supplemented diets, the ratio of E to R increased with time.

Histological and General Observations. At the end of each experiment the animals were killed and the tissues excised. When a single 5 mg oral dose of gossypol was given, there were no histological changes in the animal tissues. The rats fed the basal diet lost some weight (10%) at first and then maintained weights comparable to that of the control animals thereafter. However, rats fed the iron supplemented diet did not lose weight during the experiments.

Tissue Deposits of ^{14}C Radioactivity. Table III shows the specific activity and the ratio of E to R for various tissues and gastrointestinal tract contents of rats fed basal diet and given a single 5 mg oral dose of ^{14}C-labeled gossypol. One day after the administration, all tissues contained radioactivity with the brain having the lowest activity. The liver had the highest

TABLE III

Specific Activity[a] and Ratio (E/R) of Extractable to Nonextractable[b] Radioactivity of Various Tissues and Gastrointestinal Contents of Rats[c] Fed Basal Diet and Given a Single 5 mg Oral Dose (0.18 μCi) of ^{14}C-Labeled Gossypol

Sample	1 Day		2 Days		4 Days		9 Days		13 Days	
	Spec. Act	E/R	Spec. Act.	E/R	Spec. Act.	E/R	Spec. Act.	E/R	Spec. Act.	E/R
Tissues										
Brain	53	0.33	51	0.49	0	---	0	---	0	---
Heart	1,018	0.45	353	0.72	248	0.99	31	1.13	0	---
Blood	171	0.13	148	0.59	58	0.78	8	0.92	2	0.99
Lungs	208	1.01	161	1.34	109	1.93	26	2.09	0	---
Spleen	341	1.21	321	2.60	165	3.39	19	3.59	0	---
Kidney	855	0.87	227	1.10	184	1.89	42	3.09	27	3.15
Testes	75	0.54	99	0.83	41	1.12	0	---	0	---
Liver	1,425	0.16	747	0.28	550	0.47	92	0.68	70	1.73
Muscle	167	1.05	184	1.49	102	1.47	7	1.69	1	1.85
Adipose tissues	113	0.11	124	1.06	53	1.27	4	1.42	0	---
Stomach	3,384	0.12	2,563	0.19	1,741	0.35	112	0.51	33	0.69
Small intestine	458	0.15	329	0.18	335	0.29	25	0.47	14	0.61
Caecum	1,306	0.17	1,379	0.21	980	0.39	86	0.52	48	0.59
Colon	1,141	0.18	951	0.20	670	0.37	73	0.57	38	0.60
Gastrointestinal tract contents										
Stomach	4,556	0.15	373	2.70	186	4.21	0	---	0	---
Small intestine	130,245	0.10	95,451	0.15	49,813	0.52	11,614	0.73	1,616	0.85
Caecum	208,458	0.06	180,468	0.09	76,950	0.29	15,923	0.51	1,218	0.67
Colon	42,180	0.22	12,040	0.20	6,818	0.50	760	0.70	240	0.73

[a]DPM/g fresh tissue, or dpm/g dry gastrointestinal tract contents.

[b]Solvent system is ethanol:ethyl ether (5:2 v/v).

[c]Each value is an average of six determinations from three animals.

TABLE IV

Specific Activity[a] and Ratio (E/R) of Extractable to Nonextractable[b] Radioactivity of Various Tissues and Gastrointestinal Contents of Rats[c] Fed Ferrous Sulfate Supplemented Diet and Given a Single 5 mg Oral Dose (0.18 μCi) of ^{14}C-Labeled Gossypol

Sample	1 Day		2 Days		4 Days		9 Days	
	Spec. Act.	E/R	Spec. Act.	E/R	Spec. Act.	E/R	Spec. Act.	E/R
Tissue								
Brain	29	0.27	14	0.38	0	---	0	---
Heart	424	0.31	161	0.47	97	0.85	0	---
Blood	151	0.07	98	0.25	31	0.49	00	---
Lungs	141	0.87	1.09	1.15	45	1.57	0	---
Spleen	183	0.95	104	1.80	22	2.85	0	---
Kidney	173	0.65	155	0.93	44	2.79	11	3.01
Testes	60	0.26	46	0.51	8	0.97	0	---
Liver	797	0.11	438	0.19	202	0.31	16	0.57
Muscle	83	1.10	92	1.37	17	1.37	0	---
Adipose tissues	103	0.03	92	0.81	14	1.15	0	---
Stomach	1,164	0.09	925	0.11	410	0.27	30	0.41
Small intestine	295	0.12	210	0.16	124	0.27	12	0.42
Caecum	943	0.13	720	0.17	334	0.29	39	0.47
Colon	701	0.15	530	0.18	101	0.31	0	---
Gastrointestinal tract contents								
Stomach	1,505	0.36	416	1.96	64	3.21	0	---
Small intestine	106,160	0.04	40,765	0.12	10,999	0.39	2,530	0.57
Caecum	155,303	0.04	67,538	0.07	13,078	0.19	3,078	0.38
Colon	32,078	0.10	8,186	0.09	2,114	0.42	0	---

[a]DPM/g fresh tissue of dpm/g dry gastrointestinal contents.
[b]Solvent system is ethanol: ethyl ether (5:2 v/v).
[c]Each value is an average of six determinations from three animals.

total activity (2.4%) followed by the muscle (2.3%). However the highest specific activity was in the liver, followed by the heart. In addition to the liver, the kidney and the spleen, which are involved in the elimination of toxic materials from the body, contained high specific activities. Adipose tissues contained relatively smaller amounts of ^{14}C radioactivity. The different tissues of the alimentary canal had lower specific activities than the liver, with the exception of that of the stomach, which was higher than the liver. The total radioactivity in all tissues was 8.1% of the administered dose, one day after the administration. The same general pattern was noted in the animals that were killed at 2, 4, 9 and 13 days after the administration. However, the activity in the tissues successively decreased in those animals. Four days after the beginning of the experiment no activity was detected in the brain. After 13 days, the heart, lungs, spleen, testes and adipose tissues also contained no radioactivity. At the end of the experiment the different tissues of the alimentary canal had radioactivity but it was less than that found in the liver. The total radioactivity recorded from all tissues after 13 days was 0.2% of the administered dose. The contents of different parts of the alimentary canal contained a much larger amount of radioactivity than the tissues at all times. After one day the gastrointestinal tract contents had 57.5% of the total dose. The contents had very high specific activities after one day, with the caecum having the highest, followed by the small intestine, colon and the stomach. The radioactivity decreased throughout the experimental period and reached 0.66% of the administered dose at the 13 day, and no radioactivity was detected in the stomach contents.

Table IV shows the specific activity of various tissues and alimentary canal contents of rats fed diets supplemented with $FeSO_4$ and given 5 mg of gossypol. The apparent effect of iron supplementation was a rapid increase in the rate of elimination of gossypol from the animal body. After 9 days, 97.5% of the dose was eliminated from the animal body. Iron also decreased the radioactivity deposited in the individual tissues and gastrointestinal tract contents. However, the distribution pattern of the deposited radioactivity was almost the same as

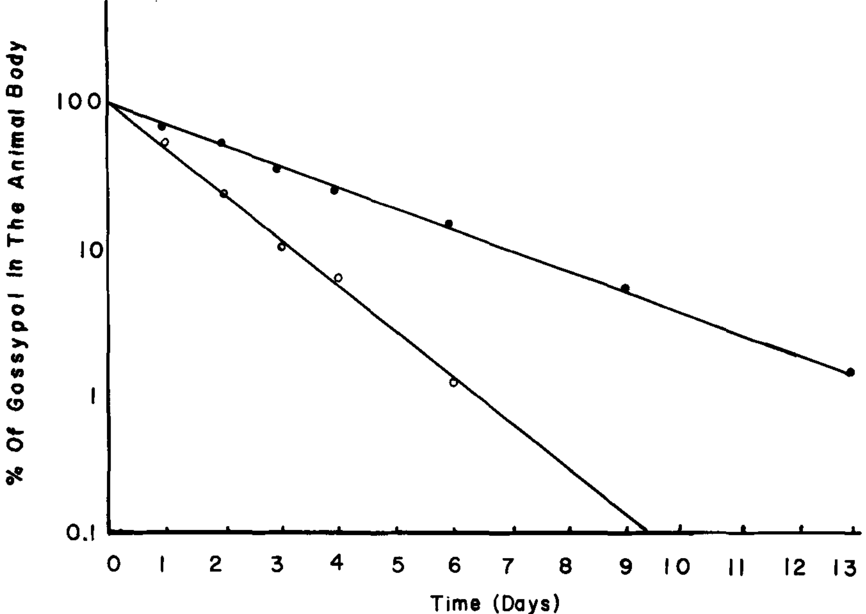

FIG. 4. Change in level of radioactivity in rat tissues following the administration of a single oral dose of formyl labeled gossypol. • Basal diet, $t1/2$ = 48 hr. ○ $FeSO_4$ supplemented diet, $t1/2$ = 23 hr.

in the rats fed the basal diet.

Tables III and IV list the ratios of E to R recovered from different tissues and tissue contents. This ratio was generally less than 1 and higher in the tissues than in the contents of the alimentary canal after one day. However, the value of the ratio progressively increased with time. After nine days the E to R ratio was much higher in the contents of the alimentary canal than in the tissues.

By plotting the percentage of ^{14}C radioactivity left in the animal body as a function of time (Fig. 4) on semi-log paper, it was possible to calculate the biological half-life ($t1/2$) of gossypol in the rats. When a single 5 mg dose of gossypol was administered, the $t1/2$ was 48 and 23 hr in rats fed basal and iron supplemented diets, respectively.

In this investigation, the distribution ratios of gossypol between organic solvents and different citrate-phosphate buffers have been determined. The results showed that increasing the pH decreases the solubility of gossypol in lipophilic solvents. In chloroform gossypol solubility was decreased from 100% at pH 2.2 to 3.76% at pH 8.9. Similar results were obtained with the more polar ethyl acetate, which showed that 49% of gossypol was soluble in ethyl acetate at pH 2.2 compared to 5.25% at pH 8.9. These results are evidence of an ionization of the gossypol to yield a base and a proton.

DISCUSSION

Decarbonylation of Gossypol. The results indicate that the decarbonylation of gossypol is an important pathway for its detoxification and elimination from the animal body. The observation that ^{14}C radioactivity of the expired air had increased sharply in rats fed iron supplemented diet suggests that iron catalyzes the decarbonylation reaction of gossypol. The decarbonylation might take place by an autooxidation process catalyzed by iron probably through a free-radical chain mechanism similar to that proposed for benzaldehyde decarbonylation (15). Such a chain reaction is accelerated by iron (capable of existing in a lower valence state), and proceeds through the formation of carboxylic acid prior to decarboxylation (16). The major part of the decarbonylation may take place in the digestive tract of the animal since $^{14}CO_2$ appeared in the expired air 1 hr after the administration of labeled gossypol. This assumption is supported by the finding that iron supplementation caused a general decrease in the absorption of gossypol by tissues. After gossypol is decarbonylated in the digestive tract, the $^{14}CO_2$ would be absorbed and excreted by respiration. The catalysis of decarbonylation of gossypol by iron may account for the reported reduced recoveries of total gossypol from an iron supplemented diet and the feces from the animals

fed this diet (6).

Excretion Via Urine. The results of the present investigation indicate that only a small portion of ingested gossypol is excreted via the urine. The observation that $FeSO_4$ in the diet reduced the ^{14}C activity in the urine is in agreement with the assumption that the added iron forms an insoluble complex with gossypol thereby decreasing its absorption and consequently its toxicity, as has been reported by several investigators (17,18). This hypothesis is supported by the results of a study to determine if factors that modify the toxicity of dietary gossypol are active when gossypol is injected (19). In that study, while dietary protein level did not alter the toxicity of injected gossypol, iron-dextran injected into the peritoneal cavity simultaneously with corn oil containing gossypol was partially effective in preventing growth depression and death losses.

Ferric and ferrous salts are reported to detoxify gossypol. Ferric ammonium citrate-treated cottonseed meal inactivated the gossypol and rendered the meal nontoxic to rabbits (20). Also in rats, the supplementation of cottonseed meal with $FeSO_4$ reduced the free gossypol and overcame the effect of gossypol (21). The complex formation of Fe^{3+} and Fe^{2+} with gossypol have been investigated in vitro. These studies showed that 1 mole of Fe^{3+} or Fe^{2+} ions combines with 1 mole of gossypol and that the perihydroxyls are the probable sites for binding of the metal ions with gossypol. The logarithm of the formation constant (pK) of Fe^{3+}-gossypol complex was calculated to be 6.75 (22) which indicates that this complex is slightly less stable than the Fe^{2+} complex since the reported pK value for Fe^{2+}-gossypol complex is 7.6 (23).

Reabsorption of gossypol from the renal tubules by nonionic absorption may account for the low ^{14}C radioactivity excreted in the urine. Mudge (24) has reported that the mechanism of nonionic diffusion in the renal tubules has a profound effect on the excretory rate of any drug that is reasonable lipid-soluble and has an appropriate pKa. Gossypol is a weak acid and may be filtered and secreted by the tubular mechanism for organic acids (25). Such compounds are reabsorbed from the tubules by nonionic diffusion. The tubular epithelium of the distal convoluted tubule is selectively permeable or more permeable to the un-ionized lipid-soluble molecule than the poorly lipid-soluble corresponding anion or cation (26). In the case of gossypol, which has a pKa value of 7 (27), reabsorption is favored by the low urinary pH (around 6 for rats) which increases the proportion of un-ionized molecules. Also the high solubility of gossypol in lipid solvents (and by inference the lipid-like cell membrane) results in a high rate of absorption.

TABLE V

Distribution Ratios of Gossypol Between Organic Solvents and Citrate-Phosphate Buffers[a]

pH	C_{CHCl_3}/C_{buffer}[b]	C_{EtAC}/C_{buffer}
2.2	∞	49.00
3.0	∞	17.52
4.0	∞	15.67
5.0	99.00	10.11
6.0	82.33	9.00
6.5	70.42	9.00
7.0	54.55	9.00
7.5	32.33	7.33
8.0	24.00	7.06
8.9	3.76	5.25

[a]0.1 M-phosphate-citrate buffers were used with chloroform or ethyl acetate (EtAC).

[b]C, concentration in the solvent indicated by the subscript.

Excretion via Feces. A study of the elimination of the radioactive material from rats fed ^{14}C labeled gossypol via expired air, urine and feces showed that most of the radioactivity appeared in the feces. The high observed rate of fecal excretion of gossypol is in harmony with the tentative conclusion (28) that compounds of high molecular weight (more than 300) and containing two or more aromatic rings tend to be excreted into the bile. It is also in agreement with the suggestion (29) that for appreciable biliary excretion in the rat, a compound should have polar anionic groups and a molecular weight more than 350 or should be able to be converted metabolically (usually by conjugation) into such a compound. Gossypol possesses these properties with 6 polar hydroxyl groups and 2 less polar carbonyl groups and a molecular weight of 518. It is suggested that the increased ratio of fecal excretion overy urinary excretion when iron is added may be partially due to the relatively nonpolar nature of the chelates. The more lipid-soluble gossypol-iron complex would have a higher rate of penetration into the liver than the free gossypol. This assumption is in harmony with the reported (30) results on the relative excretion rates of a series of chelated iron complexes. These chelates may be decomposed later in the gastrointestinal tract resulting in an increase in the ratio of free to bound gossypol in the feces. Such an interpretation would be in agreement with reports (7) that fecal excretion from dogs fed cottonseed pigments had free gossypol about 3.5 times greater than the intake. However, investigations in this

laboratory (31) indicated that gossypol in the digestive tract is largely converted to gossypol peptides. The solubility of these compounds is such that they would be classed as free gossypol (in the sense that the carbonyl groups are bound to protein fragments such compounds should be classed as bound gossypol).

Gossypol in the Tissues. The accumulation of dietary gossypol in the tissues of the rats was similar to that reported for swine (6,32), for rainbow trout (8) and for hens (12). The organs concerned with elimination of gossypol and its metabolites, liver and kidney as well as spleen, showed high concentrations. Stomach tissue had the highest concentration of radioactivity. This indicates that gossypol is readily absorbed by the epithelial lining of the stomach. This is a reasonable conclusion since gossypol is a weak acid (pKa 7.2) and would be completely in the unionized state in the gastric juice (pH 2-4), (33), thus easily absorbed by the epithelial lining of the stomach which is permeable to the lipid-soluble un-ionized form of drugs (34). This is illustrated further by our observations which show that gossypol was completely soluble in chloroform and ethylacetate at pH 2.2-4. The pattern of gossypol absorption in the rat through the epithelium or the small intestine and the colon seems to be very similar to that of the stomach.

The persistence of ^{14}C-gossypol in stomach contents might be attributed to its insolubility, its dilution by the subsequent ad lib. diet or by its binding to stomach wall or both.

Blood had a relatively high concentration of radioactivity. Due to its lipophilic character, gossypol may penetrate the erythrocyte membrane by diffusion (25). The high level of bound gossypol in the blood is in harmony with data of Lyman et al., (14) who reported that gossypol readily binds with plasma albumin and cottonseed protein. The brain had a significant but low specific activity one and two days after the administration. Apparently the blood brain barrier partially protects the brain against penetration by gossypol. In all tissues the ratios of E to R progressively increased with time throughout the experiment. This was especially noted in case of the stomach contents, in which this ratio E/R increased from 0.36% to 4.10% in animals given a 5 mg dose and fed the basal diet. These results suggest that protein bound gossypol might have been hydrolyzed in the stomach, resulting in soluble gossypol peptides which analyze as free gossypol (31).

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SHORT COMMUNICATION

Fish Cutaneous Mucus: A New Source of Skin Surface Lipid

ABSTRACT

Mucus from several species of marine fishes contained up to 20 times more lipid per unit area than human sebum. The analyses revealed free fatty acids which may provide protection against bacterial and fungal attack. Carotenoids were prominent components. The amount of phospholipids present in fish mucus seems to determine its viscosity.

The coating of mucus that makes fish so slippery is produced by epidermal mucous cells and is believed to serve primarily for protection. Two lines of evidence (1) support this view: (a) removal of mucus renders fish susceptible to attack by bacteria, fungi and parasites, and (b) the mucus coating tends to be thicker on naked or sparsely scaled fishes than on those that are heavily scaled, although there are exceptions among the more active fishes, such as the tunas. The occurrence of lipids in fish mucus has been largely overlooked, although lipids are found in mucus from other sources, such as that of the human cervix (2) and submaxillary gland (3), canine gastric juice (4), and snail epithelium (5). In 1914, Muller and Reinbach (6) reported that mucus from an eel contained 3% of a lipid mixture in which cholesterol and several phosphatides were identified. No further reports on the lipids of fish mucus appear to have been published since that time. This study confirms the occurrence of lipids in the mucus of several species of fish and reports their composition and their relative abundance.

Initially, the mucus lipids of individual specimens of 10 species of marine fish were analyzed by thin layer chromatography (TLC) (7). Since the compositions of their neutral lipids were quite similar, larger quantities of mucus were then collected for more detailed analyses from three of these species of fish selected to represent a range of scale types: the mullet, *Mugil cephalus*, is well scaled, the dusky flathead, *Planiprora fusca*, is moderately scaled, and the marine catfish, *Plotus anguillaris*, is scaleless. Mucus from the first two species was pooled from 15 and 7 specimens, respectively, obtained from a commercial market. The catfish mucus came from a single specimen, 33 cm long, which was isolated immediately after capture by hook and line.

Mucus was collected by wiping the fish with paper tissues which had been defatted in two rinses of chloroform. Lipids were extracted from the tissues by repeated elution with chloroform-methanol (2:1 v/v). Treatment of the extract and analyses followed standard techniques of chromatography on Florisil (8,9) monitored by TLC. The free fatty acids were determined by adsorption onto an ion exchange resin (10). Lecithin and phosphatidyl ethanolamine appeared to be the major components of the phospholipids. Squalene was determined by GLC as previously described (7). Small amounts of paraffinic hydrocarbons were found in all of the lipids, but a blank analysis indicated that they were contaminants. The other neutral lipids were identified by TLC before and after saponification. Carotenoids were found in all of the mucus lipids but were most prominent in the lipids of the flathead and catfish, coloring the extracts a brilliant yellow. TLC analyses of these lipids in hexane-acetone (70:30 v/v) revealed the same four carotenoids having R_f values of 0.98, 0.82, 0.64 and 0.44, the last being present in the greatest amount. In the TLC analyses of neutral lipids, a carotenoid migrated with the hydrocarbon fraction and was tentatively identified as β-carotene.

The amounts of mucus lipids per unit of surface area were calculated for the three species. Their surface areas were obtained by the weight of paper cut-outs relative to that of a known area. (Averages of lipid yield and area were used for the pooled samples.) The values corresponded to the amount of mucus on the skin and ranged from 0.67 mg/cm^2 for the mullet, which is well scaled and not slimy, to 8.5 mg/cm^2 for the catfish, which is scaleless and has large quantities of mucus. The flathead is moderately scaled and slimy and gave an intermediate value of 1.6 mg/cm^2. The mullet and flathead values are undoubtedly lower than

TABLE I

The Composition of Fish Mucus Lipids in Wt % as Determined by Adsorption of Free Fatty Acids on an Ion Exchange Resin and Column Chromatography[a]

Composition	Mullet	Flathead	Catfish	Human skin lipids, back[b]
Hydrocarbons	0.9	1.0	0.6	1.4
Squalene	0.03	0.2	0.07	11.5
Sterol and wax esters	3.9	7.9	1.2	23.8
Triglycerides	7.7	4.9	5.3	31.1
Cholesterol	13.0	16.3	13.1	4.2
Diglycerides	7.1	2.1	2.8	10.0
Monoglycerides	8.5	2.2	2.8	3.7
Free fatty acids	10.5	23.1	9.8	14.1
Phospholipids	36.1	48.8	62.4	---
Total	87.73	106.5	98.07	

[a]Human skin surface lipids are listed for comparison.
[b]Averages from four subjects (13).

they would have been had these fish not undergone handling and icing which probably removed some mucus.

The quantities of lipids present in fish mucus are surprisingly high when compared with the values for human sebum, the only other skin surface lipid for which data on quantities per unit area are available. These show that the largest amount of human skin surface lipids normally found, at saturation levels on the forehead (11), is 0.4 mg/cm^2 or only 5% of the largest amount found in fish mucus.

Since mucus lipids comprise a new type of skin surface lipid, it is of interest to compare their composition with that of mammalian sebum. Of the many sources of sebum, mucus lipids show the greatest similarity to human skin surface lipids (Table I) in having the same neutral lipids, although there are some marked differences in concentration. This spectrum of components is unique among naturally-occurring lipids because of the relatively large amounts of free fatty acids, and the presence of squalene, wax esters, diglycerides and monoglycerides. In human skin surface lipids, free fatty acids occur at levels of 10-20% of the total lipids and are known to provide protection against bacterial and fungal attack (11). Free fatty acids occur at similar levels (10-23%) in fish mucus lipids and may reach the same effective concentrations in mucus, since the larger amounts per unit area are offset by their low concentrations (ca. 3%) within the mucus. This strongly suggests that the free fatty acids perform similar protective functions in fish mucus and may partially explain why the removal of mucus from fish frequently results in bacterial or fungal infections. The free fatty acids, diglycerides and monoglycerides in mucus probably result from the same enzymatic lipolysis of triglycerides at the skin surface that occurs in sebum (12).

However, mucus lipids are far more polar than human skin surface lipids, having large amounts of phospholipids (not established as occurring in sebum) and relatively small amounts of squalene, sterol esters, wax esters and triglycerides, all of which comprise the bulk of sebum (12). This shift towards greater polarity in mucus lipids appears to be an adaptation to aquatic conditions in which the secretion of lipid occurs in an glycoprotein-lipid complex which is dispersable in water. The highly polar phospholipids could serve to link the neutral lipids to the glycoproteins. They could also have an important influence on the internal structure of the mucus. It may be noted that the phospholipid values for fish having scanty, moderate and copious amounts of mucus were 36%, 49% and 62%, respectively, of the total lipids. Although the data are limited, it appears that interaction between the phospholipids and glycoproteins may determine the relative viscosity of mucus and hence its accumulation on the surface of the skin or dispersal into water.

This view is supported by the results of Breckenridge and Pommerenke (2) who found that the phospholipid and cholesterol content of human cervical mucus varied throughout the menstrual cycle but maintained a constant ratio. During the pre- and post-ovulatory phases these components reached levels that were approximately three times greater than during the ovulatory phase. It is known that cervical mucus undergoes cyclic changes in viscosity,

being viscous during the pre- and post-ovulatory phases but fluid at the time of ovulation. Since the cholesterol content of the fish mucus samples of this study were nearly the same, despite differences in viscosity, the phospholipid content is evidently a major factor in determining the viscosity of mucus.

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LETTER TO THE EDITOR

Confusion Between C_{18} and C_{20} Fatty Acids in a Gas Chromatographic Analysis of Seed Lipids of Water Plants

Sir: It has been known for some time that an all *cis* octadecatetraenoic acid (18:4ω3) is a characteristic of marine and fresh-water algae (Klenk and Eberhagen, Z. Physiol. Chemie 328:189, 1962; Klenk et al., Ibid. 334:44, 1962; Wagner and Pohl, Biochem. Z. 341:476, 1965; Ackman et al., J. Fish. Res. Bd. Canada 25:1603, 1968; Cheucas and Riley, J. Mar. Biol. Ass. U.K. 49:97, 1969). A recent analysis of the fresh-water plant (*Myosotis scorpioides*, the water forget-me-not) identified both 18:4ω3 and 18:3ω6 (γ-linolenic) acids in moderate proportions (respectively about 5-12% and 12-16%, depending on season) (Jamieson and Reid, J. Sci. Food Agric. 19:628, 1968). Attention has been drawn to 18:4ω3 as possibly being particularly associated with water plants (Ackman, presented at the 60th AOCS Meeting, San Francisco, 1969).

The absence of 18:4ω3 from a recent publication (Lotti and Averna, La Riv. Ital. Sost. Grasse 46:668, 1969) listing the fatty acids of seed lipids of water plants seemed rather curious in view of its association with plants from aqueous habitats or environments. The publication of a gas liquid chromatogram (GLC) of the methyl esters of fatty acids from the seed of *Symphytum officinale* permitted checking of the peak identifications.

Distances (D) were measured in millimeters from the front of the solvent peak to the intercept of the frontal tangent and baseline. A plot (Ackman et al., Can. J. Biochem. Physiol. 41:1627, 1963) was drawn on semi-logarithmic paper to establish relationships among the saturated acids and among the monounsatu-

TABLE I

Comparison of Experimental ECL Values for Methyl Esters of Fatty Acids From *Symphytum officinale* and Literature Values From Analyses on Polyester GLC Columns of Comparable Polarity

Experimental			Some Literature Polyester ECL Values				
Peak marking[a]	D, mm	ECL	Fatty acid	EGSS-X[b]	CDXA[b]	EGS[c]	DEGS[d]
C_{14}	9.8	14.00	---	---	---	---	---
C_{15}	14.2	15.35	---	---	---	---	---
C_{16}	17	16.00	---	---	---	---	---
$C_{\overline{16}}$	21	16.72	16:1ω7	---	---	16.68	16.55
C_{18}	30	18.00	---	---	---	---	---
$C_{\overline{18}}$	35	18.55	18:1ω9	18.62	18.55	18.55	18.51
$C_{\overline{\overline{18}}}$	44	19.35	18:2ω6	19.45	19.23	19.23	19.30
C_{20}	52	19.93	18:3ω6	20.15	19.70	19.80	20.00
$C_{\overline{20}}$	59	20.34	18:3ω3	20.50	20.10	20.21	20.40
$C_{\overline{20}}$	61	20.50	20:1	---	---	20.50	20.44
$C_{\overline{\overline{\overline{18}}}}$	69	20.97	18:4ω3	21.13	20.73	20.85	21.00
---	---	---	20:2ω6	---	---	21.13	21.36

[a]Lotti, G., and V. Averna, La Riv. Ital. Sost. Grasse 46:668-672 (1969).
[b]Jamieson, G.R., in "Topics in Lipid Chemistry," Vol. 1, Edited by F.D. Gunstone, 1970, p. 107.
[c]Ackman, R.G., R.P. Burgher and P.M. Jangaard, Can. J. Biochem. Physiol. 41:1627-1641 (1963).
[d]Hofstetter, H.H., N. Sen and R.T. Holman, JAOCS 42:537-540 (1965).

rated acids. This plot was also used to determine ECL (equivalent chain length) values (Miwa, JAOCS 40:309, 1963). In Table I are given comparable literature ECL values for several polyesters. Although these show individual bias they effectively bracket the ECL values calculated from the published GLC figure.

The findings of this enquiry may be summarized as follows: The peaks marked C_{14}, C_{15}, C_{16}, $C_{1\bar{6}}$, C_{18}, $C_{1\bar{8}}$, and $C_{1\bar{\bar{8}}}$ are correctly identified; the peak marked C_{20} could be 20:0 or equally well 18:3ω6; the peak marked $C_{2\bar{0}}$ is perhaps a double peak as indicated by the slight skewing. Possibly 20:1 is present but 18:3ω3 is also probable; the peak marked $C_{1\bar{\bar{\bar{8}}}}$ is definitely not 18:3ω3 and elutes rather early to be 20:2ω6. It is most probably 18:4ω3.

Where fatty acids of more than one chain length are known or suspected it is usually necessary to carry out GLC analyses of even superficially simple fatty acid mixtures on chemically different liquid phases. A basis for the use of different polarities among liquid phases has been discussed elsewhere (Ackman, Lipids 2:502, 1967) and is well exemplified by the technology of the *Myosotis scorpioides* analysis (Jamieson and Reid, J. Sci. Food Agric. 19:628, 1968).

Since 18:4ω3 is rather sparsely represented in fats from the terrestrial vegetable kingdom (Jamieson and Reid, J. Sci. Food Agric. 19:628, 1968), its possible presence in a larger number of aquatic plants may be highly significant.

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ADDENDUM

Lipids 5:628-635 (1970). "The Metabolism of Linoleic and Arachidonic Acids in Rat Testis," Raymond B. Bridges and John G. Coniglio.

On page 635, Reference No. 5 should be: Bieri, J.G., K.E. Mason and E.L. Prival, J. Nutrition 97:163-172 (1969).

Succinate Dehydrogenase: III. Stability and Interaction With Phosphatides

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ABSTRACT

The stability of soluble succinate dehydrogenase and the effect of phosphatides on dehydrogenase activity were studied. Phosphatides stabilize dehydrogenase preparations solubilized in the presence of succinate but do not affect the dehydrogenase prepared in the absence of succinate, which stabilizes the enzyme independent of the action of phospholipids. Phospholipids stabilize the flavoprotein under the same conditions they need to affect succinate dehydrogenase activity. These effects probably derive from a similar interaction of phosphatides with the flavoprotein.

INTRODUCTION

In a previous work we showed the effect of phosphatides on the catalytic activity of succinate dehydrogenase (succinate, acceptor oxidoreductase E.C. 1.3.99.1) in direct and reverse reaction, and on the inhibition of the enzyme by the lipophilic iron chelator thenoyltrifluoroacetone (1,2). The effect is produced by extracting phospholipids from particulate preparations, or by splitting them with phospholipases A, C and D, and is reversed by adding total mitochondrial phospholipids or purified phospholipids to soluble, solvent extracted preparations (1,2). These results were extended by Bruni and Racker (3) who showed that phosphatides are fundamental in allowing electron transport from succinate to ubiquinone in a reconstituted integrated complex.

The effect of lipids on soluble dehydrogenase and recombination with isolated membrane constituents are observed only when flavoprotein is solubilized from the particle under reducing conditions (1,3).

Solubilization, i.e., separation from native hydrophobic environment in the inner mitochondrial membrane, greatly decreases the stability of succinate dehydrogenase. Phospholipids affect the stability of soluble flavoprotein under the same conditions they act on the catalytic activity of the enzyme (4).

EXPERIMENTAL PROCEDURES

Materials, assay methods, enzyme preparations, assays of enzyme activity and treatment with phosphatides have been described in previous papers (1,2). Electrophoresis of succinate dehydrogenase on cellulose acetate was done as reported elsewhere (5).

Activation with succinate was performed by heating the preparation 5 min at 25 C in 60 mM

TABLE I

Stability of Soluble Succinate Dehydrogenase and the Effect of Glycerol[a]

Time, hr	No addition, N_2,2 C	Glycerol, N_2,2 C	No addition, N_2,-20 C	Glycerol N_2, -20 C	No addition air, -20 C	Glycerol, air, -20 C	No addition, N_2, -190 C
1.30	120	140					
3	113	125					
22	89	88	126	141	60	80	153
65			117	138	35	50	
96	68	80					147
150			100	116	21	34	
192							132
250	58	70	88	105			
316							100
500			71	88			
1340							102

[a]Succinate dehydrogenase prepared in 40 mM succinate was used. The storage medium was 40 mM succinate in 60 mM phosphate buffer pH 7.6. In experiments with glycerol the medium was glycerol: 7.2 mM succinate in 12 mM phosphate buffer pH 7.6 (1:1 v/v). The data represent the activity at various periods as per cent of the activity assayed before storage. They were confirmed in several experiments in which enzymes were used with initial velocities (at 25 C and infinite phenazine concentration) ranging from 4 to 6 mmoles/min succinate oxidized per gram protein at the gel eluate stage, and from 18 to 22 after ammonium sulfate fractionation.

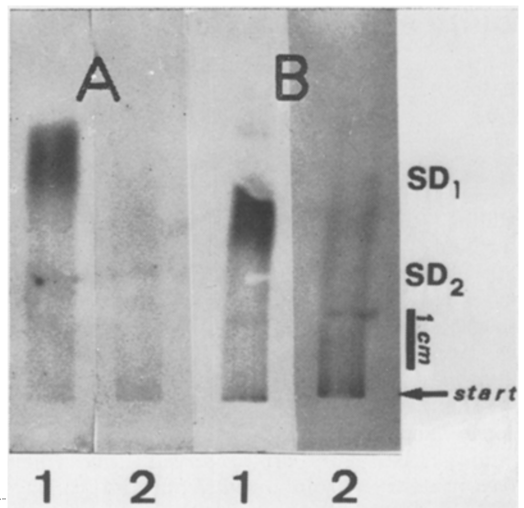

FIG. 1. Electropherogram of succinate dehydrogenase equilibrated with tris-phosphate buffer pH 7.60, I = 0.1 containing 12 mM succinate on a column of Sephadex G 25 fine (1 x 10 cm). The preparation had an activity of 12 μmoles succinate oxidized per minute per milligram protein at 25 C and 2 mM phenazine, and was applied on cellulose acetate strips (A) or after deactivating at 25 C in the air to an activity of 2.1 (B). Conditions of the run: 15 V/cm for 165 min at 2 C in a nitrogen atmosphere. At the end of the run succinate dehydrogenase was evidenced by dipping the strip in a solution of 60 mM phosphate buffer pH 7.6 containing 40 mM succinate 2 mM PMS and either 6 mM 2-(*p*-iodophenyl) 3-(*p*-nitrophenyl)-5-phenyl-tetrazolium chloride or 0.4 mM DCPI. Proteins were then colored with nigrosin. 1, formazan staining; 2, nigrosin staining; SD_1 and SD_2 indicate the two active fractions before denaturation.

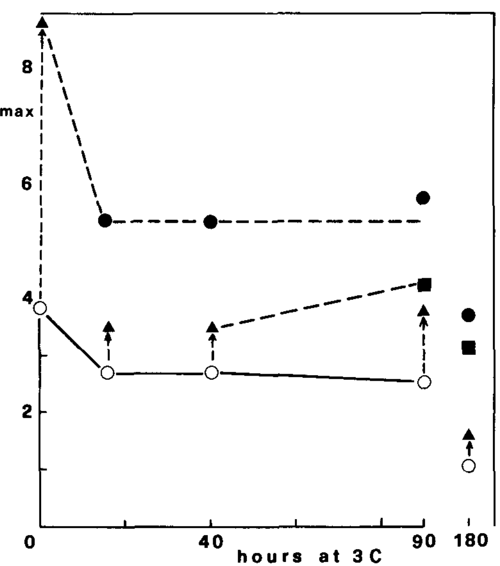

FIG. 2. Effect of lipids on the stability of soluble succinate dehydrogenase prepared in 40 mM succinate. Samples of the soluble dehydrogenase treated (dashed line) or not treated (solid line) with phosphatidylserine (25 μg P/mg protein) were kept at 2-4 C under nitrogen for the period indicated on the abscissa. The activity of samples stored without lipids was assayed also after equilibrating 30 min at 2-4 C with the same amount of phosphatidylserine (arrows). Ordinate: initial velocities at infinite phenazine concentration in mmole succinate oxidized per minute per gram protein at 25 C.

phosphate buffer pH 7.4 and 40 mM succinate, under nitrogen.

Conditions for Stability Studies

Freshly prepared soluble succinate dehydrogenase was used either at the gel eluate stage or after ammonium sulfate fractionation. Aliquots of the same preparation were placed in separate tubes, stored at the temperature indicated and opened at the moment of assay. When nitrogen was the selected gas phase, each tube was evacuated and flushed repeatedly with nitrogen and then stored under nitrogen. As a check, samples were prepared in which the flavoprotein was omitted and flavin mononucleotide (FMN) added and reduced with stoichiometric amounts of dithionite. The faint residual fluorescence was a highly sensitive index of the redox state of the compound and therefore of the entry of oxygen.

When dehydrogenase was mixed with organic solvents, the concentrated enzyme solution plus succinate was slowly added to the diluted solvent at -15 C and the temperature was then raised to 2 C.

RESULTS

One group of experiments was concerned with the stability of succinate dehydrogenase prepared and stored in the presence of succinate. A moderate increase of activity in the first hours of aging is constantly observed under good anaerobiosis (Table I). At low temperature it appears later and lasts longer. Incubation at 25 C with succinate either before storage or after various periods of storage did not increase the activity of the dehydrogenase.

The slightest traces of oxygen cause a rapid decay of succinate dehydrogenase. If air is admitted, at any storage temperature, the catalytic center activity of flavoprotein prepared and stored with succinate, drops rapidly to a steady value averaging at 25 C 1800 moles/min of succinate oxidized per mole peptide-bound flavin. The inactivation follows first order kinetics. The deactivated flavoprotein has a lower electrophoretic mobility, particularly at high ionic strength (I =

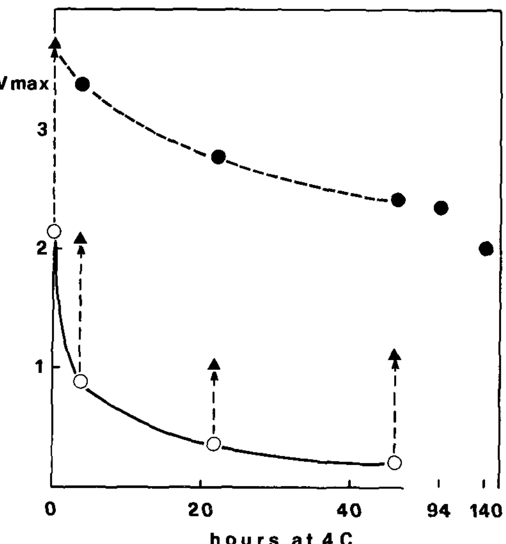

FIG. 3. Effect of succinate on the stability of soluble succinate dehydrogenase prepared in the absence of succinate. The soluble dehydrogenase was partially stored in 60 mM phosphate buffer pH 7.6, in separate tubes under nitrogen, for the period indicated in the abscissa (open circles). Other aliquots were first activated for 10 min at 22 C in 40 mM succinate and 60 mM phosphate buffer pH 7.6 and then stored in this medium (solid circles). In both cases succinate dehydrogenase activity was assayed without further activation. The activity of the enzyme stored without succinate was also assayed after activating in 40 mM succinate prior to assay (arrows and solid triangles). Ordinate: initial velocities at infinite phenazine concentration in mmoles succinate oxidized per min per gram protein at 25 C.

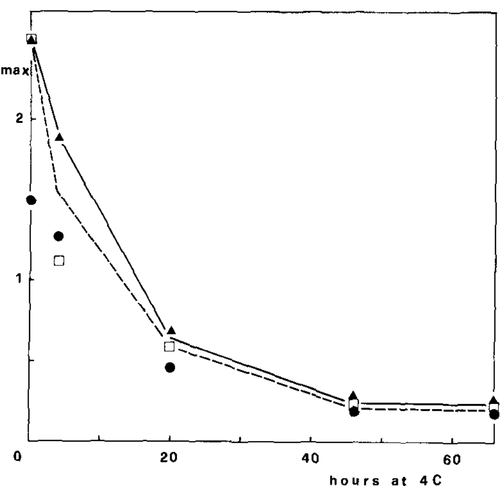

FIG. 4. Effect of phosphatides and of oxaloacetate on the stability of soluble succinate dehydrogenase prepared in the absence of succinate. Samples of the soluble dehydrogenase in 60 mM phosphate buffer pH 7.6 were kept at 2-4 C under nitrogen (solid line) or after addition of phosphatidylinositol (100 μg P/mg protein) for the period indicated on the abscissa (dashed line). The activity of samples stored without phospholipids was also assayed in the following conditions: (a) Phosphatidylinositol was added 30 min prior to the assay and equilibrated at 2-4 C with the dehydrogenase then oxaloacetate, 3.10^{-7} M final concentration in the assay, was added 30 sec prior to recording dehydrogenase activity (triangles). (b) Oxaloacetate alone was added as above (circles). The activity of samples stored with phosphatidylinositol was also assayed after oxaloacetate addition as above (squares). The presence of oxaloacetate during storage leads rapidly to complete inactivation of the enzyme. Ordinate: initial velocities at infinite phenazine concentration in mmoles succinate oxidized per minute per gram protein at 25 C.

0.08-0.10); activation with succinate does not reverse this change (Fig. 1).

The storage medium was varied to find conditions for best stability. Equal volume mixtures of water with ethanol, propanol or diglyme, in which the dielectric constant is progressively smaller, were assayed at 2 C. Sucrose 1.0 M, and saturated NaCl were also tested. None increased the stability.

An increased stability was observed with increasing glycerol concentrations, 50% glycerol being optimal (Table I). When mixing the enzyme with glycerol there is a minor deactivation, which may be due to dilution of succinate in the storage medium, but stability is thereafter improved; the increase in activity at early stages of aging is larger.

Reducing substances were also included in the storage medium, namely 5 mM dithiothreitol, 1 mM sodium dithionite or 5 mM sodium dithioglycolate. The latter does not significantly affect the dehydrogenase; dithionite inhibits and lowers the stability of the enzyme. Dithiotreitol has a moderate stabilizing effect, especially manifest in the presence of air. The spectra of the flavoprotein show that dithiotreitol does not reduce the flavin.

Phospholipids considerably improve the stability of the soluble flavoprotein prepared in succinate. After lipid addition and consequent stimulation, a slow decay during storage follows at 2 C (Fig. 2, dashed line). The activity of samples stored with phospholipids is always significantly higher than that of samples stored without phosphatides and preincubated with phosphatides at the moment of each assay. In these experiments the storage medium contained 40 mM succinate as well as lipid. Succinate has a conspicuous role on the stability of the flavoprotein. The effect of phosphatides is, however, separate from that of succinate, for the flavoprotein stored with succinate alone (Fig. 2, solid line) behaves differently from

when stored with succinate and lipids. Dehydrogenase preparations made in 40 mM succinate are not activated by succinate.

Another group of experiments considered the flavoprotein solubilized and purified in the absence of succinate. This preparation, stored without succinate, is very unstable even in a nitrogen atmosphere. Its catalytic center activity has a rapid decay and reaches a steady value at 25 C of about 900 moles/min of succinate oxidized per mole peptide-bound flavin. However, when the same flavoprotein is activated in 40 mM succinate prior to aging and is stored in a medium containing succinate, the activity before aging is increased, as expected, and the stability is very much improved (Fig. 3). Samples aged without succinate and activated prior to the assay do not reach the activity displayed by the aliquots of the same preparation which are stored after being activated.

The rate of decay in activity depends on the previous history of the enzyme (activity of the original mitochondrial preparation, rapidity of performing the isolation procedure, anaerobiosis, temperature, etc.) Less active preparations appear to have a smaller decay, for they tend towards steady state values of catalytic activity as indicated above.

The flavoprotein prepared in the absence of succinate is not stabilized by phosphatides (Fig. 4). Activation of the enzyme either before or after addition of the lipids does not bring about an effect of lipid, addition of oxaloacetate to the storage medium causes a more rapid decay of the enzyme.

DISCUSSION

The increase in activity of succinate dehydrogenase during storage under reducing conditions is not due to activation by succinate. Indeed as was shown earlier (6) and the present studies confirm, dehydrogenase preparations made and kept throughout in the presence of 40 mM succinate are already fully activated. Dervartanian and Veeger (7) reported a similar increase in activity for the soluble oxidized enzyme stored 3 hr at 25 C under nitrogen. A decrease of succinate in the medium slightly deactivates the enzyme which may then be activated up to 10-15%; the larger increase in activity during early stages of storage in glycerol is explained on this basis. Enhanced stability in prolonged storage in 50% glycerol may be due to the effect of glycerol on the structure of water: the decreased diffusion of oxygen may also play a role.

Increase in activity during early storage and decay in reconstitutive capacity (8) are probably different aspects of the adaptation of the flavoprotein to an aqueous medium. The alterations in electrophoretic pattern relate probably to a change in charge density of the molecule. They indicate more profound modifications of the flavoprotein associated with a strong decay in activity. The same holds for the loss of non-heme iron. Preparations have been described with 4 non-heme iron atoms per mole flavin (9) instead of 8 as in the native flavoprotein; with aging the flavoprotein may further lose iron and labile sulfide to a form eventually containing only two iron atoms (10).

The flavoprotein, particularly in the oxidized form, appears, therefore, to be unusually labile in aqueous medium. When the soluble dehydrogenase is recombined with particles or with isolated membrane constituents, the enzyme becomes much more stable, just as the native membrane bound dehydrogenase. Likely, interactions with membrane proteins and lipids stabilize the flavoprotein. Indeed, its native environment, the inner mitochondrial membrane, is highly hydrophobic; the flavoprotein is tightly bound to it and has high affinity for other membrane constituents in reconstitution experiments (3,10,12); its isolectric point, PI=5.7 (5), is in the same acidic range as for most lipoproteins. Proteins which in nature are associated with lipids in lipoproteins and membranes may possess some unique surface activity. They organize spontaneously at interfaces, or aggregate, also in dilute electrolyte solutions (13). A similar flexibility of structure may have a role in the modifications of succinate dehydrogenase in solution. Membrane proteins and the apoprotein part from lipoproteins interact specifically with lipid monolayers (13); the interaction of succinate dehydrogenase with phosphatides probably reflects a similar affinity.

The results of this work and our previous experience on the conditions of lipid action on dehydrogenase activity (1) exclude that the effect of phospholipids on stability may be attributed to a temporary interaction or a kinetic effect during assay of activity. Continuous interaction with phosphatides throughout the storage period is required. The stability increase, as well as the effect of phospholipids on the catalytic activity, occur under identical conditions; namely, in dehydrogenase preparations made in the presence of succinate. This suggests that they are aspects of the same phenomenon and confirms that the effect on dehydrogenase activity involves an interaction with phosphatides which modifies the protein.

In previous work we also showed that phos-

phatides remove oxaloacetate and fumarate inhibition of succinate dehydrogenase prepared in the absence of succinate (1). It was supposed that phosphatides interfere with the interactions between inhibitor and enzyme, but do not modify the flavoprotein in the same way that they do when they act on the dehydrogenase prepared in the presence of succinate. The present work confirms this hypothesis for phosphatides do not stabilize the flavoprotein prepared in the absence of succinate and interaction with the protein is not elicited by oxaloacetate. In view of similar behaviour also in these instances, the same mechanisms can be envisaged for the interactions between flavoprotein and lipids as discussed earlier (1,2,6).

Reducing conditions at the solubilization of the flavoprotein are required without exception to obtain soluble dehydrogenase preparations able to interact with dehydrogenase depleted particles (8) and with isolated membrane constituents (3). It was shown recently that reconstitutive capacity can be restored to soluble succinate dehydrogenase prepared in the absence of succinate by treating it with ferrous ammonium sulfate, sulfide and mercaptoethanol (12). The possible presence of colloidal iron in the treated enzyme makes it difficult to understand the mechanism of the treatment. The process of reconstitution may involve protein-protein interactions. Lipids are not required for the recombination of the soluble dehydrogenase with other membrane proteins (cytochrome b or factor F_4) but they are mandatory in restoring electron transfer from succinate to ubiquinone, a redox reaction in which neither cytochrome b nor F_4 apparently participate (3). This specification of the role of lipids is not feasible when particles are reacted with soluble succinate dehydrogenase in reconstitution experiments, since membrane fragments contain lipids and protein together.

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Gas Chromatographic Analysis of the Dimethylhydrazones of Long Chain Aldehydes[1,2]

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ABSTRACT

Long chain saturated and unsaturated aldehydes, prepared by the partial reduction of the corresponding fatty acid phenyl esters with lithium tri-*t*-butoxyhydroaluminate, were converted quantitatively to their dimethylhydrazones. Dimethyl acetals and 1,3-dioxolanes were prepared from the dimethylhydrazones. The dimethylhydrazones were then separated from the corresponding aldehydes by gas liquid chromatography on a semipolar column. Fatty acids were readily separated quantitatively from fatty aldehydes by formation of dimethylhydrazones of the aldehydes followed by an alkaline extraction of the reaction mixture prior to gas chromatography. A method for analysis of fatty acids and aldehydes from plasmalogens is described.

INTRODUCTION

Mixtures of long chain aldehydes have been successfully separated into their components by gas liquid chromatography (GLC) of the parent compounds (1), the dimethyl acetals (2,3) or the cyclic acetals (1,3-dioxolanes) (4). Dimethylhydrazones of ketosteroids, prepared in quantitative yields by treating the carbonyl compounds with N,N-dimethylhydrazine, have also been analyzed by GLC (5). Dimethylhydrazones are more stable than the parent carbonyl compounds (5) or the dimethyl acetals (6). Therefore, a method for the gas chromatographic separation of plasmalogen aldehydes in the form of their dimethylhydrazones is described in this paper.

EXPERIMENTAL PROCEDURES

Chromatography and Spectrometry

Either 0.25 mm (analytical) or 1.00 mm (preparative) layers of Silica Gel G (E. Merck Ltd., Darmstadt, Germany) were spread on thin layer chromatoplates (20 x 20 cm) with a Desaga applicator (Brinkman Instruments Inc., Westbury, N.Y.). Silver nitrate impregnated silica gel layers were prepared by spraying air-dried analytical plates with aqueous 10% (w/v) silver nitrate solution until the layers were just saturated with the solution (7). After the plates were air-dried they were activated at 120 C for 1 hr (analytical) or 3 hr (preparative).

Aldehydes and their derivatives were separated with an Aerograph Model 200 gas chromatograph equipped with a flame ionization detector. A 7 ft x 1/8 in. o.d. aluminum column, packed with 20% (w/v) diethylene glycol succinate polyester on 80-100 mesh Chromosorb W, was used for the separations. The column temperature was maintained at 175 C while the inlet and detector temperatures were 250 C. Flow rates of the gases were: nitrogen (carrier) 40 ml/min, hydrogen 40 ml/min, and air 500 ml/min.

Absorption of UV radiation by solutions of the unsaturated aldehydes was measured with a Beckman D.U. Spectrophotometer. IR spectra were recorded with a Perkin Elmer Model 137E Spectrophotometer, the samples being in the form of thin films between potassium bromide discs.

Preparations of Aldehydes and Their Derivatives

Saturated and *cis*-unsaturated fatty acids were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. Elaidic acid was prepared from oleic acid by nitrous acid isomerization (8).

The fatty acids were treated with oxalyl chloride to yield acid chlorides, which were converted to phenyl esters by adding them to phenol in pyridine. Attempts at direct esterification and alcohol interchange (methyl ester and phenol) were unsuccessful. Aldehydes were prepared from the corresponding fatty acid phenyl esters by reduction with lithium tri-*t*-butoxyhydroaluminate (9).

Dimethyl acetals and 1,3-dioxolanes of the aldehydes were prepared by the methods of Mahadevan et al. (10) and Venkato Rao et al. (4), respectively. Dimethylhydrazones were prepared by dissolving the aldehydes (ca. 100 mg) in an excess of N,N-dimethylhydrazine (Eastman Organic Chemicals, Rochester, N.Y.)

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and allowing the solution to stand at room temperature for 2 hr. Excess reagent was removed at 20 C by a stream of nitrogen and the dimethylhydrazones were dissolved in hexane for gas chromatographic analyses. The dimethylhydrazones were converted to dimethyl acetals and 1,3-dioxolanes by similar methods to those described by Venkato Rao et al. (4) and Mahadevan et al. (11).

Separation of Fatty Acids From Fatty Aldehydes

A mixture containing myristic, palmitic and stearic acids and the corresponding aldehydes was dissolved in an excess of N,N-dimethylhydrazine and left at room temperature for 2 hr. Excess dimethylhydrazine was removed in a stream of nitrogen. Diethyl ether was added to the reaction mixture and extracted with 2 N sodium hydroxide solution and water. Fatty acids, recovered from the alkaline aqueous solutions, were esterified with methanolic hydrochloric acid. Methyl esters and dimethylhydrazones were chromatographed under identical conditions.

Plasmalogen Analysis

The total lipids from a fresh beef heart were extracted with chloroform-methanol (2:1 v/v). Phospholipids in the extract were isolated by adsorption onto activated silicic acid (14) from which they were recovered by extraction with chloroform-methanol (1:1 v/v). Individual phospholipids in the extract were separated by preparative thin layer chromatography (PTLC) (solvent; chloroform-methanol-water-concentrated ammonia solution, 25:3:1 v/v/v). Phosphatidylcholine and phosphatidylethanolamine were recovered from the plates by elution with chloroform-methanol (1:1 v/v). The solvents were removed at 20 C under reduced pressure.

A portion of the separated phospholipid sample (ca. 20 mg) was dissolved in chloroform-methanol (1:1 v/v, 0.5 ml) in a flask to which 50% phosphoric acid (0.5 ml) was added. The flask was stoppered under nitrogen and the contents stirred vigorously for 1 hr. The reaction mixture was washed with distilled water and dried over anhydrous sodium sulfate. Aldehydes in the reaction mixture were separated from the lysophospholipid and diacylphospholipid by PTLC (solvent, chloroform). Dimethylhydrazones of the aldehydes were prepared as described abofe. The lysophospholipid was separated from the diacyphospholipid by redeveloping the thin layer plate with chloroform-methanol-water-concentrated ammonia solution (d. 0.88; 75:25:3:1 v/v/v/v). Lysophospholipids were transmethylated directly on the silica gel (12).

Identical GLC conditions were used for the analyses of the methyl esters and dimethylhydrazones. Unsaturated compounds were identified from relative retention time data of known compounds and by the disappearance of the unsaturated compounds, accompanied by an increase of saturated straight-chain compounds, in chromatograms of hydrogenated derivatives. The possibility that the dimethylhydrazones were contaminated with fatty acids was checked by extracting a hexane solution of the hydrazones with 2 N sodium hydroxide solution. No fatty acids were found in the alkaline aqueous extract.

RESULTS

Lauric, myristic, palmitic, stearic, oleic, linoleic and linolenic acids were reduced to the corresponding aldehydes with overall yields of 50-56%. IR spectra of the unsaturated aldehydes did not exhibit absorption at 965 cm^{-1} indicating that *cis-trans* isomerization did not take place during the preparation of these compounds. Results were confirmed by TLC of 1,3-dioxolane derivatives of the aldehydes on silver nitrate impregnated plates, which yielded only one spot for each compound. The 1,3-dioxolane derivatives of oleic and elaidaldehydes could be separated by this chromatographic procedure, their relative positions on the plates being similar to those of the corresponding fatty acid methyl esters (8,9). In addition, the absence of an absorption band at 233 nm in the UV spectra of linoleic and linolenaldehydes and at 268 nm in that of linolenaldehyde showed that there was no conjugation of the double bonds in these compounds. Thus, results indicated that double bond migration did not take place during the preparation of these aldehydes (13). Any absorption at 233 nm in the UV spectra of the polyunsaturated aldehydes could be attributed to autoxidation of these compounds so that care was required in their preparation and subsequent purification to minimize exposure to oxygen.

IR spectra of myristaldehyde, the dimethylhydrazone of myristaldehyde and the corresponding trisubstituted hydrazine are shown in Figure 1. A very small absorption band at 1740 cm^{-1} was present in the spectra of the hydrazone and the hydrazine. Strong absorption bands at 1610 cm^{-1}, 1360 cm^{-1}, 1260 cm^{-1}, 1140 cm^{-1}, 1120 cm^{-1} and 1030 cm^{-1} and weaker bands at 875 cm^{-1}, and 825 cm^{-1} in the hydrazone spectrum readily allowed one to distinguish this compound from the aldehyde. Differences in the IR spectrum of the dimethylhydrazone of myristaldehyde dis-

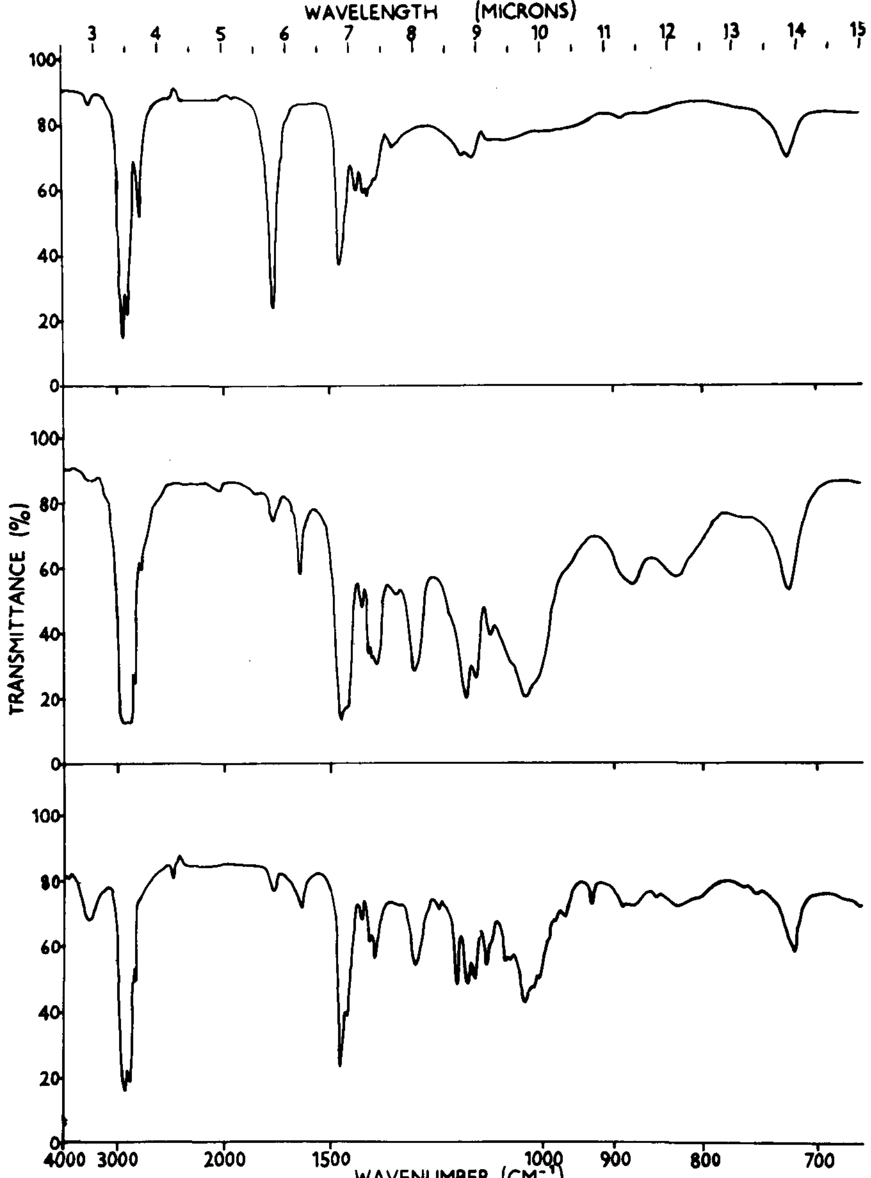

FIG. 1. IR spectra of (upper) myristaldehyde, (center) dimethylhydrazone of myristaldehyde and (lower) N,N-dimethyl -N'-tetradecylhydrazine.

cussed here and the spectra of similar compounds reported by Wiley et al. (14) probably arise from differences in sample preparations used for recording the spectra. Peaks at 1160 cm^{-1} and 1100 cm^{-1} and a shoulder at 1060 cm^{-1} were present in the spectrum of the hydrazine but not in that of the aldehyde or of the hydrazone.

The hydrazones were converted to dimethyl acetals and to 1,3-dioxolanes in 80-85% yields. The conversion products, which were contaminated (<8%) with the corresponding aldehydes and dimethylhydrazones, could be purified by TLC (solvent, toluene). The conversion products were useful for gas chromatographic identification of the aldehydes.

Retention times, relative to methylstearate, of the aldehydes, dimethyl acetals, 1,3-dioxolanes, dimethylhydrazones, dimethylhydrazines and methyl esters are shown in Table I.

TABLE I

Retention Volumes, Relative to Methyl Stearate of Some Fatty Aldehydes, Their Derivatives[a] and Methyl Esters of the Corresponding Fatty Acids

Chain length of aldehyde[b]	Aldehydes	Dimethyl acetals	1,3-Dioxolanes	Dimethyl-hydrazones	Dimethyl-hydrazines	Methyl esters
12:0	0.11	0.11	0.37	0.20	0.17	0.13
14:0	0.22	0.22	0.71	0.38	0.33	0.27
16:0	0.40	0.43	1.39	0.73	0.66	0.52
18:0	0.79	0.84	2.69	1.43	1.31	1.00
18:1	0.93	0.95	3.03	1.62	1.47	1.11
18:2	1.09	1.19	3.84	2.04	1.84	1.46
18:3	1.66	1.60	5.25	2.74	2.47	2.02

[a]Column: 7 ft x 1/8 in. o.d. containing 20% diethylene glycol succinate polyester on 80-100 mesh Chromosorb W. Column temperature 175 C, inlet and detector temperatures 250 C. Gas flow rates; nitrogen (carrier) 40 ml/min, hydrogen 40 ml/min, and air 500 ml/min.

[b]Chain length:number of double bonds.

Excessive tailing occurred when the hydrazones were chromatographed on a column containing Apiezon L grease. This aspect was not investigated further. When an equimolar mixture of methylstearate and the dimethylhydrazone of stearaldehyde was separated by GLC, the recovery in the two peaks was within experimental error. This result showed that there was no loss of the dimethylhydrazone, relative to the methyl ester, during the gas chromatographic separation.

Results of the GLC analysis of the products from the separation of fatty acids from the coresponding aldehydes are shown in Table II. The figures given in parentheses can be compared directly with those pertaining to the composition of the original mixture. Chromatograms of the fatty acid and aldehyde derivatives showed that there was no cross-contamination of these compounds in their respective separated mixtures. The small difference in the total weight of fatty acids in the original mixture (0.1013 g) and the weight of fatty acids recovered from the initial separation (0.1007 g) and the results in Table II indicate that quantitative separation of the acids and aldehydes was accomplished.

The composition of the fatty acids and fatty aldehydes of the phosphatidylethanolamine and phosphatidylcholine fractions from a sample of beef heart phospholipids are shown in Table III.

DISCUSSION

Long chain saturated and unsaturated aldehydes have been prepared by a modified Grundmann synthesis (15) and by oxidation of alcohol tosylates and mesylates with dimethyl sulfoxide (16). The partial reduction of saturated phenyl esters, as well as phenyl crotonate and phenyl cinnamate, with lithium tri-*t*-butoxyhydroaluminate to yield the corresponding aldehydes has been reported (11). Migration and *cis-trans* isomerization of double bonds was not expected to take place during the partial reduction of unsaturated fatty acid phenyl esters by lithium tri-*t*-butoxyhydroaluminate in the present study. Results of the present investigation show that isomerization did not take place during preparation of the unsaturated aldehydes. After the preparation described in the present paper was completed, a report on the partial reduction of unsaturated fatty acid chlorides with lithium tri-*t*-butoxyhydroaluminate to yield the corresponding aldehydes was published (13). Preparation of aldehydes by partial reduction of phenyl esters has no advantage over that in which fatty acid chlorides are used because the phenyl esters cannot be prepared directly from fatty acids. The acid chlorides are necessary intermediates in the preparation of the phenyl esters.

Methods have been described for estimating the plasmalogen content of phospholipids by converting the aldehydes formed by acid hydrolysis of the plasmalogens to either the corresponding *p*-nitrophenylhydrazones (17) or the 2,4-dinitrophenylhydrazones (18). Chromatographic procedures have been developed for the separation of 2,4-dinitrophenylhydrazones of short chain carbonyls, but these methods of separation are unsuitable for the resolution of complex mixtures of long-chain, 2,4-dinitrophenylhydrazones (19).

Carbonyls regenerated from the corresponding 2,4-dinitrophenylhydrazones (20), as well as derivatives of alcohols (21) and acids (22) prepared from these carbonyls, have been separated by GLC though quantitative recoveries are difficult to achieve with these

TABLE II

Analysis of a Mixture of Fatty Acids and Aldehydes

Compound	Composition of original mixture[a]	Composition of separated mixtures[b]
Myristic acid	7.5	13.0 (7.2)
Palmitic acid	18.0	32.8 (18.1)
Stearic acid	29.8	54.2 (30.0)
Myristaldehyde	5.5	12.5 (5.6)
Palmitaldehyde	14.1	31.9 (14.3)
Stearaldehyde	25.1	55.5 (24.8)

[a]Expressed as wt %.

[b]Expressed as wt % of class of compound with weight percentages corrected for the total amount of acids and the total amount of aldehydes in the original mixture given in parentheses.

TABLE III

Fatty Acid and Fatty Aldehyde Composition of Beef Heart Plasmalogens

Chain length of compound[a]	Fatty acids		Fatty aldehydes	
	PE[b]	PC[c]	PE	PC
12:0	0.2	0.2	0.9	0.9
14:0 br	---	---	Trace	Trace
14:0	0.2	0.3	1.4	1.4
14:1	---	---	0.2	0.3
15:0 br	---	---	0.2	0.8
15:0	Trace	0.1	0.4	0.6
16:0 br	0.2	Trace	0.5	0.5
16:0	37.8	8.1	39.5	69.2
16:1	2.6	1.9	1.2	2.8
17:0 br	Trace	Trace	0.2	1.1
17:0	1.0	0.2	0.8	0.8
18:0 br	Trace	Trace	Trace	Trace
18:0	8.6	5.8	47.8	19.3
18:1	33.2	26.4	6.8	2.3
18:2	12.6	48.1	0.4	Trace
18:3	2.4	3.8	---	---
20:3	Trace	1.8	---	---
20:4	1.2	3.3	---	---

[a]Chain length: number of double bonds; br, branched (tentative identification).

[b]PE, phosphatidylethanolamine.

[c]PC, phosphatidylcholine.

methods. Although long chain aldehydes dissolved in carbon disulfide show no decomposition on analysis by GLC, a simple alkaline extraction method for the separation of a mixture of fatty acids and aldehydes may result in some polymerization of the latter compounds. Quantitative conversions of free aldehydes to dimethyl acetals (10) and 1,3-dioxolanes (4) have been shown to take place under suitable conditions. The main disadvantage in the use of acetals as carbonyl derivatives is that fatty acids, if present in the sample, will also react with the alcohol. A mild saponification of the acetal reaction product, which may result in the decomposition of some of the acetals, is required to remove the esters (2). A reagent that combines preferentially with aldehydes so that fatty acids, if present, could be removed by a simple alkaline extraction would be preferred to those reagents requiring saponification to effect the separation of acids from aldehydes. Hydrazines are examples of this type of reagent.

Low molecular weight unsymmetrically disubstituted hydrazines are more stable towards oxidation than similar monosubstituted hydrazines (23). During the present investigation, the methyl hydrazone of myristaldehyde was found to be less stable than the corresponding N,N-dimethylhydrazone and was rapidly oxidized when left in a loosely stoppered flask at 5 C. Reaction of equimolar quantities of short chain carbonyls and N,N-dimethylhydrazine in the presence of an acid catalyst does not result in quantitative conversion of the carbonyls to hydrazones (17). Absence of an aldehyde peak in the gas chromatograms of the reaction products from the dimethylhydrazone preparations described in the present paper, as well as absence of a significant carbonyl absorption band in the IR spectra of the hydrazones, indicates that the aldehyde-hydrazone conversions went to completion.

The dimethylhydrazones were well separated by GLC from the corresponding aldehydes, but emerged from the column before the corresponding 1,3-dioxolanes. The greater retention times of the dimethylhydrazones relative to those of the aldehydes was useful for measuring the extent of conversion of the aldehydes to their dimethylhydrazones. Aldehydes and dimethylacetals were inseparable on the column as were most dimethylhydrazones and hydrazines.

The composition of the saturated aldehydes of beef heart plasmalogens in the present study was similar to those reported by Gray (24) and Venkato Rao et al. (4). Compounds corresponding to branched-chain aldehydes were less prominent and fewer in number in the present investigation than previous studies (4,24). The unsaturated components in the mixture were separated from branched-chain components though peaks corresponding to the latter in the gas chromatograms may have represented more than one compound. Conversion of the dimethylhydrazones to dimethyl acetals or 1,3-dioxolanes followed by GLC separation of the products gave results similar to those in Table III.

Results reported herein show that dimethyl-

hydrazones are suitable derivatives for GLC analysis of long chain aldehydes. Contamination of the derivative reaction products by unreacted aldehydes was negligible. Saturated straight chain aldehydes may be identified by comparison of the relative retention times of their dimethylhydrazone, dimethyl acetal and 1,3-dioxolane derivatives on a polar column with those of compounds of known structure under identical conditions. Application of other techniques of lipid chemistry is required for the location of double bonds and branching in unsaturated and branched-chain aldehydes, respectively.

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Intracerebrally Injected Monohydroxy and Other C_{24} Steroid Acids as Demyelinating Agents in the Guinea Pig

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ABSTRACT

Sodium salts of lithocholic acid (3α-hydroxy-5β-cholanoic acid), 5β-cholanoic acid, 3β-hydroxy-Δ^5-cholenoic acid and 3-keto-5β-cholanoic acid injected intracerebrally into guinea pigs in doses of 1 mg or higher produced periventricular demyelination. 24-^{14}C-sodium lithocholate was rapidly released from the brain (only traces remained 2 hr after injection) if injected in quantities ranging from 2 μg to 5 mg. This rapid elimination is believed to account for the relatively high dose of lithocholate required for producing demyelination, and may also account for the limited demyelinating capacity of the other acids injected intracerebrally.

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INTRODUCTION

Sodium cholate (1) and its taurine conjugate (2) are known to produce demyelination in vivo and in vitro respectively. Lithocholic acid and some other unidentified steroid acids were present in detectable amounts in the demyelinating brains of guinea pigs afflicted with experimental allergic encephalomyelitis (3). In addition, this acid has recently been detected in multiple sclerosis brain tissue (4). On the basis of these observations it was suggested that the presence of bile acids in EAE brain may have implications in the development of demyelinating diseases (3). Since the main steroid acid isolated from EAE brain was lithocholic acid, it seemed of interest to determine if lithocholic acid and some of its possible precursors (5) and metabolites (6) produce demyelination when injected into the brain. Previous work has indicated that radioactive sodium cholate was not retained very long by the brain following intracerebral injection (1). In addition (Fig. 1), since the sodium salts of lithocholic acid (I), 5β-cholanoic acid (II), 3β-hydroxy-Δ^5-cholenoic acid (III) and 3-keto-5β-cholanoic acid (IV) did

TABLE I

Intracerebral Administration of Sodium Lithocholate in the Guinea Pig: Dose Level and Brain Histology

Amount of Bile salt injected[a] mg	Time killed after injection	Histological changes at autopsy[b]
0.1	3 weeks	None
0.1	8 weeks	None
0.2	3 weeks	None
0.2	8 weeks	None
1.0	3 weeks	None
1.0	8 weeks	Periventricular demyelination, 2 plus
2.0	3 weeks	None
2.0	8 weeks	Periventricular demyelination, 2 plus
3.0	8 weeks	Periventricular demyelination, 3 plus
5.0	Died night of injection	Acute ventriculitis, Periventricular demyelination
5.0	10 days	Periventricular demyelination, moderate
5.0	8 weeks	Periventricular demyelination, 2 plus

[a]There were two to three animals in each group.

[b]1 plus: localized hemorrhage and/or microglial reaction; 2 plus: localized swelling and degeneration of myelin sheath; 3 plus: generalized myelin sheath swelling, microglial reaction or myelin degeneration; 4 plus: marked changes, either general or localized. Severe demyelination.

TABLE II

Intracerebral Administration of Sodium 3β-Hydroxy-Δ^5-Cholenoate in the Guinea Pig: Dose Level and Brain Histology

Amount of bile salt injected[a] mg	Time killed after injection	Histological changes at autopsy[b]
0.1	3 weeks	None
0.1	8 weeks	None
0.2	3 weeks	None
0.2	8 weeks	None
1.0	3 weeks	None
1.0	8 weeks	Periventricular demyelination, 1 plus
2.0	3 weeks	None
2.0	8 weeks	Periventricular demyelination, 1 plus
3.0	8 weeks	Periventricular demyelination, 2 plus
5.0	2 days	Periventricular demyelination, equivocal
5.0	8 weeks	Periventricular demyelination, 3 plus

[a]There were two to three animals in each group.

[b]1 plus: localized hemorrhage and/or microglial reaction; 2 plus: localized swelling and degeneration of myelin sheath; 3 plus: generalized myelin sheath swelling, microglial reaction on myelin degeneration; 4 plus: marked changes, either general or localized. Severe demyelination.

produce demyelination when injected intracerebrally (see Results) it became of interest to determine the dose level sufficient to produce this effect. 24-^{14}C-sodium lithocholate was used as a model bile salt to trace the fate of the injected salts. The term bile-salt refers to sodium salts of C_{24} steroid acids.

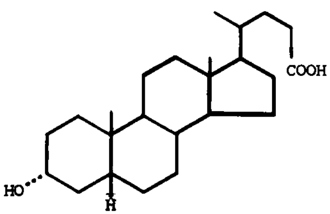
Production of demyelination by intracerebral injection of some bile salts and the study of elimination of these salts from the brain is the subject of this paper.

EXPERIMENTAL PROCEDURES

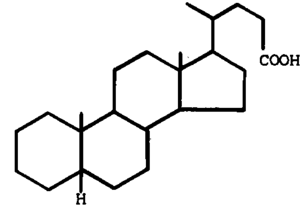
Materials and Methods

Animals. Guinea pigs of the Hartley strain weighing 400-500 g were obtained either locally or from the Animal Farm Division, Department of the Army, Fort Dietrick, Frederick, Md. They were maintained on a standard diet of Purina chow occasionally supplemented with fresh lettuce.

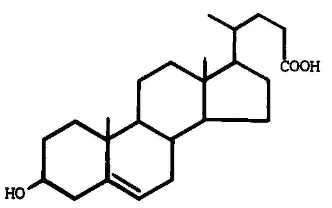
After intracerebral injections the animals



I. Lithocholic Acid



II. 5β-Cholanoic Acid



III. 3β-Hydroxy-Δ^5-cholenoic Acid

IV. 3-Keto-5β-cholanoic Acid

FIG. 1. Structural formulas of compounds investigated.

TABLE III

Radioactivity Excreted[a] in Feces After Intracerebral Administration of 24-^{14}C-Sodium Lithocholate Into Guinea Pig[b]

Days after injection	DPM/g 1[b]	DPM/g 2	DPM/g 3	DPM/g 4	DPM/g 5	DPM/g 6	DPM/g 7
0-2	852 (14.0)[c]	473 (6.3)	2,132 (13.4)	1,485 (10.1)	3,560 (28.7)	5,707 (12.2)	11,385 (10.1)
2-4	2,001 (33.2)	3,984 (52.8)	12,195 (76.5)	12,195 (83.0)	7,574 (61.1)	39,195 (84.1)	96,750 (85.3)
4-6	1,004 (16.5)	---	334 (2.1)	---	---	1,102 (2.3)	---
6-8	347 (5.7)	730 (9.6)	182 (1.1)	690 (4.7)	446 (3.7)	23 (0.04)	1,973 (1.7)
8-10	1,054 (17.5)	---	207 (1.3)	---	159 (1.2)	110 (0.2)	835 (0.7)
10-12	740 (12.3)	738 (9.7)	472 (2.9)	87 (0.5)	165 (1.3)	67 (0.1)	2,173 (1.8)
12-14	---	1,337 (17.7)	210 (1.3)	91 (0.5)	468 (3.8)	347 (0.7)	242 (0.2)
14-16	38 (0.6)	276 (3.6)	199 (1.2)	99 (0.6)	12 (0.1)	---	---

[a]In all cases the excreted radioactivity accounted for 80-90% of the injected dose. The 20% loss may be due to the inefficiency of the extraction procedure or perhaps to extra-cerebral retention by the carcass.

[b]Animal number.

[c]Numbers in parentheses represent the per cent of total excreted radioactivity.

were kept in individual cages and in experiments where labeled acid was administered, urine and feces were collected at 48 hr intervals.

Steroid Acids. Lithocholic acid, 5β-cholanoic acid and 3β-hydroxy-Δ^5-cholenoic acid were obtained from chemical supply houses. The acids were purified by preparative thin layer chromatography (TLC) and crystallization. They were shown to be pure by melting point, TLC, optical rotations and gas liquid chromatography (GLC) of a methylated sample. The purified acids were converted to sodium salts by treatment with sodium carbonate and precipitation with excess ethyl ether.

24-^{14}C-lithocholic acid (50 μc/mg; New England Nuclear Corp., Boston, Mass.) was purified by preparative TLC, diluted with cold lithocholic acid and the sodium salt prepared as above. The salt was dissolved in a suitable amount of water and 0.1 ml of this solution used for intracerebral injections; the total amount and the activity of the labeled bile salt varied in different experiments and will be mentioned at appropriate places.

Intracerebral Injections. Bile acids were injected intracerebrally as aqueous solutions of their sodium salts as previously described in detail for the rat (7). Controls consisted of similar injections of 0.1 ml normal saline and 0.1 ml distilled water. After a suitable time interval (see Tables) the animals (controls and bile salt injected) were decapitated under ether anesthesia. Brain and spinal cord were removed and stored in 10% formalin for histological examination. In experiments where radioactive bile salt was injected the brains were removed and extracted as described later.

Procedure for Histological Study. The brain tissue was examined by making two coronal sections from the region of the pre-motor area transecting the basal ganglia and lateral ventricules, and one section of the cerebellum and pons. Spinal cord samples were taken at four levels about the cervical, thoracic, lumbar and sacral areas. Sections, 12-15 μ thick, were stained with modified Luxol Fast Blue-PAS myelin stain. Sections were also cut at 5μ and stained with Harris' Hematoxylin and Eosin.

Determination of Radioactivity in Brain. In experiments with labeled sodium lithocholate the animals were decapitated at time periods varying from ½ hr to three weeks after injection. Brains were homogenized and boiled with acetone-methanol-water. The mixture was cooled and acidified with dilute HCl, and again heated on a steam bath. The cooled mixture was filtered, the liquid layer concentrated under reduced pressure, diluted with 100 ml water

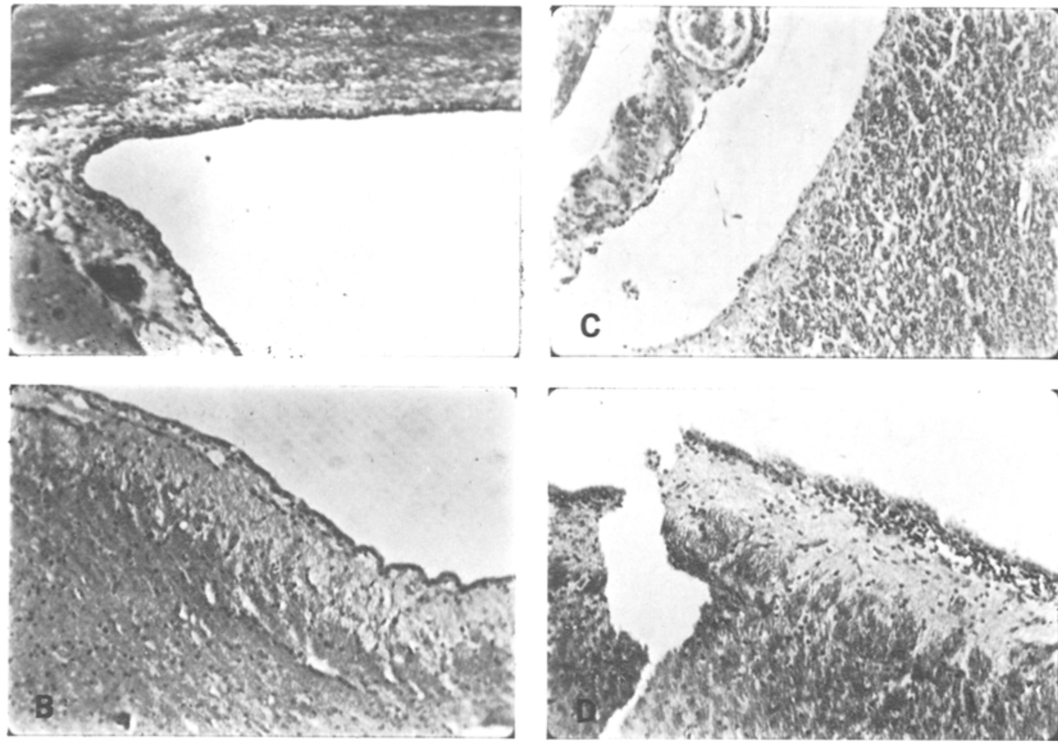


FIG. 2. Luxol fast blue x 125. Guinea pig brain after intracerebral administration of steroid acids. Coronal sections through lateral ventricle. (A) Sodium lithocholate; periventricular demyelinization, 3 plus. (B) Sodium 5β-cholanoate; moderate periventricular demyelinization. (C) Sodium 3β-hydroxy-Δ^5-cholenoate; periventricular demyelinization, 3 plus. (D) Sodium 3-keto-5β-cholanoate; moderate periventricular demyelinization.

and extracted with ether (3 x 100 ml). The ether layer was washed, dried over anhydrous Na_2SO_4 and evaporated. An aliquot was used for radioactivity estimations by liquid scintillation spectrometry (Ansitron Liquid Scintillation spectrometer; efficiency for ^{14}C, 92%).

The tissue residue left after acetone-methanol-water treatment and the water layer from the above extraction were combined and treated with 5% NaOH in 50% ethanol at 120 C for 4 hr in an autoclave. The cooled alkaline layer was acidified and extracted with ether and the ether extract examined for radioactivity.

Determination of Radioactivity in Feces. Each collection of the feces specimens was dried, weighed and hydrolyzed with 5% NaOH in 50% ethanol by autoclaving at 120 C overnight. The nonsaponifiable material was extracted with petroleum ether (4 x 250 ml) and then ether (2 x 200 ml). The aqueous layer was acidified and extracted with ether (4 x 200 ml); the ether layer was washed, dried, evaporated and one tenth of the residue was used for radioactivity estimations in a thin-window gas flow counter (Picker Nuclear; efficiency for ^{14}C, 22%).

RESULTS

Neurology

None of the animals receiving doses 3 mg or less in amount exhibited any neurological symptoms (paralysis or tremor) or any extensive weight loss during the experimental period. Some of those which received 5 mg exhibited tremor and a wobbling gate a day or so before killing.

Histological Study of the Brain After Intracerebral Injections

The brains and spinal cords from guinea pigs injected with distilled water or saline and killed after different time intervals did not show any pathologic changes. The brains from bile salt injected animals showed uniformly periventricular lesions. No significant mononuclear cell reaction was seen in demyelinated areas. Neutrophilic reaction with disorganization along the needle tract was frequent. No

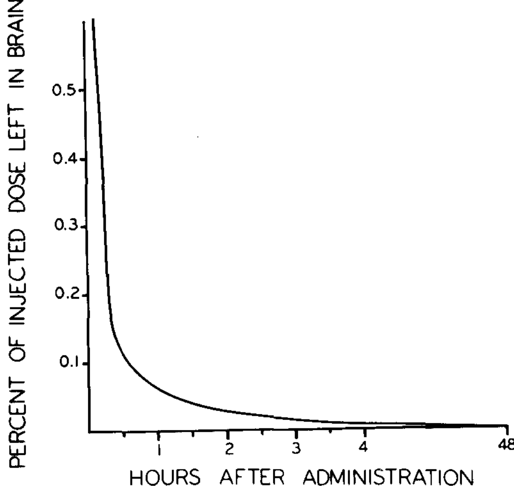


FIG. 3. Plot showing the residual radioactivity in the brain after intracerebral administration of 24-^{14}C-sodium lithocholate. The doses given were: 2 μg (0.1 μc), 1 mg (1 μc), 1.25 mg (1.25 μc), 2.5 mg (2.5 μc) and 5 mg (15 μc). The pattern of excretion was the same with all these doses.

demyelination was found in any of the spinal cords examined. Typical demyelinating lesions produced by sodium salts of lithocholic, 5β-cholanoic, 3β-hydroxy-Δ^5-cholenoic and 3-keto-5β-cholanoic acids are shown in Figure 2.

Table I and II show the extent of demyelination produced by different doses of the bile salts, I and III respectively; neither of the acids produced demyelination when administered in 0.1 and 0.2 mg quantities. [Sodium 3-keto-5β-cholanoate injected intracerebrally in 5 mg quantities (two experiments) produced periventricular demyelination in both cases where the animals were killed after six weeks of injection. We did not evaluate other dose levels of this compound. The sodium salt of 5β-cholanoic acid also produced similar demyelination when given in doses of 1 mg or higher.] At doses of 1 mg and higher the type of demyelination was the same. There was no correlation between the severity of demyelination and the amount of bile salt injected within the limits of 1-3 mg of the dose; with higher doses the mortality rate as well as the severity of demyelination increased.

Intracerebral Injection of 24-^{14}C-Sodium Lithocholate

Radioactivity in Brain. Figure 3 shows that the intracerebrally administered bile salt almost disappeared from the brain within 2 hr; less than 0.1% of the total dose was retained in the brain for some length of time. The pattern of elimination was the same with different doses varying from 0.1 μg (0.1 μc) to 5 mg (15 μc).

All but a negligible amount of radioactivity was extractable with acetone-methanol-water treatment; the hydrolyzed residue did not show significant amounts of radioactivity.

Preliminary results on the characterization of the radioactivity in the brain after the intracerebral injection indicated that the injected bile salt was metabolized to products less polar and also more polar than the injected salt. About 25% of the injected bile salt was converted to products less polar and more polar than lithocholic acid. These products have not yet been identified. There was similar indication of metabolism by the brain when sodium 3-keto-5β-cholanoate was injected. However, lithocholic acid may undergo auto-oxidation under certain circumstances, and it has not yet been ascertained whether the products indicated were real metabolites of lithocholic acid or auto-oxidation products.

Radioactivity in Feces. Table III shows that most of the intracerebrally injected bile salt was excreted in the feces within four days. However, the feces retained some radioactivity for more than two weeks, probably due to absorption and recirculation of the bile salt.

The total amount of radioactivity in the feces accounted for 80-90% of the injected dose. Preliminary TLC and GLC data showed radioactive products in the regions of mono-, di- and trihydroxy bile acids and also in the less polar region. The products have not been conclusively identified.

DISCUSSION

The present study shows that several C_{24} steroid acids, when injected intracerebrally in doses 1 mg or higher, cause demyelination. One must raise the question, what is the significance of such apparently unphysiological doses in view of the fact that only trace quantities of lithocholic acid were detected in EAE guinea pig brain (3) or in multiple sclerosis brain tissue (4)? Actually no comparison should as yet be made between the amounts detected in brain tissue and the dose required to produce demyelination in the present experiments, for a number of reasons. For example, as indicated in Figure 3, at all dosage levels lithocholic acid was rapidly excreted from the brain after intracerebral injection. It seems probable that the other acids behave in the same manner. The small amount retained is probably the material responsible for the pathological effect. The larger the dose, the greater the amount retained

within the brain. The animals injected with 1 or 2 mg of the bile salt (Tables I and II) showed demyelination after eight weeks; no histological change was apparent within three weeks yet the brain retained only a negligible fraction of the injected salt after 2 hr (Fig. 3). Correlating these two observations it seems that demyelination can proceed in the presence of only a fraction of the injected dose.

These results do not exclude the possibility that the bile salts induce a secondary process which, after a certain period of time, is responsible for the demyelination. Further we do not know whether the injected bile salts were demyelinating per se or by conversion into some more potent metabolite, or perhaps by complexing with some other material.

The capacity of brain tissue to metabolize a number of steroids is now well established (8-12). It has been found in this laboratory that yet another steroid can be metabolized by this tissue; ^{14}C-3-keto-5β-cholanoic acid on incubation with guinea pig brain tissue gave ^{14}C-lithocholic acid and other unidentified products (13). These observations suggest tht the lithocholic acid isolated from demyelinating brain (3,4) could have been formed within the brain. Whether it or similar acids have a role in the etiology of demyelinating diseases must await further investigation.

ACKNOWLEDGMENTS

Technical assistance by Miss Angela Rios and S. Tathir Husain Naqvi. This work was supported by Grant 506-E-5 from the National Multiple Sclerosis Society. 3-Keto-5β-cholanoic acid was given by W.H. Elliott.

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Metabolism of 1-^{14}C-Methyl Linoleate Hydroperoxide in the Rabbit

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ABSTRACT

The metabolism of 1-^{14}C- methyl linoleate hydroperoxide (1-^{14}C-MLHP) by the rabbit was investigated. Administration of 1.1-1.9 mg of 1-^{14}C-MLHP by ear vein injection proved lethal to four of the nine experimental animals. After 2 hr the lungs and liver contained 3.3% and 7.2%, respectively, of the dose. This radioactivity was found to be associated primarily with intact 1-^{14}C-MLHP. The triglycerides from these tissues also contained ^{14}C-trienoic and ^{14}C-dienoic fatty acids. Of the dose, 68% was recovered as $^{14}CO_2$ in 2 hr compared to 39% after 1-^{14}C-methyl linoleate injection. The triglycerides from kidney adipose tissue contained a small amount of ^{14}C-hydroxy fatty acid, providing confirmation of previous evidence for the presence of a fatty acid hydroperoxide reductase in animal tissues.

INTRODUCTION

Whether fatty acid peroxides are formed in animal tissues in vivo has been a controversial question for some years (1-6). Substances which presumably are derived from peroxides or their degradation products have been detected in tissue extracts (7-10), but it is difficult to prove their existence in the live organism. It seems apparent that only minute concentrations of peroxides, if any, occur in vivo, and in the absence of information on their turnover rate, it is impossible to draw conclusions as to the amounts formed. Although the evidence is largely circumstantial, present indications favor the view that small amounts of lipid peroxides are formed in vivo and that vitamin E, serving as a natural antioxidant, is an important factor in their metabolism. Infusion of small quantities of methyl linoleate hydroperoxide has been reported to induce incipient symptoms of vitamin E deficiency, including encephalomalacia in chicks (11) and creatinuria and hemolysis in rabbits (12). Lipid peroxides also have been implicated in aging, CCl_4 hepatotoxicity and ionizing radiation damage.

Studies on the toxicity of lipoperoxides indicate that only trace quantities in the tissues are tolerated. Fatty acid hydroperoxides are highly toxic when administered intraperitoneally (13,14). When administered orally they are considerably less toxic, and failure to

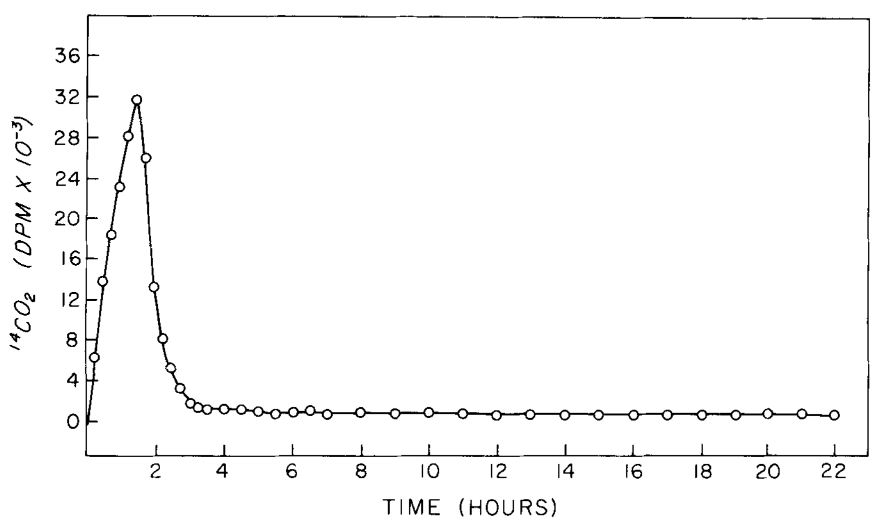

FIG. 1. $^{14}CO_2$ expiration during 22 hr following 1-^{14}C-MLHP injection.

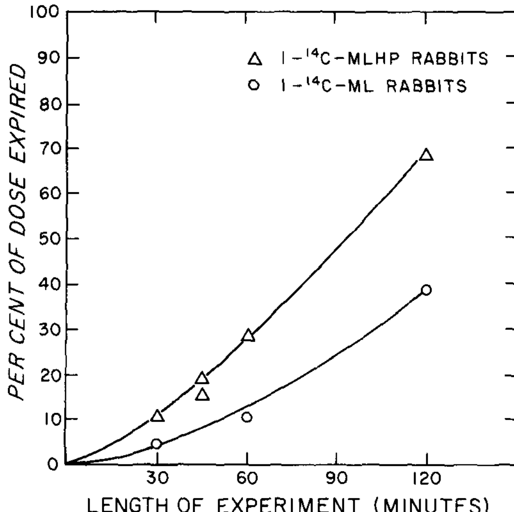

FIG. 2. Per cent of injected dose expired as $^{14}CO_2$. Each point represents the total $^{14}CO_2$ expired by a single animal.

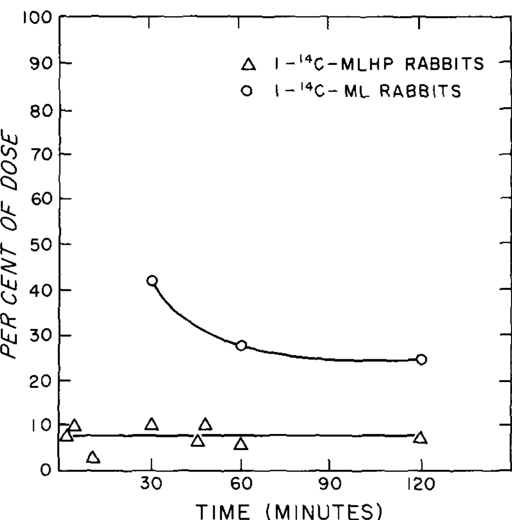

FIG. 3. Levels of radioactivity in liver following 1-^{14}C-MLHP or 1-^{14}C-ML administration.

find them in the tissues subsequently (15) indicates that their oral toxicity is due either to damage to the gut wall or to a systemic effect of decomposition products formed in the intestine. Methyl linoleate hydroperoxide is apparently not converted to linoleic acid in the tissues (16).

The present study was designed to investigate the metabolism of intravenously administered 1-^{14}C-methyl linoleate hydroperoxide in the rabbit.

EXPERIMENTAL PROCEDURES

Skelly Solve solvents were distilled twice before use; other reagents and solvents were of analytical grade. 1-^{14}C-Methyl linoleate (1-^{14}C-ML) with a specific activity of 9.3 mc/mM was obtained from Tracerlab (Waltham, Mass.). 1-^{14}C-Methyl linoleate hydroperoxide (1-^{14}C-MLHP) was prepared by controlled oxidation of 1-^{14}C-ML according to a modification of the method of Banks et al. (17). The oxygenation tube (2.6 x 30 cm) was loaded with 25 ml of Skelly Solve C (bp 88-100 C), 83 μc of 1-^{14}C-ML and 52.4 mg of methyl linoleate. Crude 1-^{14}C-MLHP obtained by stripping the solution with 87% ethanol was purified by the method of Kokatnur et al. (18).

The purity of the hydroperoxide was established using unlabeled ML. A peroxide number of 6022-6145 meq/kg was obtained by the Wheeler (19) method as modified by Kokatnur et al. (18). The theoretical value for pure MLHP is 6125 (18-20). A single spot (Rf = 0.52) was obtained by thin layer chromatography (TLC) on Silica Gel G using a 1% methanolic benzene solvent system. IR analysis revealed a hydroperoxy group (2.9 μ) and *trans,trans* conjugation (10.15μ). UV spectroscopy yielded a peak at 233 mμ for conjugated double bonds which gave a calculated molar extinction coefficient of 28,455. Banks et al. (17) have reported a value of 29,000 and Kokatnur et al. (18) found 27,873. The peroxide obtained by this procedure has been characterized as a mixture of 9-hydroperoxy-10,12-*trans,trans*-octadecadienoate and 13-hydroperoxy-9,11-*trans,trans*-octadecadienoate (17).

1-^{14}C-MLHP, 1.1-1.9 mg, was dissolved in 0.5 ml of ethanol and emulsified with 0.5 ml of physiological saline containing 2 mg/ml Tween 80. This material was injected slowly, over a period of 1.5-2 min, into the marginal ear vein of nine New Zealand White rabbits. The rabbits, weighing 1.7-2.3 kg, either died or were killed at 2, 4, 11, 30, 45, 47, 60, 120 min or 22 hr after injection. Three additional rabbits were injected with a like amount of 1-^{14}C-ML as a reference compound and were killed after 30, 60 or 120 min. Each animal was deprived of food for 16 hr prior to injection and was placed in a metabolism unit immediately after injection. $^{14}CO_2$ was monitored at 5 min intervals by passing the expired gases through 2 ml of Hyamine for 30 seconds. The $^{14}CO_2$ samples were counted in a Packard Tri-Carb liquid scintillation spectrometer, model 3003, after adding 15 ml of 0.3% 2,5-diphenyloxazole (PPO) in toluene.

TABLE I

Distribution of Radioactivity in the Tissues and Excreta of the 1-^{14}C-MLHP and 1-^{14}C-ML Injected Rabbits (% of Dose)

^{14}C-Compound administered	MLHP	MLHP	MLHP	ML	ML
Time after dose, hr	1	2	22	1	2
Tissues					
Liver	6.1	7.2	3.3	28.2	26.2
Lung	0.9	3.3	<0.1	8.0	0.6
Heart	<0.1	0.6	<0.1	---	---
Kidney	<0.1	1.0	0.4	---	---
Kidney adipose	<0.1	0.3	0.4	---	---
Brain	<0.1	0.2	0.1	---	---
Blood[a]	2.8	3.0	1.1	6.5	11.7
Excreta					
Urine	0	1.6	3.8	---	---
Feces	0	0	0.2	---	---
$^{14}CO_2$	29.0	68.0	78.0	10.0	39.1

[a]Calculated on basis of 6.1% body weight.

At the termination of the experiment various tissues were removed for analysis. Radioactivity in the blood serum was determined using Cab-O-Sil and 15 ml of 0.3% PPO in toluene. The excised tissues and red blood cells were ground with Na_2SO_4 in a mortar and extracted with $CHCl_3/CH_3OH$ (2:1) for 12 hr on a mechanical shaker. The extract was washed with 20 vol of water (21) and the residue was fractionated using silicic acid column chromatography (22). Trimethyl silyl (TMS) derivatives were formed using Tri•SIL/BSA (Pierce Chemical Company) (23). Separation of the silyl mono- and diglycerides and starting compounds was carried out on silica gel using a Skelly Solve B (bp 60-80 C)-diethyl ether-acetic acid (65:35:0.25) solvent system.

The lipid fractions were saponified with 1 N ethanolic KOH for 16 hr at room temperature (24). Fatty acids were decarboxylated with NaN_3 and H_2SO_4 (25). An ethereal solution of diazomethane was used to methylate the free fatty acids. Methyl esters of normal fatty acids were separated from the esters of polar acids using the 1% methanolic benzene TLC system. This system also was used for the separation of the methyl esters of polar acids and their silyl derivatives. Fractionation of normal fatty acid methyl esters according to their degree of saturation was accomplished by TLC on Silica Gel G impregnated with 5% $AgNO_3$ using a Skelly Solve F (bp 30-60 C)-diethyl ether (85:15) system (26).

Pure ricinoleic acid (12-hydroxy-9-*cis*-octadecaenoic acid) and dimorphecolic acid (9-hydroxy-10,12-*trans,trans*-octadecadienoic acid) were used as standard hydroxy fatty acids. MLHP was reduced to the corresponding hydroxy fatty acid using KBH_4 (27).

TABLE II

Distribution of Radioactivity in Lipid Fractions Isolated by Silicic Acid Column Chromatography (% of total DPM)

Tissue	Lung			Liver			
^{14}C-Compound administered	ML		MLHP	ML		MLHP	
Time after dose, hr	1	2	2	1	2	1	2
Cholesterol esters	1.1	0.2	0	1.1	1.8	0.2	0.2
Triglycerides	15.0	14.0	0.5	24.0	41.1	10.5	24.7
Free fatty acids	11.0	4.1	10.5	11.2	10.0	13.1	6.4
Cholesterol							
Diglycerides	6.4	6.5	37.4	6.1	8.8	25.1	27.6
Monoglycerides	4.4	5.4	34.8	10.2	3.3	40.7	32.1
Phospholipids	62.0	68.0	16.8	47.3	34.6	9.9	9.0

RESULTS AND DISCUSSION

$^{14}CO_2$

Figure 1 illustrates the rate of $^{14}CO_2$ expiration following intravenous injection of 1-^{14}C-MLHP. Peak expiration occurred at 85 min, 68% of the injected radioactivity being expired by the end of the second hr and 78% by the end of 22 hr. By comparison, after 1-^{14}C-ML injection only 39% of the dose was expired as $^{14}CO_2$ during the initial 2 hr period and peak expiration occurred 50 min after administration of the dose.

Figure 2 gives a comparison of the total $^{14}CO_2$ expired after administration of either 1-^{14}C-MLHP or 1-^{14}C-ML. Obviously 1-^{14}C-MLHP is much more rapidly oxidized to $^{14}CO_2$.

Toxicity of 1-^{14}C-MLHP

Four of the nine rabbits injected with 1-^{14}C-MLHP (3.5-6.0 μM) developed torpidity and died at 2, 4, 11 and 47 min, respectively, following administration of the hydroperoxide. No such reaction was observed in the animals injected with 1-^{14}C-ML. On an equivalent body weight basis the amount of MLHP administered was 0.83% and .17% of the LD_{50} dose reported for mice (13) and rats (14), respectively, following intraperitoneal injection. Differences in routes of administration, species, chemical form and purity of compounds administered are factors which may contribute to this wide range in toxicity values. It also was observed that those animals to which the dose proved fatal all contained high concentrations of radioactivity in the lungs. This observation, in addition to the nature of the symptoms induced, suggests that an impairment of the respiratory function may have been the cause of death. Lung tissue is known to be sensitive to small concentrations of gaseous oxidants and the lungs represented the first capillary bed encountered by the administered dose in these experiments.

Distribution of Radioactivity in the Tissue

Table I shows the distribution of radioactivity in the tissues and excreta at various times after 1-^{14}C-MLHP or 1-^{14}C-ML administration. The only tissues except blood which contained a significant amount of activity were the liver and lungs. Figure 3 illustrates the relatively constant level of radioactivity found in the liver during the initial 2 hr after 1-^{14}C-MLHP administration and the greater activity found following 1-^{14}C-ML injection.

Identification of ^{14}C-Compounds in Liver and Lung

Table II gives the distribution of radioactivity among the lipid classes of lung and liver 1 and 2 hr after 1-^{14}C-ML and 1-^{14}C-MLHP injection. As expected, the major concentrations of activity following 1-^{14}C-ML administration were in the TG and PL fractions. Saponification of these fractions, TLC of the fatty acid methyl esters on $AgNO_3$-impregnated Silica Gel G and subsequent decarboxylation of the fractionated acids indicated that 90% of this radioactivity was present in 1-^{14}C-linoleic acid.

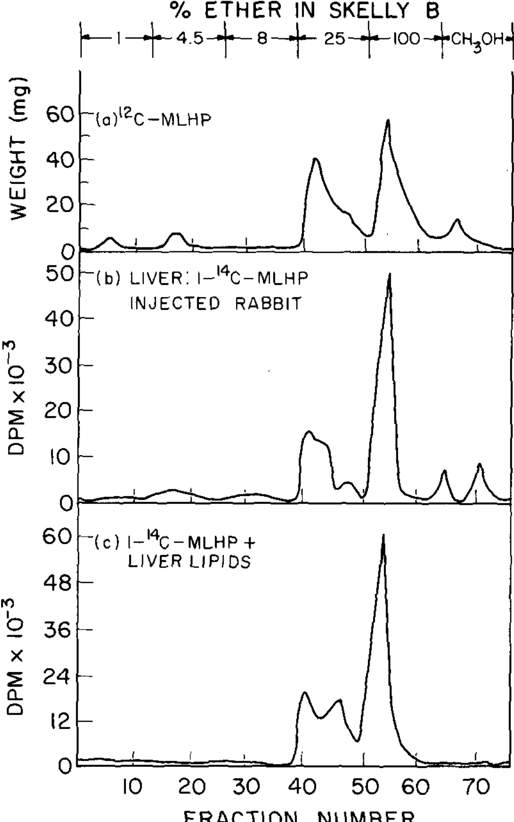

FIG. 4. Comparative silicic acid column elution patterns.

In contrast, following 1-^{14}C-MLHP injection the radioactivity in both lung and liver lipids was located predominately in the DG and MG fractions which were eluted with 25% and 100% diethyl ether in Skelly Solve B, respectively. However, the ^{14}C compounds in the DG and MG fractions did not form silyl derivatives, indicating that the radioactivity in these two fractions was not present in esters of glycerol but in compounds with similar polarity. The ^{14}C compounds in the DG and MG fractions from the animals injected with 1-^{14}C-ML could be easily silylated.

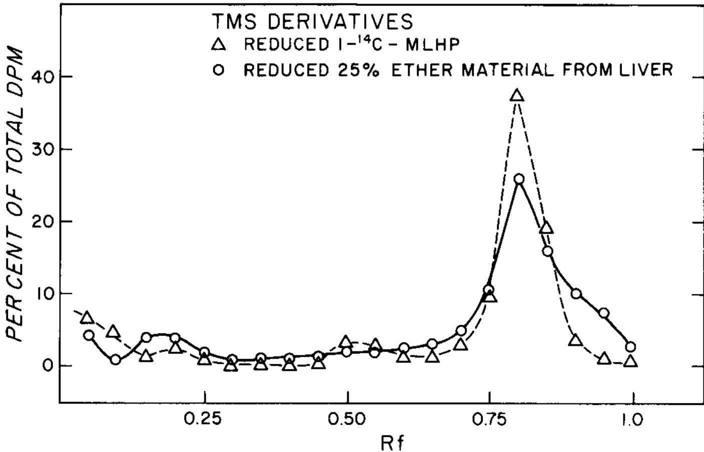

FIG. 5. TLC of pure 1-^{14}C-MLHP and the 25% ether fraction from the liver of a 1-^{14}C-MLHP-injected rabbit after KBH_4 reduction and silylation. The developing solvent was 1% methanolic benzene.

Standardization of the silicic acid column with radioactive and nonradioactive MLHP revealed that intact hydroperoxide was eluted with 25% diethyl ether in Skelly Solve B and that 100% diethyl ether eluted degradation products of the peroxide which formed on the column (Fig. 4). After applying a 230 mg sample of ^{12}C-MLHP (peroxide number 6000) to the column it was observed that the material eluted with the 25% diethyl ether comprised 42% of the sample weight, had a peroxide number of 5400 and an IR spectrum indistinguishable from that of the original peroxide. The 100% diethyl ether eluate represented 36% of the original mass and had a peroxide number of 2800 (Fig. 4a). Comparison of the IR spectrum of this fraction with that of the original sample revealed numerous discrepancies. When 1-^{14}C-MLHP was combined with liver lipids and chromatographed on a silicic acid column, the radioactivity was recovered in the same two solvents in similar proportions (Fig. 4c). The 25% diethyl ether eluate contained 41% of the radioactivity and the 100% diethyl ether fraction contained 49%. Comparison of these two elution patterns with that of the liver lipid after 1-^{14}C-MLHP injection (Fig. 4b) showed that all three elution profiles were similar and suggested that the ^{14}C materials eluted with the DG and MG from the injected animals were unhydrolyzed 1-^{14}C-MLHP and products of its degradation on the column.

Exposure of 1-^{14}C-MLHP and the 25% diethyl ether fraction from the liver of a 1-^{14}C-MLHP-injected rabbit to Tri•SIL/BSA and TLC of the products showed that neither 1-^{14}C-MLHP nor the isolate from liver formed a silyl derivative. After reduction of the two samples both formed a silyl derivative (Fig. 5), confirming the chromatographic evidence that they were identical. Decarboxylation of the 25% and 100% diethyl ether fractions showed that the materials present were entirely carboxyl-labeled. Consequently it was concluded that a large portion of the radioactivity remaining in the liver and lung lipids 2 hr after 1-^{14}C-MLHP injection was still present in the form of unhydrolyzed hydroperoxide. These results support the results of earlier studies (28) in which intact peroxides were observed in rat liver 48 hr after intravenous administration of ethyl linoleate hydroperoxide. Evidently these hydroperoxy fatty acid esters can survive in biological systems longer than generally presumed.

After saponification of the TG fraction from lung lipids the fatty acid methyl esters were chromatographed using the 1% methanolic benzene system and then $AgNO_3$-impregnated Silica Gel G. It was observed that following 1-^{14}C-MLHP injection this fraction contained labeled trienoic fatty acids. Although only 0.5% of the lung radioactivity was present in the TG 2 hr after injection, 75% of this activity was found in the trienoic and another 22% was present in the dienoic acids. In addition, 19% and 6% of the radioactivity in the liver TG was associated with the dienoic and trienoic acids, respectively. It is noteworthy in this connection that in an associated study on the metabolism of the hydroxy acid obtained by reduction of 1-^{14}C-MLHP, labeled dienoic and trienoic acids were detected in liver triglycerides (29).

Saponification and TLC of the fatty acid methyl esters of kidney adipose tissue (1% methanolic benzene) revealed that 24% of the activity in this tissue was associated with polar fatty acids. Silylation of the methyl esters indicated that a hydroxy fatty acid comprised a portion of this fraction. Unfortunately, the low levels of radioactivity present, coupled with a comparatively large tissue weight, made further analysis impossible. A subsequent paper in this series indicates that hydroxy and trienoic acids are formed from 1-^{14}C-MLHP during absorption (30).

The results of these experiments show that intravenous MLHP is rapidly oxidized to CO_2 in rabbit tissues and that the peroxide is not incorporated into tissue lipids. No evidence was obtained for the presence of free peroxide. However, substantial amounts of MLHP were found in the lungs and liver 2 hr after administration. This finding indicates that the limiting factor in MLHP oxidation was the hydrolysis of the ester, and suggests that fatty acid peroxides formed in vivo might be even more rapidly oxidized. The detection of a hydroxy fatty acid in the kidney TG supports the proposals of O'Brien and Little (31) and Christophersen (32) that a reductase is involved in peroxide metabolism.

ACKNOWLEDGMENT

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Absorption and Metabolism of 1-^{14}C-Methyl Linoleate Hydroperoxide

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ABSTRACT

The absorption and metabolism of 1-^{14}C-methyl linoleate hydroperoxide by rats was investigated. After intubation with 2 mg of peroxide, peak $^{14}CO_2$ production occurred at 90 min and 25% of the dose was expired in 24 hr. Forty-five per cent still remained in the gastrointestinal tract after 24 hr, most of it bound to the stomach epithelium in the form of intact peroxide. Lymph was collected from the thoracic duct 2 hr after intubation and examined for labeled metabolites. Seven per cent of the radioactivity in the lymph was present in a free 1-^{14}C-hydroxy fatty acid and 31% in its methyl ester. Fifty-seven per cent occurred in lymph triglycerides where it was equally distributed between a 1-^{14}C-trienoic fatty acid and an unidentified 1-^{14}C-oxy acid. The radioactivity in liver lipids was associated mainly with randomly labeled normal fatty acids. No ^{14}C-hydroxy acids were detected in liver lipids and no evidence was obtained for the absorption of unchanged peroxide. The hydroxy and trienoic acids appear to be formed during absorption by a reduction-dehydration reaction sequence.

INTRODUCTION

Although lipoperoxides are known to occur in foods, little is known about their metabolic fate. It has been generally concluded that the amounts of lipoperoxides normally present in dietary fat are relatively harmless (1); however, extensively peroxidized oils have been shown to have a pronounced toxic effect on animals. Whether fatty acid peroxides are absorbed from the lumen of the intestine is a controversial question. Andrews et al. (2) were unable to find evidence for the absorption of peroxides and therefore concluded that their toxic action took place on the intestinal wall. Other investigators also have concluded that intact peroxides are not absorbed (3-4); still others (5-6) have reported that feeding peroxides to rats leads to appreciable concentrations in the tissues. Nishida and Kummerow (7) obtained spectral evidence of diene conjugation in the lymph of rats dosed with methyl linoleate hydroperoxide and concluded therefore that some peroxide was absorbed.

Recent improvements in methods for the preparation of pure radioactive fatty acid hydroperoxides (8-9) make metabolic studies on these compounds more feasible. In the present study the absorption and metabolism of 1-^{14}C-methyl linoleate hydroperoxide (1-^{14}C-MLHP) was investigated.

TABLE I
Distribution of Radioactivity in Tissues and Excreta
24 Hr After 1-^{14}C-MLHP Intubation

Tissue and excreta	DPM x 10^{-3}	Per cent of recovered $^{14}C^{a}$
Heart	0.8	$<$ 0.1
Kidneys	3.9	0.1
Liver	42.0	1.2
Lung	2.6	$<$ 0.1
Spleen	0.4	$<$ 0.1
Testes	1.4	$<$ 0.1
Epididymal adipose tissue	0.1	$<$ 0.1
Kidney adipose tissue	3.1	0.1
Mesenteric adipose tissue	6.3	0.2
GI tract and contents		
Stomach	1,713.0	48.0
Small intestine	332.5	9.3
Cecum	59.2	1.7
Large intestine	75.4	2.1
Feces	30.9	0.9
Urine	207.0	5.8
$^{14}CO_2$	1,087.3	30.5

[a]Recovery of dose, 73.2%.

EXPERIMENTAL PROCEDURES

General Procedures

The method used for the preparation of 1-^{14}C-MLHP is described in a previous paper (8). Sprague-Dawley male rats weighing 220-270 g, and fed a commercial feed (Rockland Rat Diet), were used. Two milligrams of pure 1-^{14}C-MLHP (1 μc/mg) was suspended in 100 mg triolein and administered by stomach tube. The animals then were placed in a metabolism cage and $^{14}CO_2$ expiration was monitored (8). At the termination of the experiment (either 2 or 24 hr) the gastrointestinal tract and viscera were excised and lipids were extracted with $CHCl_3/CH_3OH$ (2:1) (8).

Procedures used for saponification, methylation, reduction, decarboxylation and thin layer chromatography (TLC) have been described elsewhere (8). Trimethyl silyl (TMS) derivatives were prepared using hexamethyldisilazane (10). Unsaturated fatty acids were hydrogenated using Pd-$CaCO_3$ catalyst (11).

Collection of Lymph

Lymph was collected from three rats after ligation of the thoracic duct. To facilitate the operation, the rats were placed on a 50% restricted dietary intake for 24 hr prior to a scheduled experiment. Ad lib. feeding was resumed after the operation. Ligation of the thoracic duct was carried out through an abdominal incision by the procedure of Bollman et al. (12) after anesthesia with Diabutal. Six hours after regaining consciousness, the animals were intubated with 0.6 mg of 1-^{14}C-MLHP (1 μc/mg) suspended in 400 mg triolein. The lymphatic fluid was collected for 2 hr and extracted with 20 vol of $CHCl_3/CH_3OH$ (2:1).

RESULTS AND DISCUSSION

$^{14}CO_2$ Production

The $^{14}CO_2$ expiration curve (Fig. 1) of an intact rat intubated with 1-^{14}C-MLHP indicates that maximum $^{14}CO_2$ production occurred about 90 min after intubation. The secondary peak was associated with a period of increased physical activity and is not considered to be of metabolic significance. Ligation of the thoracic duct resulted in a 41% decrease in $^{14}CO_2$ expiration in 2 hr. The $^{14}CO_2$ expired by the ligated animals may have arisen from oxidation of water-soluble decomposition products formed by bacterial degradation in the gut or from compounds which diffused out of the ligated duct.

Distribution of ^{14}C-Compounds in the Tissues of Intact Rats

Table I lists the distribution of radioactivity

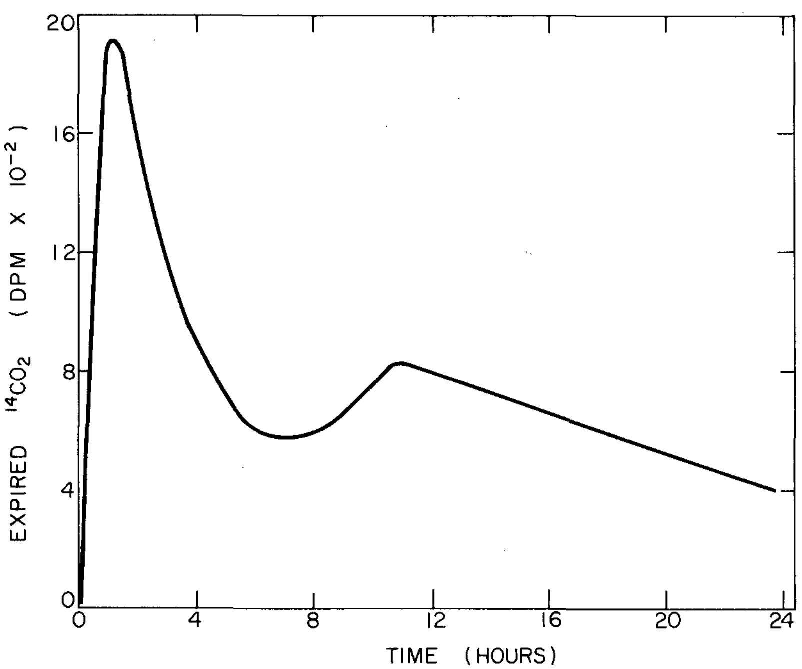

FIG. 1. $^{14}CO_2$ production after 1-^{14}C-MLHP intubation of intact rat.

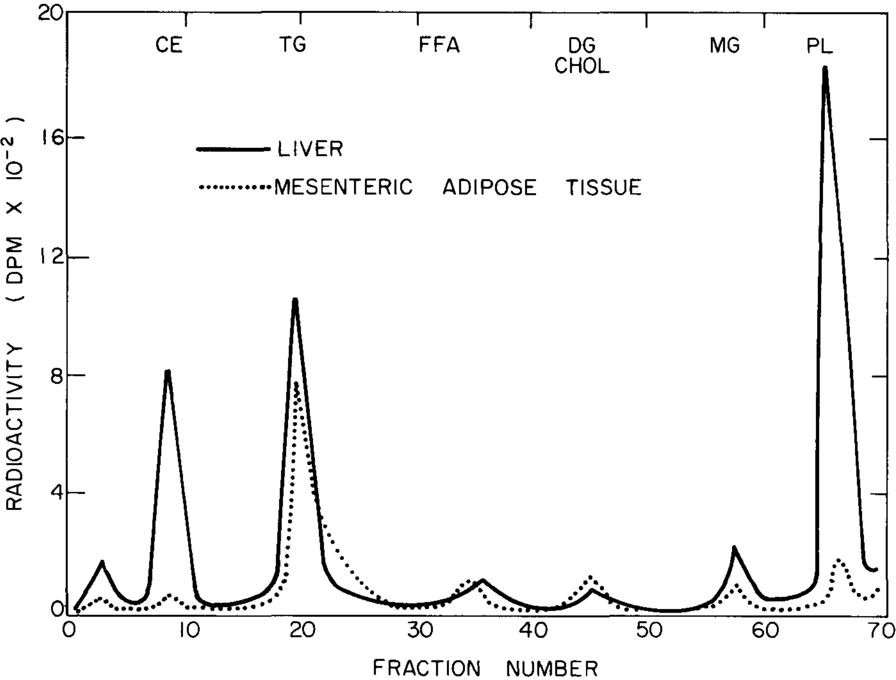

FIG. 2. Silicic acid column chromatography of lipids from liver and mesenteric adipose tissue 24 hr after intubation with 1-^{14}C-MLHP.

in the tissues and excreta 24 hr after intubation. Most of the dose (91.6% of the radioactivity recovered and 67.1% of that administered) was recovered from the gastrointestinal tract and expired $^{14}CO_2$, indicating that little activity was deposited in tissue lipids.

The lipid extracts from liver and mesenteric adipose tissue, which contained the greatest amounts of radioactivity, were fractionated into lipid classes by silicic acid chromatography (13). The radioactivity in the liver lipids (Fig. 2) occurred mainly in the cholesterol esters (CE), triglycerides (TG) and phospholipids (PL) but in the mesenteric adipose tissue it was associated primarily with the TG fraction.

The three liver lipid fractions were saponified at room temperature overnight, methylated and chromatographed on thin layers of Silica Gel G using a benzene-methanol (99:1) solvent system. All the radioactivity migrated to an Rf of 0.7, corresponding to the methyl esters of normal fatty acid standards, indicating that no radioactive polar fatty acids were incorporated into liver lipids. Chromatography on Silica Gel G impregnated with 5% $AgNO_3$ showed that the monoenoic fatty acids of the TG and CE contained much of the radioactivity (61.9% and 66.5%, respectively). In the PL fraction the dienoic acids contained more radioactivity than the monoenes (42.6% vs. 31.9%). Decarboxylation of the saturated, monoenoic and dienoic acids in the CE and PL fractions (14) showed that 11-33% of the radioactivity was located in the carboxyl group and indicated that these acids were randomly labeled by incorporation of ^{14}C-acetate. In contrast, the dienoic and trienoic fatty acids from the TG fraction contained 83% and 100%, respectively, of their radioactivity in the carboxyl group. This labeling pattern indicated that a dienoic and a trienoic fatty acid were formed from 1-^{14}C-MLHP by reduction and dehydration reactions. These acids represented 23.8% and 4.8%, respectively, of the total activity in the liver TG fraction.

It was concluded that no polar fatty acids were deposited in the liver after 1-^{14}C-MLHP intubation and that most of the radioactivity in this organ resulted from ^{14}C-acetate incorporation. However, small concentrations of carboxyl-labeled dienoic and trienoic fatty acids were present in the TG which arose by direct conversion of the hydroperoxide. These compounds may provide an important clue to lipoperoxide metabolism in the animal.

The TG fraction from the mesenteric adipose tissue was saponified and characterized by the procedures used for the liver lipids. TLC

TABLE II

Distribution of Radioactivity in the Gastrointestinal Tract After 1-^{14}C-MLHP Intubation

Organ	Per cent of dose, 2 hr	Per cent of dose, 24 hr
Stomach	34.8	35.2
Small intestine	15.8	6.8
Cecum	0.1	1.2
Large intestine	0.4	1.5
Total	54.3	44.7

using the benzene-methanol (99:1) system revealed the presence of both polar and nonpolar radioactive fatty acid methyl esters(Rf = 0.3 and 0.7, respectively). Fractionation and decarboxylation of the randomly labeled ^{14}C-nonpolar fatty acids showed that they were primarily monoenoic and dienoic. In contrast, decarboxylation of the polar fatty acid methyl ester (approximately 40% of the total TG radioactivity) showed that it was entirely carboxyl-labeled and consequently must have been formed directly from 1-^{14}C-MLHP. After silylation and rechromatography in the benzene-methanol system, the radioactive compound moved to Rf 0.9 opposite the silyl derivative of dimorphecolic acid methyl ester (9-hydroxy-10,12-*trans,trans*-octadecadienoate). Since MLHP does not form a silyl derivative under the conditions employed, it was concluded that the labeled metabolite was a hydroxy acid. However, since the mesenteric adipose tissue contained lymph as well as blood, it was not clear from these results whether the hydroxy acid was present in the adipose tissue or in these contaminants.

Distribution of Radioactivity in the Gastrointestinal Tract of Intact Rats

Table II shows the distribution of radioactivity in the gastrointestinal tract and its contents 2 and 24 hr after intubation. Approximately 46% of the intubated sample was absorbed in the first 2 hr and only 10% in the next 22 hr. The amount of sample remaining in the stomach after 2 and 24 hr was approximately the same (35%). In a related study (15), it was observed that following intubation of 1-^{14}C-methyl linoleate and 1-^{14}C-methyl hydroxy octadecadienoate these acids were almost completely eliminated from the gastrointestinal tract by the end of 24 hr. Apparently the fatty acid hydroperoxide was bound to the stomach epithelium, perhaps in the same way that lipoperoxide binds to protein to form ceroidal and lipofuscin pigments. This binding appears to be quite stable and may be reversed only through turnover and regeneration of new cells in the stomach lining.

TABLE III

Radioactivity Remaining in the Ester Form 2 and 24 Hr After 1-^{14}C-MLHP Intubation

Organ	2 hr, %	24 hr, %
Stomach	59	48
Small intestine	40	33
Cecum	---	7
Large intestine	32	7

In addition to the total radioactivity remaining in the gastrointestinal tract, the degree of hydrolysis of the esterified sample was determined. The lipid extracts were chromatographed on Silica Gel G using a chloroform-acetone-acetic acid (88:12:0.25) TLC system in which triglycerides and methyl esters of normal and hydroxy fatty acids and peroxides migrate to the solvent front. Free fatty acids and peroxides move to an Rf of 0.4. As a means of confirming their identity the free acids were methylated and rechromatographed.

The radioactivity in all gastrointestinal organs except the cecum was present in two peaks with Rf values of 0.4 and 1.0 for free and esterified acids, respectively. The cecum contained considerable amounts of origin material whose Rf was not altered by methylation. Table III gives the fraction of radioactivity present in the ester form 2 and 24 hr after 1-^{14}C-MLHP intubation. Approximately 41% of the peroxide present in the stomach at the end of 2 hr was in the free form and an additional 11% was hydrolyzed during the next 22 hr. The proportion of unhydrolyzed ester present in the small intestine was 40% and 33% at the end of the 2 and 24 hr experiments, respectively, despite the fact that less than half

TABLE IV

Distribution of Radioactivity Among Lymph Lipids After 1-^{14}C-MLHP Intubation

Band	Rf	Per cent[a]	Lipid class
I	0.00-0.20	7	Free fatty acids
II	0.25-0.55	31	Methyl polar fatty acids
III	0.75-1.00	57	Triglycerides

[a]Per cent of radioactivity on chromatogram (Silica Gel G; benzene-methanol, 99:1).

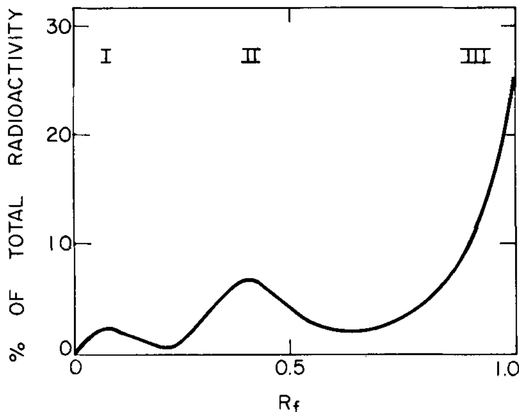

FIG. 3. Distribution of radioactivity after TLC (Silicic Gel G; benzene-methanol, 99:1) of lymph lipid 2 hr after intubation with 1-^{14}C-MLHP.

as much total radioactivity remained in this organ at the end of the 24 hr period. The results suggest that the rate of absorption of the peroxide or its degradation products was dependent on the rate of hydrolysis of the ester. Hydrolysis and absorption apparently proceeded at a rate which left a relatively uniform fraction of unhydrolyzed sample in the small intestine. It is also evident that the methyl ester of linoleic acid hydroperoxide is slowly hydrolyzed in the gut. Similar results have been reported for the ethyl ester of the hydroperoxide (4). The corresponding methyl hydroxy fatty acid, on the other hand, has been found to be completely hydrolyzed within 24 hr (15).

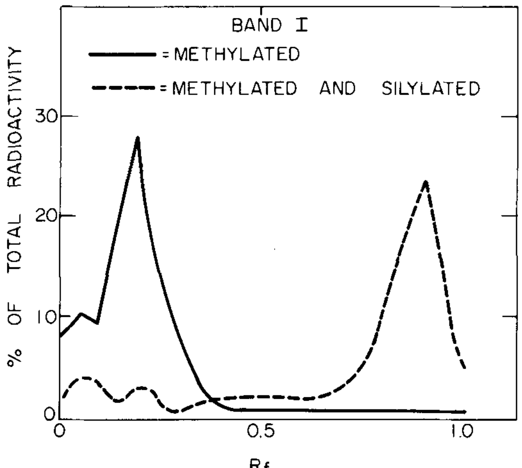

FIG. 4. Distribution of radioactivity after TLC (Silica Gel G; benzene-methanol, 99:1) of silylated and methylated band I material (Fig. 3) from lymph.

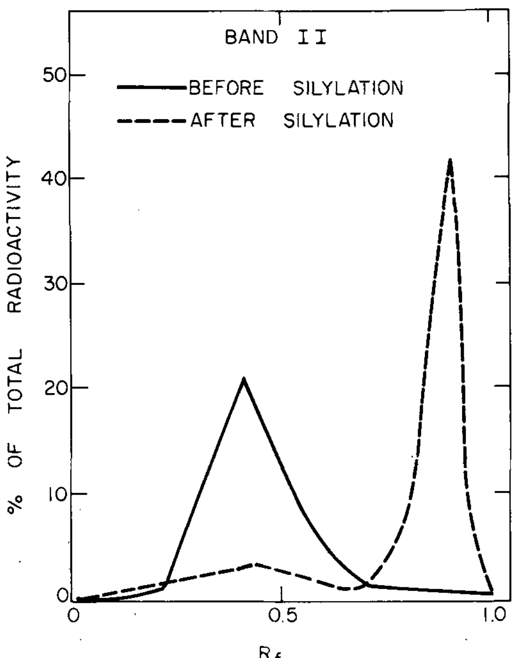

FIG. 5. Distribution of radioactivity after TLC (Silica Gel G; benzene-methanol, 99:1) before and after silylation of band II material (Fig. 3).

Characterization of Radioactivity in Lymph

Lymph was collected from three rats for analysis of ^{14}C-lipids present 2 hr after intubation with the peroxide. One to 2 ml of lymph were collected containing 1.1-1.5% of the dose. The amount of radioactivity found in the lymph indicated that a large portion of the radioactivity previously detected in the mesenteric adipose tissue was actually present in the lymphatic vessels of that tissue. Extraction of the lymph extract with water (16) removed only 4.3% of the activity. Treatment of the lipid extract with H_2SO_4-NaN_3 led to recovery of 92% of the label from the carboxyl carbon of the fatty acid components. This finding showed that the radioactive compound(s) present in the lymph were formed directly from 1-^{14}C-MLHP. When the lymph lipid was chromatographed using the benzene-methanol (99:1) TLC system, three distinct radioactive bands were observed on the chromatogram (Fig. 3). The distribution of radioactivity among the bands is listed in Table IV. The Rf of band I, containing 7% of the total radioactivity, was consistent with that of a free fatty acid, either polar or nonpolar; band II, containing 31% of the total activity, had the Rf characteristics of a polar methyl fatty acid; band III, which contained the remaining 57% of

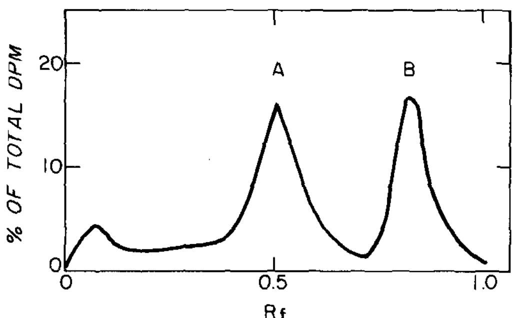

FIG. 6. Distribution of radioactivity after TLC (Silica Gel G; benzene-methanol, 99:1) of saponified and methylated band III material (Fig. 3).

the total radioactivity, was identified with triglyceride.

These labeled components were further characterized. Methylation and rechromatography of the band I material yielded results indicative of a methyl polar fatty acid (Fig. 4). Treatment of the methyl ester with silylating reagent followed by chromatography (Fig. 4) showed that the compound formed a silyl derivative. Consequently, it was concluded that the radioactivity present in band I was due to a 1-^{14}C-hydroxy fatty acid which arose by direct conversion of the intubated hydroperoxide.

Silylation and chromatography of the band II material revealed that this compound also formed a silyl derivative (Fig. 5). The activity in this band therefore was also attributed to a hydroxy fatty acid which, unlike the acid in band I, was present as the methyl ester. Reduction (17) with KBH_4 did not alter its TLC characteristics either before or after silylation, thereby eliminating the possibility that it was a peroxide. Hence at least 38% of the radioactivity in the lymph after 1-^{14}C-MLHP intubation was present in 1-^{14}C-hydroxy acids.

Chromatography of band III material after saponification and methylation showed that it was an equal mixture of two compounds (Fig. 6). For purposes of identification, the two radioactive components were designated compound A (Rf = 0.50) and compound B (Rf = 0.75).

The Rf for compound B was indicative of a methylated normal fatty acid. This compound failed to form a silyl derivative and was resistant to reduction with KBH_4. Decarboxylation showed that it was entirely carboxyl-labeled. TLC on Silica Gel G impregnated with 5% $AgNO_3$ using Skelly Solve F-diethyl ether (85:15) as solvent yielded an Rf corresponding to that of a trienoic fatty acid. This identifica-

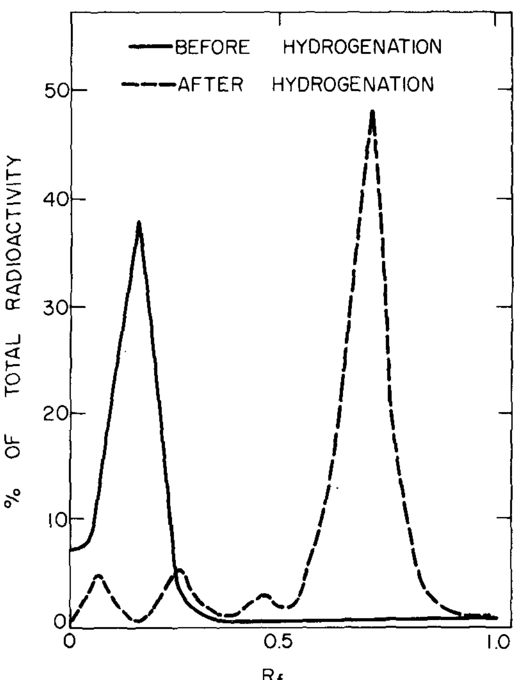

FIG. 7. Distribution of radioactivity after TLC (Silica Gel G impregnated with 5% $AgNO_3$; Skelly Solve F-diethyl ether, 85:15) of compound B (Fig. 6) before and after hydrogenation.

tion was confirmed by catalytic hydrogenation which converted the compound to a saturated fatty acid (Fig. 7).

The Rf value for compound A (Fig. 6) was intermediate between that of a normal fatty acid methyl ester and the ester of a polar acid such as a hydroxy or hydroperoxy fatty acid. This compound was reducible with KBH_4 and when chromatographed in the benzene-methanol system, the reduction product migrated to an Rf similar to that of compound B. TLC of compound A on Silica Gel G impregnated with 5% $AgNO_3$ using Skelly Solve F-diethyl ether (85:15) as solvent yielded an Rf suggestive of a trienoic fatty acid. However, in contrast to compound B, catalytic hydrogenation of compound A failed to yield a saturated fatty acid. Decarboxylation resulted in a complete loss of activity. The compound failed to form a silyl derivative. The identity of compound A remains unknown; it is a long chain oxy fatty acid in which the oxygen function is reducible but is not susceptible to silylation.

Hence band III, comprising 57% of the radioactivity in the lymph, was shown to consist of a triglyceride containing equal amounts of two radioactive compounds. One component (compound B) was identified as a 1-^{14}C-

TABLE V

Distribution of Radioactivity in Lymph Lipids 2 Hr After 1-^{14}C-MLHP Intubation

Lipid	Per cent of total DPM
1-^{14}C-free hydroxy fatty acid	7
1-^{14}C-methyl hydroxy fatty acid	31
Triglyceride	
1-^{14}C-polar fatty acid (Unidentified)	28
1-^{14}C-trienoic fatty acid	28

trienoic fatty acid, while the other was identified only as a 1-^{14}C-oxy fatty acid. The formation of a trienoic fatty acid from ingested MLHP is explainable in terms of a reduction-dehydration reaction sequence in which a hydroxy acid is an intermediate. These reactions apparently occur in the mucosa of the small intestine during absorption.

Table V summarizes the distribution of radioactivity found in the labeled compounds isolated from lymph following 1-^{14}C-MLHP intubation. 1-^{14}C-hydroxy fatty acids comprised 38% of the total radioactivity absorbed into the lymph. No radioactive hydroxy fatty acids appeared to be incorporated into the lymph triglycerides. The TG contained equal amounts of radioactivity in the form of trienoic fatty acid and a slightly polar oxy fatty acid of unknown identity. The existence of a 1-^{14}C-hydroxy fatty acid in the lymph after 1-^{14}C-MLHP intubation supports the conclusion that peroxides are reduced during the absorption process (2,4). The presence of a ^{14}C-trienoic fatty acid further indicates that the reduction product may undergo dehydration during absorption. The quantity of the unidentified ^{14}C-oxy fatty acid present in the TG indicates that it is of considerable importance as a metabolite of ingested fatty acid peroxides but the mechanism of its formation is unknown.

Although hydroxy fatty acids were found in the lymph after ingestion of methyl linoleate hydroperoxide, no hydroxy acids were deposited in the liver. This observation is consistent with the results of a study on the metabolism of ^{14}C-hydroxy octadecadienoic acid in the rat (15). Hydroxy fatty acids appear to be rapidly oxidized in the liver and only in long term feeding experiments is any appreciable amount found in the depot fat (15,18). Consequently, measurable amounts of hydroxy fatty acids may not occur in the tissues unless peroxides were ingested at a relatively high level over a prolonged period of time.

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Absorption and Metabolism of 1-^{14}C-Hydroxy Octadecadienoate in the Rat

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ABSTRACT

The metabolism of 1-^{14}C-9(13)-hydroxy octadecadienoic acid methyl ester (1-^{14}C-HAME) by the rat was investigated in vivo and in liver slices. A 1.5 mg dose of 1-^{14}C-HAME administered by stomach tube was efficiently hydrolyzed and absorbed from the intestinal tract. In comparison with 1-^{14}C-methyl linoleate (1-^{14}C-ML), 1-^{14}C-HAME was more extensively oxidized to $^{14}CO_2$ in vivo and in vitro. After 1-^{14}C-HAME administration as much as 50% of the radioactivity in the adipose tissue triglycerides was associated with ^{14}C-hydroxy fatty acids. The remaining activity was present in randomly labeled normal fatty acids. No evidence was obtained for the incorporation of ^{14}C-hydroxy acids into liver lipids; most of the radioactivity from 1-^{14}C-HAME in this organ was recovered in saturated and monoenoic fatty acids. About 10% of the radioactivity 24 hr after 1-^{14}C-HAME administration was associated with triglyceride trienoic acids, indicating that at least a portion of this acid was dehydrated in the liver. An unidentified polar acid was detected in the urine of the 1-^{14}C-HAME-treated animals.

INTRODUCTION

Interest in the metabolism of dienoic hydroxy acids stems from their presence in certain seed oils and from evidence that they may be formed in animal tissues by reduction of linoleic acid hydroperoxide. Previous experiments in this series (1) indicated that after oral administration of 1-^{14}C-methyl linoleate hydroperoxide (MLHP) to rats, a hydroxy fatty acid and a trienoic normal acid constituted major products of absorption in the lymph. Evidence also was obtained for the presence of small concentrations of hydroxy and trienoic acids in rabbit tissues after intravenous administration of labeled MLHP (2). Other investigators (3-5) have reported the presence of a glutathione peroxidase in liver which catalyzes the reduction of linoleic and linolenic hydroperoxides to their corresponding hydroxy acids. Both the 9- and 13-hydroperoxy isomers of the linoleic acid peroxide were reduced by the enzyme (5).

Most studies on the metabolism of hydroxy fatty acids have pertained to ricinoleic acid. This acid has been shown to be absorbed into the lymph system and deposited in depot fat but was not discernible in phospholipids (PL) or liver triglycerides (TG) (6-8). It appears to be less efficiently absorbed and more rapidly oxidized than normal fatty acids (7).

In the present experiments, the metabolism of 9(13)-hydroxy octadecadienoic acid by the rat was investigated.

EXPERIMENTAL PROCEDURES

1-^{14}C-Hydroxy Acid Synthesis

1-^{14}C-9(13)-Hydroxy octadecadienoic acid methyl ester (1-^{14}C-HAME) was prepared by reduction of 1-^{14}C-methyl linoleate hydroperoxide. (1-^{14}C-MLHP) with KBH_4. The preparation of the hydroperoxide has been described in a previous paper (2). Since the peroxide produced by this procedure is a mixture of 9- and 13-hydroxy *trans,trans* isomers, the reduction product is a mixture of the corresponding hydroxy esters: 1-^{14}C-9-hydroxy-*trans,trans*-10,12-octadecadienoate and 1-^{14}C-13-hydroxy-*trans,trans*-9,11-octadecadienoate. The former is synonymous with methyl dimorphecolate (9) which is a major constituent of *Dimorphotheca aurantiaca* seed oil. This acid was isolated from the oil of *aurantiaca* as a reference standard.

The purity of the labeled acid (1 μc/mg) was established by thin layer chromatography (TLC) on Silica Gel G (benzene-methanol, 99:1), chromatography on paraffin-coated paper (85% ethanol), gas liquid chromatography (GLC) and formation of a silyl derivative.

Animal Experiments

The experimental animals were adult male rats of the Sprague-Dawley strain weighing 435-555 g. For four days before use, they were fed a linoleate-free synthetic diet containing 4% triolein as the only source of fat. 1-^{14}C-HAME (1.5 mg) or 1-^{14}C-methyl linoleate (1-^{14}C-ML) (1 μc/mg) was dissolved in a

TABLE I

Distribution of Radioactivity in the Tissues and Excreta of the 1-^{14}C-HAME and 1-^{14}C-ML-Treated Rats (% of Dose)

Tissue and Excreta	Rat					
	1	2	3	4	5	6
^{14}C-Compound administered	HAME	HAME	HAME	ML	HAME	ML
Route of administration	ST[a]	ST	ST	ST	IP[b]	IP
Time after dose, hr	4	8	24	8	6	6
Tissues						
Liver	1.7	2.1	2.1	5.3	7.7	7.3
Lung	0[c]	0	.1	.2		
Heart	0	0	0	.2		
Brain	0	0	0	0		
Fat pads	.1	.1	.7	.5		
Kidney Adipose	.2	.3	.6	.6	1.9	4.0
Kidneys	.1	.1	.1	.2		
Blood[d]	1.8	2.1	1.8	7.2	5.5	4.0
GI tract contents						
Stomach	30.0	21.0	.2	12.1		
Small intestine	9.8	1.5	.1	11.2		
Large intestine and cecum	.1	.8	4.3	4.6		
Excreta						
Urine	0	.6	5.4	0	.2	0
Feces	0	0	0	0		
CO_2	8.0	27.5	40.9	10.9	13.8	8.8
Total	51.8	56.1	56.3	53.0	29.1	24.1

[a]Stomach tube.
[b]Intraperitoneal injection.
[c]<0.1%.
[d]Calculated on the basis of 6% of body weight.

minimum of ethanol, mixed with 0.5 ml of triolein and dried under a stream of nitrogen. Three rats were dosed with 1-^{14}C-HAME by stomach intubation and one with 1-^{14}C-ML. One additional rat was dosed intraperitoneally with similar amounts of each compound; for this purpose the esters were dissolved in 0.5 ml ethanol and emulsified with 0.5 ml of 0.9% NaCl containing 2 mg Tween 80/ml.

The rats were killed 4 to 24 hr after administration of the dose. Procedures for monitoring expired $^{14}CO_2$, extraction of the tissues and measurement of radioactivity were the same as those used previously (2).

Lipid Analysis

The lipids recovered from liver, epididymal fat pad and kidney adipose tissue were fractionated into major lipid classes by TLC on 1.5 mm layers of Silica Gel G. A solvent mixture consisting of $CHCl_3$, acetone and acetic acid (88:12:0.25) was used to resolve all classes except triglycerides (TG) and cholesterol esters (CE); these two components were separated using hexane, diethyl ether and acetic acid (70:30:0.25). The most extensively labeled fractions were saponified in 1 N ethanolic KOH at room temperature for 8 hr. Heat was avoided to preclude dehydration of hydroxy acids (9). Nonsaponifiable lipids were removed with diethyl ether after addition of 2 vol. of water. The saponifiable fraction was acidified to pH 1 with HCl and the fatty acids were extracted with ether. Water was removed with Na_2SO_4 and the solvent was evaporated under a stream of nitrogen. Methods used for the preparation and isolation of normal and hydroxy acid methyl esters have been described elsewhere (2).

In Vitro Experiments

Liver slices were used to compare the rates of oxidation of free 1-^{14}C-hydroxy acid and 1-^{14}C-linoleic acid and their rates of incorporation into liver lipids. The in vitro system employed was identical with that described by O'Hea and Leveille (10). Slices (135-196 mg) were prepared from the livers of stock rats.

TABLE II

Proportions of Free and Esterified Acids in the Contents of the Gastrointestinal Tract (% of Total Radioactivity)

	Rat			
	1	2	3	4[a]
Time after intubation (hr)	4	8	24	8
Stomach				
Free acid	15	14	b	3
Methyl ester	85	86		97
Small intestine				
Free acid	68	64		59
Methyl ester	32	36		41
Large intestine + cecum				
Free acid		85	99	30[c]
Methyl ester		15	1	13

[a]Intubated with 1-^{14}C-ML; all others with 1-^{14}C-HAME.

[b]Negligible radioactivity present.

[c]Remaining activity present in unidentified products.

After incubation, the tissues were extracted with $CHCl_3/CH_3OH$ (2:1) and analyzed for radioactivity.

RESULTS AND DISCUSSION

Distribution of Radioactivity

The distribution of radioactivity in the tissues and excreta of the experimental animals is summarized in Table I. The data for the stomach and small intestine contents indicate that after 1-^{14}C-HAME intubation nearly all the radioactivity traversed the gastrointestinal tract within 24 hr (rats 1-3). Labeling of the adipose tissues increased with time, but negligible activity was recovered from the lungs, heart, brain and feces. Liver, kidney and blood radioactivity remained relatively constant. Comparison of the data for rats 2 and 4 indicates that more radioactivity was deposited in the liver and adipose tissues during an 8 hr period after 1-^{14}C-ML intubation and less was oxidized to $^{14}CO_2$. A similar difference was observed 6 hr after intraperitoneal administration (rats 5 and 6). The faster rate of 1-^{14}C-HAME oxidation is also evident from the $^{14}CO_2$ expiration curves shown in Figure 1. A further difference in the metabolism of the ^{14}C-hydroxy and normal fatty acid esters was that significant amounts of radioactivity from 1-^{14}C-HAME were excreted in the urine.

Hydrolysis and Absorption of 1-^{14}C-HAME

The extracts of the gastrointestinal contents of rats 1-4 were analyzed for free and esterified ^{14}C-acids by TLC on Silica Gel G using benzene-methanol (99:1) (2). In this system free acids remain at the origin whereas the methyl esters of normal and hydroxy acids migrate to Rf 0.75 and 0.25, respectively. The identity of the free acids was confirmed by methylation and rechromatography. The proportions of free and esterified acids present in different segments of the tract at various time intervals are shown in Table II. The data show that there was a progressive increase in the proportion of free ^{14}C-hydroxy acid present as the intubated material moved through the gut and that hydrolysis went almost to completion. Comparison of the data for rats 2 and 4 (Tables I and II) indicates that there was little difference in the rates of hydrolysis and absorption of the normal and hydroxy acid esters. The apparent discrepancy between this finding and previous observations that ricinoleic acid and its esters are more slowly absorbed than normal fatty acids (6,8) may lie in the much smaller amounts of esters administered in the present study.

TABLE III

Comparison of 1-^{14}C-Hydroxy Octadecadienoic Acid and 1-^{14}C-Linoleic Acid Metabolism by Liver Slices[a]

	1-^{14}C-Hydroxy acid[b]			1-^{14}C-Linoleic acid[b]		
Per cent DPM	1	2	3	1	2	3
Oxidized to $^{14}CO_2$	7.0	12.6	13.4	1.1	2.1	3.2
Recovered from liver lipids	26.3	16.2	13.5	23.0	28.0	32.0
Metabolized	33.3	28.8	26.9	24.1	30.1	35.2
Metabolized/mg tissue (x 10^2)	18.9	17.4	16.4	14.8	19.5	20.9

[a]Averages of duplicate determinations, 0.2 μg of substrate (1 μc/mg) and 135 to 196 mg of tissue used per determination.

[b]Incubation time, hr.

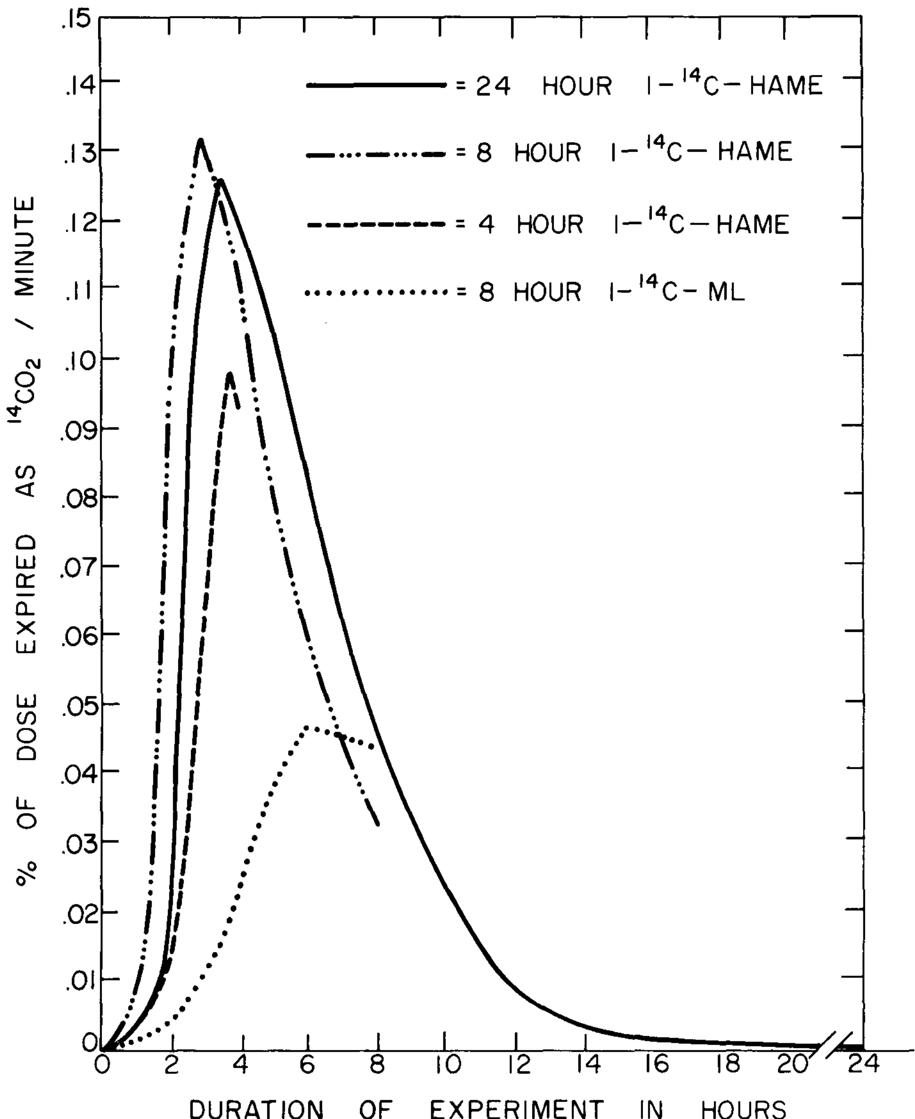

FIG. 1. $^{14}CO_2$ expiration curves for rats intubated with 1-^{14}C-HAME or 1-^{14}C-ML.

No additional ^{14}C-labeled compounds were detected in the gut contents except in the large intestine and cecum of the rat intubated with 1-^{14}C-ML. Most of the radioactivity in this organ was located in an unidentified polar fatty acid ester which migrated to an Rf of 0.15 in the benzene-methanol (99:1) system. This compound probably was a product of microbial oxidation of the linoleate ester.

^{14}C-Compounds in Liver Lipids

Most of the radioactivity (77-93%) in the liver lipids of both the 1-^{14}C-HAME and 1-^{14}C-ML-dosed animals was associated with the TG and PL. Separation of the normal and hydroxy fatty acid methyl esters revealed that this radioactivity was present exclusively in the esters of normal acids. No evidence was obtained for the presence of labeled hydroxy acids in the livers of the animals given 1-^{14}C-HAME. This finding is in agreement with the observation that ricinoleic acid is not deposited in liver TG or PL (11).

Fractionation of the normal fatty acid esters on $AgNO_3$-impregnated Silica Gel G (2) revealed that 78-90% of the activity in the livers of the 1-^{14}C-HAME-treated rats was associated with monoenoic and saturated acids. Hence

most of the radioactivity in the liver lipids of these animals arose through biosynthesis of normal acids from ^{14}C-acetate. In the case of rat 6, given 1-^{14}C-ML, 88.7% of the activity in the liver lipids was present in the dienoic acids.

In the liver of rat 3, killed 24 hr after intubation with 1-^{14}C-HAME, and of rat 5, killed 6 hr after 1-^{14}C-HAME injection, 11.2% and 10.0%, respectively, of the total radioactivity, chromatographed with the trienoic acids. These observations support the results of previous studies (1,2) which indicated that labeled hydroxy acids and trienes were formed from 1-^{14}C-methyl linoleate hydroperoxide in rabbit and rat tissues. The presence of a labeled triene suggests that one route of hydroxy dienoic acid metabolism in animals entails a dehydration reaction.

^{14}C-Compounds in Adipose Tissues

As anticipated, most of the radioactivity (70-90%) in the epididymal and kidney adipose tissues of the 1-^{14}C-HAME-treated rats was recovered in the TG fraction. In contrast to the liver lipids, however, a substantial portion of the activity in this fraction was found to reside in ^{14}C-hydroxy fatty acids. Ten per cent, 37% and 12% of the activity present in the epididymal fat pad TG of rats 1, 2 and 3 was associated with hydroxy acids. The corresponding values for the kidney adipose tissue TG were 42%, 41% and 13%. In rat 5, given 1-^{14}C-HAME by injection, 50% of kidney adipose tissue TG radioactivity was recovered in the hydroxy acid fraction. The remaining activity in the TG of these animals was present in normal fatty acids.

The observation that dietary dienoic hydroxy fatty acids are deposited in stored fat extends similar findings for saturated and monoenoic hydroxy acids (6,7,11). No clear evidence was obtained for the presence of labeled trienoic acids in adipose tissue (1).

Radioactivity in Urine

As shown in Table I, significant amounts of radioactivity were recovered in the urine 6 to 24 hr following 1-^{14}C-HAME administration. After acidification of the urine to pH 1 with HCl the activity was extractable with $CHCl_3$. When the acid-hydrolyzed material was chromatographed on Silica Gel G using 1% methanol in benzene as solvent, the labeled compound remained at the origin, suggesting that it was a free acid. Methylation increased its Rf value to 0.5. These observations indicate that the compound has a polarity intermediate between long chain hydroxy fatty acids and normal acids. Its presence in the urine after 1-^{14}C-HAME injection (rat 5) suggests that it is not a product of bacterial metabolism in the gut.

In Vitro Experiments

The results of the in vitro experiments are summarized in Table III. The data show that the oxidation of the ^{14}C-hydroxy acid was four to six times greater than that of ^{14}C-linoleic acid at all three incubation times. This observation is in agreement with the greater $^{14}CO_2$ production recorded in the in vivo experiments (Fig. 1). The amount of substrate activity recovered from the liver slices decreased with time in the case of the ^{14}C-hydroxy acid experiments, whereas it increased in the ^{14}C-linoleic acid experiments. These trends reflect the greater tendency of the tissue to combust the hydroxy acid substrate. Total ^{14}C-hydroxy acid radioactivity metabolized per milligram of tissue was relatively uniform over the 3 hr incubation period, while that from ^{14}C-linoleic acid tended to increase with time as a result of its limited conversion to $^{14}CO_2$.

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Plasma Free Fatty Acid Incorporation Into the Outer and Inner Myocardium of Unanesthetized Dogs[1,2]

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ABSTRACT

^{14}C-Palmitate (16:0) and ^{3}H-oleate (18:1) were infused into unanesthetized dogs for 90 min. Lipid and isotopic analyses were then performed on the left ventricular outer and inner walls. Average values in μmoles per gram wet weight tissue for both outer wall triglyceride (TG) (7.0 ± 2.6 (S.E.)) and phospholipid (PL) (17.3 ± 2.4) were higher then the inner wall TG (1.4 ± 0.3) and PL (12.0 ± 2.4). TG fatty acid distribution was similar in the outer and inner myocardium. Thus those factors regulating incorporation of various fatty acids may operate at comparable rates in both heart segments. The same observation and relationship was observed for PL, but the two classes had markedly different fatty acid spectra. The transmural gradients for these classes may be related to relatively hypoxic conditions in the inner wall. Uniform ^{14}C DPM concentrations were found in both TG and PL of the outer and inner myocardium. A similar distribution pattern was found for ^{3}H. This may indicate that an individual species of plasma free fatty acid (FFA) undergoes uniform initial incorporation into TG and PL despite the existence of transmural gradients and fatty acid distribution differences. The mean outer and inner wall TG and PL ^{3}H to ^{14}C DPM concentration ratio (range of 3.9 to 4.8) was similar to a calculated plasma ^{3}H - 18:1 to ^{14}C - 16:0 specific activity ratio of 4.23. This indicates that net incorporation of fatty acids into TG and PL over 90 min was proportional to their plasma FFA concentrations, rather than to endogenous tissue lipid concentrations. The lipid gradients and fatty acid spectrum differences observed may thus be caused by recycling and catabolic pathways rather than to direct control of plasma FFA entry into TG and PL.

INTRODUCTION

As early as 1914, Evans (3) demonstrated fat utilization by the heart. A series of papers published in the 1930's (4-8) strongly implicated fat as a significant source of energy for the myocardium. In 1941 (9) Cruickshank and Kosterlitz published evidence indicating the uptake of plasma free fatty acids (FFA) and their utilization by the heart muscle. The studies of Dole (10), Gordon and Cherkes (11) and Frederickson and Gordon (12) showed the myocardium to incorporate plasma FFA into its lipid components. Subsequently, extensive investigations (13) have been conducted to resolve the role that FFA plays as a source for both energy and endogenous lipids for the heart. Most of these studies have utilized isolated perfused heart preparations, arteriovenous difference measurements in anesthetized animals or in situ perfusions of hearts. All of these preparations have necessarily involved the hazards of an altered physiological system. The most desirable method is one in which the animal is intact and unanesthetized.

Some studies have indicated the possible existence of metabolic gradients across the heart wall. Kirk and Honig (14) reported the existence of a blood supply gradient decreasing towards the endocardium that may cause a concomitant decrease in oxidative metabolism. Such a suggestion is supported by Jedeikin (15) who reported a greater concentration of glycogen and phosphorylase b in the inner wall.

There is a paucity of knowledge concerning the fate of incorporated plasma FFA into the myocardium of unanesthetized animals. To provide more information about this area, the uptake and distribution of labeled plasma free palmitate and oleate into myocardial lipids of unanesthetized dogs were studied. As part of these experiments, the outer and inner halves of left ventricular walls were examined and compared to ascertain if any transmural differences

[1]The research presented here was performed in partial fulfillment of the requirements for the M.Sc. degree in Physiology at Hahnemann Medical College by J.S. Steinberg.

[2]Two abstracts related to this study were published previously (1,2).

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TABLE I

Lipid Composition of Canine Left Outer and Inner Myocardium

Dog	Tissue wet wt., g	Lipid % of wet wt.	Triglyceride μmoles/g wet wt.	Phospholipid μmoles/g wet wt.
46. LOV[a]	60.0	3.2	13.7	23.8
LIV[b]	53.0	2.8	1.1	13.3
47. LOV	42.5	3.0	12.7	15.5
LIV	31.5	1.9	1.8	7.9
48. LOV	78.2	3.8	1.4	12.8
LIV	67.0	2.6	0.6	6.1
50. LOV	32.5	2.3	3.6	12.4
LIV	24.0	2.0	2.5	12.6
51. LOV	41.0	2.0	3.5	22.0
LIV	24.0	2.0	0.8	20.2
Mean ± S.E.M. LOV	50.8 ± 8.2	2.9 ± 0.3	7.0 ± 2.6	17.3 ± 2.4
LIV	39.9 ± 8.6	2.3 ± 0.2	1.4 ± 0.3	12.0 ± 2.4

[a]LOV, left outer ventricle.
[b]LIV, left inner ventricle.

in lipids were present. The results of these investigations are reported in the present communication.

METHODS

The research plan consisted of four phases: (a) the infusion of ^{14}C-labeled palmitic and ^{3}H-labeled oleic acids into unanesthetized dogs; (b) myocardial tissue removal and lipid extraction; (c) chemical analysis of lipid classes; and (d) radioactive analysis of lipid classes. In dogs 49 and 50, phase (a), and consequently, phase (d), were omitted. These dogs thus served as controls when considering the effects of radioactive infusion on lipid content.

Male mongrel dogs weighing from 20 to 27 kg, and fed ad lib. on Purina Laboratory Dog Chow, were used. The day prior to infusion a dog was anesthetized with sodium pentothal and a catheter was inserted into a leg vein. The dog was then fasted overnight.

The following day the dog was placed in a resting position and infused with 20 ml of a FFA albumin mixture over a period of 90 min (infusion rate of 0.2 ml/min). The infusion mixture, prepared as described previously (19), consisted of 11.4 μc/ml of 1-^{14}C-palmitate (Tracerlab) and 84.4 μc/ml of 9,10-^{3}H-oleate (Tracerlab) bound to a 20% solution of human serum albumin (Fraction V). The molar ratio of fatty acid added to albumin was 0.8 for palmitate and 0.5 for oleate.

Ten minutes before the end of the infusion the dog was anesthetized with phenobarbital, 30 mg/kg, and exsanguinated at the end of infusion. The heart was removed, accessory tissues trimmed off and the left myocardium cut into inner and outer portions by estimating the half-thickness level and slicing with a scapel. These two tissue segments were weighed (see Table I) and their lipids extracted with 20 vol. of chloroform-methanol (2:1) according to Folch et al. (16). A 0.1% solution of santoquin (1,2-dihydro 6-ethoxy-2,2,4-trimethyl quinoline: K and K Labs) was added as an antioxidant (17) to the lipid extract which was stored at -20 C.

Duplicate lipid class analyses were then performed on each lipid extract. Classes were separated on Silica Gel G coated thin layer plates, using a solvent system of petroleum ether-acetone (85:15) as described by Christophe and Matthijs (18).

Methyl esters of fatty acids and dimethylacetal derivatives of fatty aldehydes from the individual lipid classes were prepared. They were separated on a 10% diethylene glycol succinate polymer column, identified by comparison with retention distances of standards obtained from Applied Science Laboratories (State College, Pa.), and quantitated by means of a flame ionization detector in a Beckman GC4 gas chromatograph (19). Micromoles of triglyceride and phospholipid fatty acids were calculated by the use of heptadecanoic acid as an internal standard (19).

Radioactive analyses of lipid classes were accomplished as follows. Thin layer bands containing each lipid class were obtained as described above. These were scraped into glass vials containing Bray's solution (20) and their radioactivity determined. ^{14}C and ^{3}H were measured in a Packard Tri-Carb Liquid Scintillation Counter; DPMs were calculated from

TABLE II

Triglyceride and Phospholipid Fatty Acid Distribution in Mole Per Cent of Dog Outer and Inner Myocardium (Mean ± S.E.M.)

Myocardium	Fatty acid	14:A[a]	14:0	14:1	16:A	16:0	16:1	18:A	18:0	18:1	18:2	18:3	20:0	20:3	20:4
LOV[b]	TG	--	2.7 ± 0.4[c]	--	--	23.4 ± 0.8	5.0 ± 0.4	0.2 ± 0.2	9.0 ± 0.2	44.9 ± 1.5	13.3 ± 1.7	0.7 ± 0.3	0.6 ± 0.5	--	0.2 ± 0.2
	PL	1.5 ± 0.3	1.1 ± 0.2	0.1 ± 0.1	7.6 ± 1.1	8.9 ± 0.7	0.6 ± 0.2	2.8 ± 0.4	17.0 ± 0.9	14.3 ± 0.9	21.8 ± 1.6	0.2 ± 0.1	0.1 ± 0.1	0.6 ± 0.2	22.3 ± 1.1
LIV	TG	--	0.8 ± 0.5	--	--	22.4 ± 1.8	5.0 ± 0.4	--	10.6 ± 1.2	45.0 ± 0.7	17.0 ± 1.1	0.6 ± 0.2	0.2 ± 0.2	--	0.3 ± 0.3
	PL	1.4 ± 0.4	1.0 ± 0.3	--	8.0 ± 0.5	10.1 ± 1.0	0.5 ± 0.1	3.2 ± 0.6	16.6 ± 0.5	14.4 ± 1.0	21.7 ± 0.7	0.2 ± 0.1	0.1 ± 0.0	0.5 ± 0.1	22.6 ± 0.8

[a]Number of carbon atoms:number of double bonds; "A" indicates the aldehyde form of the fatty acid.

[b]Abbreviations: LOV, left outer ventricle; LIV, left inner ventricle; TG, triglyceride; PL, phospholipid.

[c]Mean ± S.E.M. for five dogs.

efficiency, overlap and correction factors. From the aliquot size used and the original stock of lipid extract, the DPMs per gram wet weight of tissue for each isotope in the various lipid classes were calculated. Specific activities of individual fatty acids were computed from the gas chromatography and liquid scintillation data. A detailed presentation of the methodology used may be found in a report by Gold (19).

RESULTS

The wet weight, per cent lipid composition, total phospholipid and total triglyceride composition of the left ventricles, outer and inner segments, are shown in Table I. In four of the five dogs studied, the outer walls had more total lipid than did the inner walls. The overall range was 1.9% to 3.8% lipid in the tissues. This is somewhat similar to the total lipid composition of beef ventricle of 2.7% to 3.7% as reported by Das and Rouser (21).

The triglyceride levels were consistently lower in the inner walls of these dogs. There were however considerable fluctuations in the outer wall concentrations. Particular care had been taken to trim off excess adipose tissue, so that this factor probably did not cause the variations. Age differences or other unknown sources might account for the values observed.

Phospholipid accounted for most of the fatty acid-containing lipids in these hearts. In three dogs it was twice as concentrated in the outer walls as in the inner walls. However, two other dogs did not have marked phospholipid differences. Again, the reasons for the concentration fluctuations within the outer or inner walls are not immediately evident (see Discussion). The data do not indicate that the infusions, per se, caused fluctuations. Of the other lipid classes separated cholesterol ester, diglyceride, FFA and monoglyceride were present in only trace amounts, i.e., less than 2%.

Table II contains average values with standard errors for the fatty acid distribution of triglyceride and phospholipid in the outer and inner myocardial wall. A comparison of the outer and inner segments indicated that except for myristic acid of triglyceride no difference existed in fatty acid composition with respect to triglyceride or phospholipid transmurally. A similar observation could be made between animals. However, obvious differences existed between triglyceride and phospholipid fatty acid composition. The major triglyceride fatty acids in decreasing concentrations were 18:1, 16:0, 18:2 and 18:0 (number of carbon atoms: number of double bonds). The major phospho-

lipid fatty acids in decreasing order were 20:4, 18:2, 18:0, 18:1 and 16:0.

Average values and standard errors for the radioactivity distribution among the lipid classes is shown in Table III. Most of the radioactivity (> 90%) was found in phospholipid and triglyceride. The distribution of ^{14}C and ^{3}H was similar in both triglyceride and phospholipid in both the inner and outer walls. The micromolar and DPM concentrations per gram wet weight for palmitic and oleic acids and ^{14}C and ^{3}H found in outer and inner myocardial tissues are given in Table IV.

Specific activities of individual fatty acids were calculated for triglyceride and phospholipid and are listed in Table V. In each animal, the triglyceride of the inner wall had a higher specific activity for both palmitate and oleate than did the outer wall. For phospholipid, this same directional difference existed except for the palmitate of one dog. This general trend may be noted from the mean values. The ratio of ^{3}H to ^{14}C DPM concentrations for triglyceride and phospholipid were similar in the outer and inner walls. This pattern holds for the specific activity ratios also.

DISCUSSION

Due to the limited number of animals used, interpretations of the data are best made with caution. However, this group of animals demonstrated a larger amount of triglyceride in the outer wall of the left ventricle as opposed to the inner portion (Table I). Paired analysis of the outer and inner wall data by the t-test indicated that at a P of 0.05 there was a significant difference between the triglyceride content of the outer and inner wall. Using a one-tailed test with four degrees of freedom, the paired triglyceride data gave a value of 2.22, versus the critical t value of 2.13. A similar analysis indicated that a statistically significant phospholipid gradient existed, giving a t value of 2.69. It is still of interest to note that one dog (50) did not have a phospholipid gradient. Total lipid did not have a significant gradient as determined by this test. Possibly cholesterol, which was not measured, accounted for this.

That the inner wall had decreased amounts of lipids may be due to the interaction of a number of factors. Kirk and Honig (14) observed a decreased blood flow in the inner wall and Jedeikin (15) measured more glycogen and phosphorylase b in the inner than in the outer wall. These results indicate the possibility of decreased oxygen availability in the inner wall and of a concomitant increase in anaerobic metabolism. Such factors may contribute to the

TABLE III

Per Cent Distribution of Radioactivity in Lipid Classes of the Left Outer and Inner Myocardium[a]

Myocardium	Phospholipid		Triglyceride		Diglyceride		Free fatty acid		Cholesterol		Cholesterol ester		Monoglyceride	
	^{14}C[b]	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
LOV[c]	48.1 ± 3.0	46.6 ± 5.1	45.3 ± 5.5	49.2 ± 6.4	0.8 ± 0.4	0.3	2.2	1.3	1.7 ± 0.4	1.1 ± 0.2	1.6 ± 0.8	1.2 ± 0.5	---	---
LIV	50.9 ± 2.3	46.7 ± 4.8	44.7 ± 1.9	50.1 ± 4.4	0.7 ± 0.4	0.4	---	---	1.6 ± 0.2	1.3 ± 0.2	2.2 ± 1.1	1.5 ± 0.8	---	---

[a]Mean ± S.E.M. for three dogs.

[b]^{14}C (1-^{14}C-palmitate), ^{3}H (oleate-9,10-^{3}H).

[c]LOV, left outer ventricle; LIV, left inner ventricle.

TABLE IV

Fatty Acid and DPM Concentrations in Canine Left Outer and Inner Myocardial Triglyceride and Phospholipid

		Triglyceride fatty acid µmoles/g[a]		Phospholipid fatty acids µmoles/g		Triglyceride DPM/g[b]		Phospholipid DPM/g	
Dog No.	Myocardium	16:0[c]	18:1	16:0	18:1	^{14}C	^{3}H	^{14}C	^{3}H
46	LOV[d]	10.46	19.8	4.85	7.47	2,077	9,460	2,752	11,240
	LIV	.92	1.45	3.57	3.96	2,066	11,175	2,358	14,210
47	LOV	8.87	15.60	2.90	4.88	2,550	9,690	3,180	11,885
	LIV	1.05	2.53	1.84	2.69	1,599	6,291	2,095	6,052
48	LOV	0.99	1.87	2.17	3.40	1,655	7,660	1,256	4,477
	LIV	0.43	0.85	1.65	3.08	2,176	8,935	2,119	5,885
Avg.	LOV	6.77	12.42	3.30	5.25	2,094	8,937	2,396	9,201
	LIV	0.80	1.61	2.35	3.24	1,847	8,800	2,191	8,716

[a]Micromoles per gram wet weight tissue.

[b]DPMs per gram wet weight tissue.

[c]Number of carbon atoms:number of double bonds.

[d]LOV, left outer ventricle; LIV, left inner ventricle.

maintenance of a reduced triglyceride level in the inner wall, since fatty acids depend upon β oxidation in order to be utilized for energy.

Several interesting observations may be made when the isotopic data are considered in the following manner. As shown in Table IV the average DPM concentrations (expressed as DPM/g wet weight tissue) were similar for a particular isotope. This relationship holds for the outer versus the inner wall where lipid gradients exist within a class. It also holds for triglyceride versus phospholipid within a tissue, even though the two classes are present in different concentrations and have different fatty acid distributions. An explanation that could account for these results in that net incorporation of an individual plasma free fatty acid over 90 min into these lipid classes is similar and therefore independent of the endogenous lipid gradients and fatty acid spectrum differences.

A comparison of tissue DPM ratios with plasma specific activity ratios indicates a correlation between plasma FFA levels and net fatty acid incorporation. For example, based upon the ^{3}H to ^{14}C infusion ratio of 7.4 and an assumed plasma oleate to palmitate concentration ratio of 1.75 (22), the expected oleate to palmitate specific activity ratio utilized in these experiments would be 4.23. If net incorporation of oleate to palmitate into tissue lipids was proportional to their plasma levels then their tissue DPM ratios would also be 4.23. As indicated in Table V, the average tissue oleate to palmitate DPM ratio was, indeed, very close to this value, exhibiting a ratio of 3.8 to 4.6. Transmural differences were minimal. This supports the concept that initial incorporation of individual plasma free fatty acids into phospholipid and triglyceride is proportional to fatty acid distribution in plasma.

The origins of triglyceride, phospholipid and fatty acid concentration levels observed (see Tables I and IV) can be considered further. Table II shows that fatty acid per cent distribution in either triglyceride or phospholipid was the same in the outer as well as the inner wall. Thus the fatty acid differences measured were apparently due to control of total lipid class concentration rather than a preferential accumulation of a particular fatty acid or acids into the outer wall. This observation also implies that the enzymes controlling fatty acid distribution in triglyceride and phospholipid operated at proportional rates in both wall segments, despite the lipid class gradients.

The lipid concentration differences may be partly explained as follows. It can be assumed that within the short time of 90 min plasma FFA incorporation primarily accounted for the new lipids formed. As concluded above, initial incorporation of fatty acids was proportional to their plasma levels and independent of endogenous fatty acid levels within triglyceride or phospholipid, and was therefore uniform transmurally and between classes for a particular fatty acid. Apparently, therefore, a time period of greater than 90 min was required for the radioactivity to be redistributed and to reach an equilibrium comparable to the lipid levels observed. One may then conclude that the myocardial lipid gradients as well as fatty acid distribution differences were formed over an

TABLE V

Fatty Acid Specific Activities and Ratios of Canine Left Outer and Inner Myocardial Triglyceride and Phospholipid

Dog No.	Myocardium	Triglyceride fatty acid specific activities		Phospholipid fatty acid specific activities		Triglyceride		Phospholipid	
		^{14}C DPM[a] / µmole Palmitate	^{3}H DPM / µmole Oleate	^{14}C DPM / µmole Palmitate	^{3}H DPM / µmole Oleate	^{3}H DPM / ^{14}C DPM	18:1 SA[b] / 16:0 SA	^{3}H DPM / ^{14}C DPM	18:1 SA / 16:0 SA
46	LOV	198	478	567	1,506	4.6	2.4	4.1	2.6
	LIV	2,251	7,717	660	3,593	5.6	3.4	5.9	5.6
47	LOV	287	621	1,098	2,436	3.8	2.2	3.7	2.2
	LIV	1,521	2,487	1,141	2,252	4.0	1.6	2.9	2.0
48	LOV	1,668	4,107	580	1,316	4.6	2.5	3.6	2.3
	LIV	5,103	10,494	1,288	1,912	4.2	2.0	2.8	1.5
Avg.	LOV	718	1,735	748	1,753	4.3	2.4	3.8	2.4
	LIV	2,958	6,899	1,030	2,586	4.6	2.3	3.9	3.0

[a]DPMs per gram wet weight tissue divided by micromoles fatty acid per gram wet weight tissue.
[b]SA, specific activity.

extended length of time. They may be due primarily to the regulation of catabolic or fatty acid recycling systems, or both, rather than to direct control of plasma FFA entry into triglyceride and phospholipid. A possible example is the lyso-phospholipid pathway.

Finally, it might be added that since total myocardial tissue consists of a variety of triglyceride and phospholipid containing cellular and subcellular particles, it is difficult to assess the incorporation of a fatty acid into individual cell types or organelles from the analysis of a total outer or inner myocardial lipid extract. The present findings represent the net results of the actions of a number of fatty acid pools and pathways.

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Partial Purification and Conversion of the Particulate-Bound Diglyceride Kinase of *Escherichia coli* to a Water Soluble, Detergent Free State

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ABSTRACT

Alkali (pH 11.5) treatment of a washed, ammonium sulfate-precipitated residue derived from a triton-X-100 extract of a 30,000 X *g* particulate diglyceride kinase preparation of *E. coli* converts much of the enzyme to a 100,000 X *g* soluble, detergent free state. This procedure improves the enzyme as an analytical tool for the structural analysis of triglycerides, and may be applicable for the solubilization of the many other glyceride- and phosphoglyceride-forming enzymes which have all resisted further purification because of insoluble, particulate properties.

INTRODUCTION

With probably only one exception (1), glyceride- and phosphoglyceride-synthesizing enzymes are always isolated from broken cell preparations of various forms of life as particulate-bound, water insoluble entities. These properties have been a major barrier to the further purification of these enzymes. While studying ways in which this obstacle might be overcome we discovered that the phosphatidic acid-forming enzyme, the diglyceride kinase of *Escherichia coli* (2), could be converted from a 30,000 X *g* particulate-bound state to a detergent free, 100,000 X *g* water soluble form by treatment with alkali. The steps leading to this conversion also yielded a 15-to 30 fold purification of the enzyme and aided in the removal of endogenous glyceride type lipids from the diglyceride kinase. The further purification of this enzyme, which is stereospecific for 1,2-diglycerides (3,4) and which has been used in the structural analysis of triglycerides (3), should improve it as an analytical tool.

EXPERIMENTAL PROCEDURES AND RESULTS

Partial Purification and Solubilization

A washed 30,000 X *g* particulate fraction obtained from 5.7 g of whole cells of *E. coli* grown to mid log phase (purchased from General Biochemicals) was prepared according to the procedure of Pieringer and Kunnes (2). (Each step is presented sequentially in Table I.) This particulate pellet was resuspended in 20 ml of 0.1% cysteine hydrochloride and 0.01 M dibasic sodium phosphate (final pH of solution was 7) and lyophilized for 15 hr. The lyophilized material was then mixed for 10 min at room temperature with a 1% aqueous solution of triton-X-100 (isooctyl phenoxypolyethoxy ethanol from Rohm and Haas) in a ratio of 1 ml of solution to 10 mg of lyophilized preparation. This mixture was centrifuged at 30,000 X *g* for 30 min at 4 C. The pellet from this centrifugation was resuspended in 1.0% triton-X-100 in a volume equal to the volume of the suspension before centrifuging (Table I, Fraction 4). To the 30,000 X *g* supernatant (triton-X-100 extract) (Table I, Fraction 5), ammonium sulfate in a concentration of 0.058 g/ml of extract (approximately 10% of saturation) was added with mixing at 4 C. The mixture was immediately centrifuged at 30,000 X *g* for 30 min at 4 C. The residue from this step was resuspended in 1 ml cold distilled water (1 ml water per 20 ml of original triton-X-100 suspension). To the supernatant from the above centrifugation, 0.058 g of ammonium sulfate per milliliter was again added to produce a concentration of 0.116 g/ml (approximately 20% of saturation). This mixture was centrifuged at 30,000 X *g* and the resultant residue resuspended in 1 ml water.

The concentration of ammonium sulfate in the supernatant from the above step was increased to 0.174 g/ml (approximately 30% of saturation) and the mixture was then recentrifuged at 30,000 X *g* for 30 min. This centrifugation produced an insoluble residue that now floated or layered on top of a relatively clear solution. The solution was carefully removed with a Pasteur pipette leaving the residue, which adhered to the wall of the tube. This residue was resuspended in distilled water, mixed and centrifuged at 30,000 X *g*. The washed residue, which no longer floated in the absence of salt and detergent, was resuspended in pure water, in a volume of 1 ml water per 20 ml of original triton-X-100 solution (Table I, Fraction 8).

TABLE I

Fractionation of Diglyceride Kinase Activity

Fraction	Protein conc., mg/ml	Total protein, mg	Specific activity, nmoles formed per mg protein per hr	Total activity, nmoles per total protein per hr
1. Disrupted whole cells	24.39	770.00	32.8	25280
2. Washed 30,000 x *g* pellet	9.28	181.0	154.8	28000
3. Lyoph 30,000 x *g* pellet in T-X-100 before centrifuging	4.77	95.4	562.8	53600
4. Pellet from 30,000 x *g* Spin (T-X-100)	3.60	71.3	369.2	26700
5. Super from 30,000 x *g* spin (triton-X-100)	0.86	17.0	1002.0	17050
6. 10% $(NH_4)_2SO_4$ pellet	20.736	0.736	87.2	64
7. 20% $(NH_4)_2SO_4$ pellet	0.861	0.861	69.7	60
8. 30% $(NH_4)_2SO_4$ pellet	3.41	8.52	495.5	4082
9. 30% $(NH_4)_2SO_4$ super	0.465	8.97	2.6	23
10. Base-treated 30% pellet in suspension[a]	2.37	7.75	64.2	497
11. 100,000 x *g* pellet	0.493	0.493	3.8	2
12. 100,000 x *g* super	2.13	6.65	120.0	800
13. First 45 ml from Biogel 200 col.	0.1	4.04	10.0	40.0
14. First 25 ml from .02 M Tris-HCl eluted from DEAE cellulose col.	2.7	1.08	32.6	35.2

[a]Washed free of detergent.

Treatment with mild alkali was then carried out on this suspension by the sequential addition of 0.05 ml of 2 N potassium or sodium hydroxide with 2 min mixing on a magnetic stirrer between each addition at 4 C until a pH of 11.5 was reached. The solution was stirred and kept at this pH and at 4 C for 30 min. The pH was then returned to 7 by the serial addition of 0.05 ml aliquots of 2 N acetic acid (Table I, Fraction 10). Centrifugation of this base-treated, neutralized suspension was then carried out at 100,000 X *g* for 30 min. The residue from this step was resuspended in a small volume of water (Table I, Fraction 11). The activity of the diglyceride kinase in each fraction was measured as described by Pieringer and Kunnes (2) and is shown in Table I. Protein was determined by the method of Lowry et al. (5).

Supernatant (100,000 x g) Enzymes as a Soluble Protein

Although the instability of the enzyme appeared to increase at each point beyond the triton-x-100 extraction step (Table I, Fraction 5) several further procedures were attempted to determine the workability of the 100,000 x *g* enzyme (Table I, Fraction 12) as a soluble entity. A portion of the 100,000 x *g* supernatant was placed on a Biogel 200 column, with dimensions of 1.5 x 16 cm and eluted with 250 ml of 0.02 M Tris-HCl buffer, pH 7.6. Five milliliter fractions from this elution were collected at 4 C. A second portion of the 100,000 x *g* supernatant was placed on a DEAE cellulose column 1.6 x 11 cm prepared in 0.02 M Tris-HCl buffer pH 7.6 and serially eluted with 100 ml each of the following solutions: 0.02 M Tris-HCl pH 7.6; 0.05 M Tris-HCl pH 7.6; and 0.075 M Tris-HCl pH 7.6. Five milliliter fractions were collected at 4 C.

Fractions eluted from the DEAE cellulose and Biogel columns were tested qualitatively for protein by spotting 0.01 ml of each fraction on Whatman No. 1 paper and staining with 0.002% Nigrosin in 2% acetic acid. Protein was detected by this method in the first 45 ml (tubes 1 through 9) eluted from the Biogel 200 column and in the final 100 ml (tubes 30 through 50). Protein was detected in the first 25 ml (tubes 1 to 5 eluted from the DEAE cellulose column by 0.02 M Tris-HCl, the first 10 ml eluted by 0.05 M Tris-HCl, and the first 10 ml eluted by 0.075 M Tris-HCl. The fractions containing protein were lyophilized and then taken up in a small volume of water. Diglyceride kinase activity was found only in the first 45 ml from the Biogel 200 column, and in the first 25 ml of 0.02 M Tris-HCl fraction of the DEAE cellulose column (Table I). These results indicate that the 100,000 X *g* supernatant enzyme can be handled as a water soluble substance.

Furthermore the 100,000 X *g* soluble en-

zyme was amenable to electrophoresis on polyacrylamide gels. In a typical experiment 60 μg of protein of the 100,000 X *g* supernatant were found to move 0.3 cm toward the anode in a Tris-HCl buffer of pH 9.5 in 2 hr at 5 mamps per tube; whereas four bands of protein from bovine serum albumin (50 μg) moved 1.25, 1.4, 2 and 3 cm under the same conditions. Only one band of the 100,000 X *g* supernatant material was detected using a buffalo black stain for protein.

The fact that the enzyme is eluted early from the columns and does not move rapidly on polyacrylamide gels may indicate that the 100,000 X *g* soluble enzyme is a protein of relatively high molecular weight.

Indication of Removal of Endogenous Glyceride-Containing Lipids

The purification procedure when taken through the alkali treatment step appears to assure the removal of endogenous glycerides of which diglyceride and phosphatidic acid interfere slightly by increasing the blank value of the assay for triglyceride structure (3). This removal is demonstrated in the following experiment. Phosphatidic acid, 171,000 CPM, labeled with ^{14}C in the glycerol moiety was added in a 5 μl aliquot of chloroform to a dry, lyophilized 30,000 X *g* particulate fraction of *E. coli*. After evaporating the 5 μl of solvent, the enzyme was subjected to the previously described procedure up to and including the alkali treatment step. The 100,000 X *g* supernatant was extracted with chloroform and methanol. The chloroform extract, after having been washed with water, contained no radioactivity. Thus the ^{14}C-phosphatidic acid did not survive the treatment and did not appear in the 100,000 X *g* water soluble enzyme preparation. Although the ^{14}C-phosphatidic acid used here can not be exactly equated with endogenous phospholipid, it is unlikely that an intimate association with protein would offer much protection at pH 11.5 to fatty acylglycerides. The fatty acid ester bonds of glycerides of all types are extremely labile to alkali (6-8).

DISCUSSION

To our knowledge, this is the first demonstration of the conversion of a glyceride- or phosphoglyceride-synthesizing enzyme from a particulate to a truly soluble state. However, alkali has been used in the past to successfully solubilize particulate proteins such as cytochrome oxidase (9), and certain cell membranes (10). This technique may be applicable for the solubilization of one or more of the many other particulate, lipid-forming enzymes found in nature. The greatest fault of the procedure, which we have not as yet circumvented (even by the addition of a crude lipid extract of *E. coli* to the enzyme) is the reduction of the activity of the diglyceride kinase beyond the triton-X-100 extraction step. However, this difficulty may not be encountered with other lipid-forming, insoluble enzymes.

For use of the diglyceride kinase as an analytical tool, the triton-X-100 extraction step, which produces a 30-fold increase in specific activity, may have the greatest applicability. However, the soluble enzyme does offer the advantage of being free of interfering lipid.

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Conjugation of Polyunsaturated Acids[1,2]

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ABSTRACT

The isomerization reaction of methyl linoleate and methyl linolenate with potassium *t*-butoxide has been investigated. The compositions of the reaction products formed at three temperatures have been determined and the relationships between these analyses and observed differences in absorptivities by UV spectrometry are discussed. Conclusions concerning the reaction mechanisms are based on compositional analysis and results of experiments using radioactive or stable isotope labeled reagent. Double bonds in molecules which are not conjugated during the reaction retain the original *cis* configuration. The double bond in the Δ12 position is the most susceptible to positional isomerization to form the conjugated system. With the diene, this selectivity is small, while with the triene, the shifting of the Δ12 bond is the preponderant initial reaction. Isotopic experiments yielded direct evidence for the postulated carbanion mechanism of reaction. An activated methylene group is generally required for the formation of the carbanion. While the UV spectra of the reaction products formed from methyl linolenate at 140 C showed no peak in the diene region, 34% conjugated diene-triene was present. The intact conjugated systems can migrate when the reaction is sufficiently energentic to produce conjugated trienes with double bonds other than the 10, 12, 14 system. The conjugation of triene is a stepwise reaction through the conjugated diene-triene.

INTRODUCTION

Alkali isomerization of polyunsaturated acids is an important step in the procedure for spectrophotometric estimation of these acids in fats and oils (1). Davenport et al. (2) determined that solutions of potassium *t*-butoxide in *t*-butanol, a strong base, readily produce conjugation in diene and triene acids. Simultaneously, Sreenivasan and Brown (3) were also studying this isomerization reaction utilizing UV spectrophotometry to measure the conjugated acids. The latter investigators reported that potassium *t*-butoxide reagent could be successfully used in the spectrophotometric method.

Screenivasan and Brown (3) performed the isomerization at 90 C, and a plot of the extinction coefficient vs. time of reaction showed that there was a crossover in the values determined at wavelengths characteristic of diene conjugation (233 mμ) and triene conjugation (268 mμ). They contended their observation indicated that the isomerization of linolenate occurs stepwise, i.e., diene conjugation occurs first followed by conjugation to triene. Since absorptivity value continues to increase even after long periods, this effect is considered as evidence that the reaction at this temperature is not complicated by side reactions such as polymerization and deisomerization.

White and Quackenbush (4) performed a time study on the extent of the isomerization reaction of polyunsaturated acids with potassium *t*-butoxide reagent at 60 C. Their results with linoleic acid duplicated those reported by Sreenivasan and Brown (3). However, with linolenate, experimental evidence indicated there was no crossover point on the absorptivity value graph; i.e., the absorptivity value at wavelength 233 mμ at all times exceeded that found at 268 mμ. Based on this evidence they contended there was no reason to believe that conjugated diene is a precursor of conjugated triene. Furthermore, while Sreenivasan and Brown observed that the absorptivity value at 268 mμ increased indefinitely, White and Quackenbush (4) observed maxima at both 233 and 268 mμ and these values simultaneously decreased with time.

Sreenivasan and Brown (5) investigated the possibility that, at higher temperatures, further and possibly complete conjugation of the poly-

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[1]This paper reports in part research submitted to satisfy thesis requirements for a Master's Degree at Bradley University.

[2]Bond Award paper. Award presented at the 43rd AOCS Fall Meeting, Minneapolis, October 1969.

[3]No. Utiliz. Res. Dev. Div., ARS, USDA.

TABLE I

Conjugation of Methyl Linoleate With Potassium *t*-Butoxide (Analysis of Products)

Reaction temp., C	UV abs. 233 mμ	UV abs. Lit. values	GLC analysis, wt %: Nonconjugated diene	GLC analysis, wt %: Conjugated diene *cis,trans*	GLC analysis, wt %: Conjugated diene *trans,trans*
60	42.1	76.7 (4)	34.3	55.1	10.6
90	84.5	94.0 (3)	1.5	98.5	---
140	84.1	98.0 (5)	0.0	51.0	49.0

unsaturated acids might occur. Using a sealed tube they isomerized linolenic acid at 140 C for 2 hr. UV absorptivity measurements indicated that there was no peak in the diene region of the spectra. They concluded that complete conjugation of linolenic acid had been obtained.

The AOCS standard method of isomerization involves the use of potassium hydroxide-ethylene glycol reagent at 180 C for 25 min (1). Products of this reaction were first examined by Nichols et al. (6) in 1951. Essentially a qualitative investigation, their identification of geometric and positional isomers formed during the isomerization of linoleic acid facilitated the presentation of theoretical considerations of the double bond shift mechanism.

Products of isomerization of polyunsaturated acids with potassium hydroxide-ethylene glycol reagent at 180 C and above have since been investigated by Scholfield et al. (7) using countercurrent distribution and capillary gas-liquid chromatography (GLC); Cartoni et al. (8) using capillary GLC; Jamison and Reid (9) using packed column GLC; and Capella and Strocchi (10) using GLC-mass spectrometry. A limited examination has been made of the products of isomerization of polyunsaturated acids with potassium *t*-butoxide reagent (11).

This paper reports the results of comprehensive analysis of products formed by isomerization of linoleic and linolenic acids with potassium *t*-butoxide as the base.

EXPERIMENTAL PROCEDURES

Materials

Methyl Linoleate. This ester was obtained pure by countercurrent distribution procedures (12) from soybean methyl esters. Preconjugated diene <1.0%; no hydroxyl by IR; GLC, one peak with relative retention time identical to that for methyl linoleate standard; thin layer chromatography (TLC), single spot elutes with methyl linoleate standard.

Methyl Linolenate. This ester was obtained by countercurrent distribution procedures (12) from linseed methyl esters. Preconjugated diene <1.0%; preconjugated triene, <1.0%; no hydroxyl by IR; GLC, one peak with relative retention time identical with that for methyl linolenate standard; TLC, single spot elutes with methyl linolenate standard.

Methanol. Purity same as for standard spectrophotometric procedure (1).

Tertiary Butanol. Fractionally distilled, constant boiling 83 C fraction collected and used.

Potassium. Bakers purified lump.

Tritiated Water. From Nuclear Chicago Corporation. Specific activity 11 C/mole.

Deuterated t-Butanol. From Bio-Rad Laboratories. Deuterium purity, 98%.

Tritiated t-Butanol. Prepared by the addition of a small amount of high specific activity tritiated-water to *t*-butanol. After 100 μl of HTO (5 mc/μl) was added to 1.0 ml of *t*-butanol, the solution was thoroughly mixed and allowed to equilibrate for eight days. The tritiated-*t*-butanol was dried over calcium hydride and microdistilled at 82-83 C. The activity of the tritiated *t*-butanol was 0.455 mc/μl.

Method

Reagent. A piece of potassium was cleaned while immersed in heptane by shaving off the sides, and the quantity desired was weighed. The weighings were all done in heptane and are accurate to ±0.1 g. After lifting the pieces out and allowing the heptane to drain away, they were added to 100 ml of tertiary butanol in a 125 ml stoppered flask. The flask was covered and allowed to stand overnight as suggested by Sreenivasan and Brown (3). Approximately 5 g of potassium was dissolved in the 100 ml of tertiary butanol, a molar concentration of 1.3 M potassium-*t*-butoxide in *t*-butanol. Three separate batches of reagent were prepared from unlabeled, tritium-labeled and deuterium-labeled *t*-butanol.

Apparatus. Isomerizations were performed

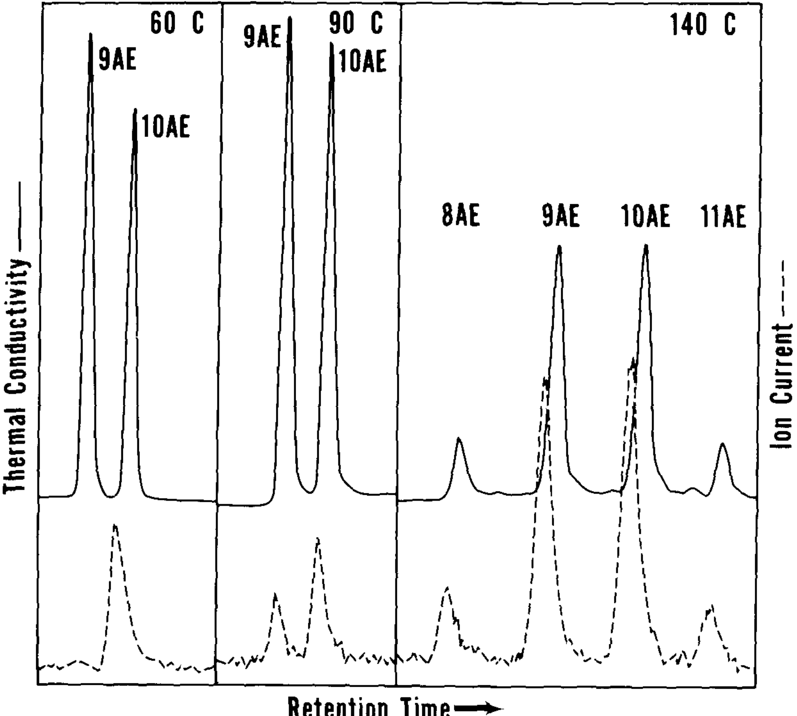


FIG. 1. Gas liquid radiochromatograms of aldehyde esters produced during ozonolysis-pyrolysis of *cis,trans*-conjugated dienes formed at 60, 90 and 140 C; 6 ft x 1/4 in. aluminum column packed with 11% EGSS-X; temperature programmed 80-200 C at 4 C/min; ZnO precolumn used to remove free acids; radioactive peak is displaced owing to time lag between detectors.

in stoppered 40 cc centrifuge tubes at 60 C, in 40 cc centrifuge tubes equipeed with air condensers at 90 C and in a sealed tube (30 cc) at 140 C. A 4 in. deep dish, obtained by sawing off the top of a 2 liter pyrex beaker, containing silicone oil served as the heating bath. Heating was thermostatically controlled to give the designated temperature (±5 C), and the bath was equipped with a magnetic stirrer.

Procedure

Approximately 500 mg of the sample was weighed into the isomerization flask. After the flask was flushed with nitrogen, 10 ml of the reagent was added. Three experiments were run, one each with the separate batches of reagent described above. Isomerizations were performed at 60 C for 20 hr, 90 C for 4 hr and 140 C for 2 hr. After the reactions continued for the desired period, the centrifuge tubes were lifted out of the heating bath and placed in a water bath to cool the contents to room temperature. Each solution was washed into a 100 ml volumetric flask with purified methanol. After appropriate dilutions, absorbance measurements were performed with a Beckman model DU continuous recording spectrophotometer. The methanol solution of the reaction products was acidified with HCl, diluted with water and extracted with hexane. The hexane solution, containing the extracted fatty acids, was dried over anhydrous sodium sulfate. After removal of the solvent on a rotary evaporator, the fatty acids were methylated with diazomethane and analyzed by gas liquid radiochromatography (GLRC). (Separatory column: 6 ft X 1/4 in. aluminum column packed with 11% EGSS-X, Applied Science Laboratory.) GLRC analyses were obtained on a Model 70 Cary-Loenco radiochromatography system, which provides for thermal conductivity detection of mass with simultaneous radioactivity determination by means of a spherical ion chamber contained in the detector oven (13).

Separation and Analysis

Initially, conjugated polyunsaturated material was separated from nonconjugated material on a silver nitrate (Ag) treated resin column [50 cm X 13 mm (ID)] (14). Nonconjugated material was eluted with methanol from

TABLE II

GLC Analysis of Fragments Produced by Ozonolysis-Pyrolysis of Conjugated Dienes

Temperature C	Configuration	Aldehyde ester, wt % 8	9	10	11
60	*Cis,trans* and *trans,cis*	---	49.6	50.4	---
	trans,trans	---	58.1	41.9	---
90	*Cis,trans* and *trans,cis*	---	52.9	47.1	---
	trans,trans	---	---	---	---
140	*Cis,trans* and *trans,cis*	9.8	43.0	40.2	7.0
	trans,trans	12.5	39.3	38.7	9.5

this short column after the conjugated material as observed by Emken (unpublished results). After composition of the column fractions was determined by GLRC, those containing conjugated materials were combined and were rechromatographed on a larger Ag resin column [213 X 2 cm (ID)] to isolate isomeric conjugated materials. The composition of the individual fractions collected from the column was again determined as described above.

Pure materials were analyzed by IR to determine the configuration of the double bonds. Approximately 100 mg of material was dissolved in 1.0 ml of carbon disulfide. IR analysis was accomplished with a Perkin-Elmer 621 grating IR spectrophotometer equipped with sodium chloride cells (cell width 0.025-1.0 mm as appropriate). Transmittance at the 10-11 μ region was utilized to determine the *cis, trans* configuration of the double bonds (15,16).

Double bonds in the fatty acid molecule were located by ozonolysis-pyrolysis techniques based on the microreactor apparatus described by Bitner et al. (17). Further information concerning double bond location was provided by nuclear magnetic resonance (NMR) spectral analysis of the samples (18). Products formed during isomerization reactions with deuterated *t*-butanol were analyzed by mass spectrometry to determine the amount of deuterium exchange and by deuteron magnetic resonance (19) to locate the deuterium in the molecule.

RESULTS AND DISCUSSION

Isomerization of Methyl Linoleate

Absorptivity values calculated according to the AOCS Standard Method (1) are presented in Table I. All our values are averages of the values determined for the three experiments performed at each temperature, and differences between these values are less than 5.0%. Calculated absorptivities indicate that the degree of conjugation in these samples generally approximates that which was reported by previous investigators (3,4).

After the methyl esters of the products were regenerated, they were analyzed by GLC to give a preliminary identification. Table I also presents the preliminary analysis of the products of the diene reactions at 60, 90 and 140 C (3-5). Baseline resolution was achieved between the *cis,trans*- and *trans,trans*-conjugated dienes. IR spectra of these geometric isomers showed two peaks, at 10.16 and 10.55 μ for *cis,trans* configuration (15.16); a single peak at 10.12 μ was generated by the *trans,trans* configuration. IR analysis was per-

TABLE III

Conjugation of Methyl Linolenate With Potassium *t*-Butoxide (Analysis of Products)

Reaction temp., C	UV 233 mμ	Lit. values	268 mμ	Lit. values	GLC analysis, wt % Nonconjugated triene	Conjugated diene triene	Conjugated triene α-type	β-type
60	55.8	67.2	50.6	64.4 (4)	8.4	72.7	4.3	14.6
90	66.6	63.2	72.7	74.2 (3)	---	73.0	6.4	20.6
140	25.3	30.5	123.5	138.4 (5)	---	34.5	65.5	

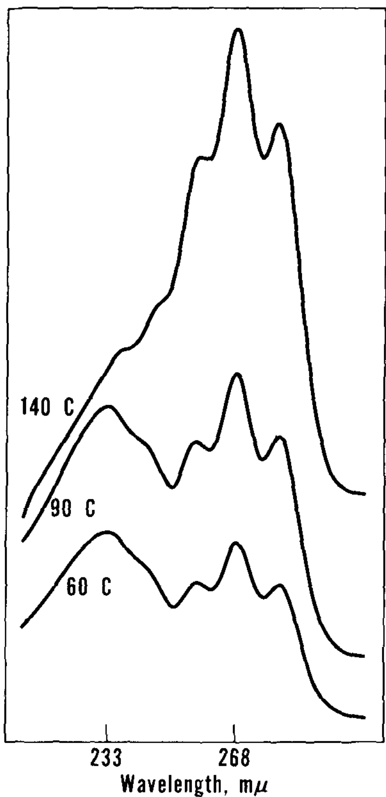


FIG. 2. UV spectra of products formed by isomerization of methyl linolenate at 60, 90 and 140 C. Diene conjugation, 233 mμ; triene conjugation, 268 mμ.

formed on the pure compounds obtained by successive liquid column fractionations on the short and long Ag resin columns.

Nonconjugated material, which amounts to 34% of the products of the 60 C reaction, was examined by IR and UV after recovery from the short Ag resin column. UV indicated that this material was free of conjugated material as had also been indicated by GLC. IR showed that there was no *trans* isomerization of the double bonds in the nonconjugated material.

GLRC chromatograms of the fragments produced during pyrolysis of the ozonides from the *cis,trans* products are shown in Figure 1. The peak area relationships of the 9- and 10-aldehyde esters provide a quantitation of the amount of 9,11- and 10,12-conjugated diene present in the sample. The lower curve in Figure 1 is the radioactivity analysis of the effluent and indicates that in the 60 C reaction only the 10-aldehyde ester is labeled. This result would be predicted from the carbanion theory of the mechanism of reaction. Carbon atom 11 is the activated methylene group in the

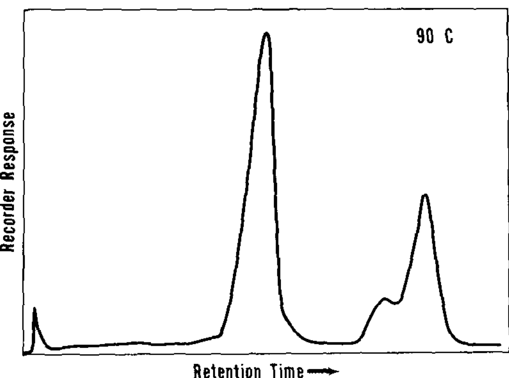


FIG. 3. GLC chromatogram of products formed by isomerization of methyl linolenate at 90 C. Conditions same as for Figure 1.

diene system. With the shifting of the double bonds to form the conjugated system, protonation of the molecule occurs at carbon atom 9 to produce the 10,12-conjugated system and at carbon atom 13 to give the 9,11-conjugated system. Ozonolysis-pyrolysis of the former yields an aldehyde ester fragment labeled with tritium on the carbon atom α to the carbonyl group. With the 9,11 system there is no radioactivity in the aldehyde ester fragment. Deuteron magnetic resonance spectra of the conjugated diene formed during isomerization in deuterated *t*-butanol at this temperature (60 C) confirms that the deuterium is exclusively α to the double bond system.

The ozonolysis-pyrolysis analysis of the *cis,trans* products of the 90 and 140 C reaction are also presented in Figure 1. While at 60 C only one radioactivity peak was detected, at higher temperatures the GLRC curve indicates that more than one fragment is labeled. Labeling of the molecule apparently also occurs by isotopic exchange at these temperatures. This increased labeling is also indicated by mass spectrometry, which showed that the conjugated diene produced in the reaction at 140 C contained an average of three deuterium atoms per molecule. Double bond position analysis showed that there was no scattering of the conjugated system when the reaction was performed at 60 or 90 C. However, at 140 C there was significant scattering of the double bonds. Apparently at this elevated temperature the reaction is highly activated resulting in both increased isotopic labeling and shifting of the conjugated system.

Ozonolysis-pyrolysis analysis of the conjugated dienes is given in Table II. The results would seem to indicate that the Δ12 double bond is shifted slightly more selectively in the

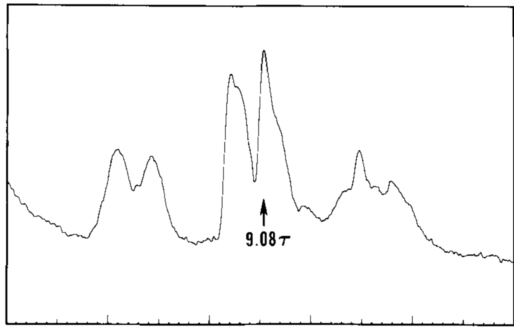


FIG. 4. Expanded NMR spectrum of conjugated diene triene isolated from products formed by isomerization of methyl linolenate at 90 C. Spectrum range 8.8-9.3 τ.

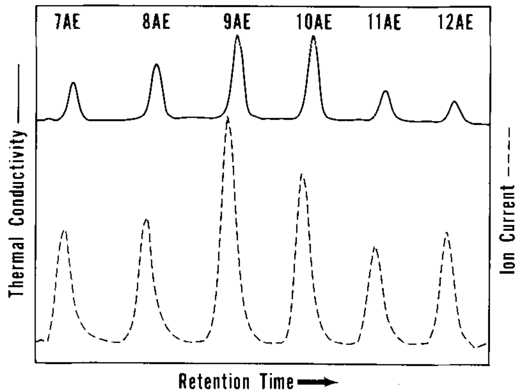


FIG. 5. Gas liquid radiochromatogram of aldehyde esters (AE) produced during ozonolysis-pyrolysis of conjugated trienes formed by isomerization of methyl linolenate at 140 C. Conditions same as for Figure 3.

initial reaction and that the elaidinization of the Δ9 double bond proceeds more readily than that of the Δ12 in the conjugated system.

It can be postulated that elaidinization of bonds which are not shifted proceeds simultaneously with the conjugation reaction through a mechanism which is subordinate to the primary prototropic shift mechanism. This secondary mechanism appears to require additional energy since *trans,trans* dienes were produced only in the 60 C reaction, which was prolonged, and in the 140 C reaction. The conjugated carbanions formed by proton extraction and the prototropic shift mechanism (9-*cis*,11-*trans*)– and (10-*trans*,12-*cis*)– would exhibit, according to Cram (20), considerable geometric stability. He states that evidence indicates the collapse of the anion is a kinetically controlled process favoring the retention of the geometric configuration. However, as the energy of the reaction is increased the *trans* configuration of the double bonds is favored, in this instance the *trans,trans*-conjugated dienes. The differences in amounts of *trans* formed in the reactions at 60 and 140 C apparently arises from the increased energy in the 140 C reaction. Strocchi and Capella (21) have reported a similar increase in all-*trans* conjugated diene with increased reaction time. The energy required to produce the *trans,trans* isomer was not provided in the 90 C reaction.

Isomerization of Methyl Linolenate

The UV absorbance curves for the triene conjugation reaction products are shown in Figure 2. It should be noted that in the 140 C reaction there is no peak at 233 mμ in agreement with the findings of Sreenivasan and Brown (5); however, there are two shoulders on the short wavelength side of the triene absorption band corresponding to approximately 249 and 239 mμ. The shoulder at 239 mμ appears to be significant and the average absorptivity value calculated for this wavelength was 38.5.

Average absorptivity values calculated for the products of reactions with methyl linolenate are presented in Table III. The values indicate that the reactions approximate results obtained by previous investigators. As reported by White and Quackenbush (4) for the reaction at 60 C the value at 268 mμ does not exceed the value determined for the peak at 233. Their data indicated that this relationship would remain constant even at long reaction times. The absorptivity values determined for the products of the 90 C reaction confirm the findings of Sreenivasan and Brown (3) who found a crossover of values resulting in a higher value at 268 mμ than that found at 233 mμ. Finally, the absorptivities for products of the 140 C reaction approximate those recorded by Sreenivasan and Brown (5).

GLC analysis of the reaction products is also given in Table III. Nearly complete isomerization of the methyl linolenate is achieved at 60 C compared to 34% nonconjugated methyl linoleate retained at this temperature. This result is in agreement with the contention of Davenport et al. (2) that 1:4:7 trienes have a lower Arrhenius activation energy for conjugated diene production than do 1:4 dienes. The GLC curve of the products of reaction at 90 C (Fig. 3) illustrates the separation achieved between conjugated diene-triene (CDT) and conjugated triene (CT) constituents.

CDT was isolated free of CT by Ag resin chromatography. IR analysis indicated that the isolated constituent contained both *cis* and *trans* double bonds. UV indicated that the CDT

TABLE IV

GLC Analysis of the Fragments Produced by Ozonolysis-Pyrolysis of Conjugated Diene-Triene

Temperature, C	Aldehyde ester, wt %				
	8	9	10	11	12
60	---	82.2	7.1	11.7	---
90	1.2	95.1	1.8	1.9	---
140	11.4	76.1	2.5	2.3	7.7

absorbed only at wavelength 233 mμ. Ozonolysis-pyrolysis analysis (Table IV) characterized the CDT as an unsaturated compound having a double bond mainly in the 9 position. The attenuated NMR spectrum of the CDT (Fig. 4) is characteristic of a sample possessing a double bond β to the terminal methyl group. This result places the double bond in the 15 position. The normal triplet expected to arise from the interaction of the protons of the terminal methyl group with those of the α-methylene appears as a double triplet. This phenomenon is thought to be due to the presence of two isomers of the CDT, where one has a conjugated system α to the methylene and the second has an isolated double bond occupying this position (22). The ratio of the two isomers, which possess 9,11,15 and 9,13,15 double bond systems, is estimated to be approximately 50:50.

The reaction products formed at 140 C (Table III) present a different picture from that indicated by analysis of the other reaction products. Whereas Sreenivasan and Brown (5) had presupposed complete or nearly complete conjugation was attained at 140 C, present evidence indicates that 34% CDT remains. The CDT and CT constituents were isolated. UV analysis indicated CDT had one peak at 233 mμ and CT had the triplet configuration with maxima at 268 mμ. The absorbance curve for isolated CT did not exhibit a shoulder at 239 mμ. With the large amount of CT material present in this sample, it is reasonable to expect that the CDT maxima would shift and exhibit a shoulder (239 mμ) on the greater peak of the major component.

Absorptivity values calculated from these absorbance curves are: CDT, wavelength 233 mμ, 94.5; wavelength 239 mμ, 81.1; wavelength 268 mμ, no value. CT, wavelength 233 mμ, 40.5; wavelength 239 mμ, 55.2; wavelength 268 mμ, 194.0. The absorptivity value calculated from the absorbance curve for the products of triene reaction was a_{268} = 123. This value approximates that which would be expected from the per cent composition, as resported in Table III, and the absorptivity value reported for pure CT above:

$$65.5\ (\%CT) \times 194.0\ (a_{268}) = 127\ [a_{268}\ (theo.)]$$

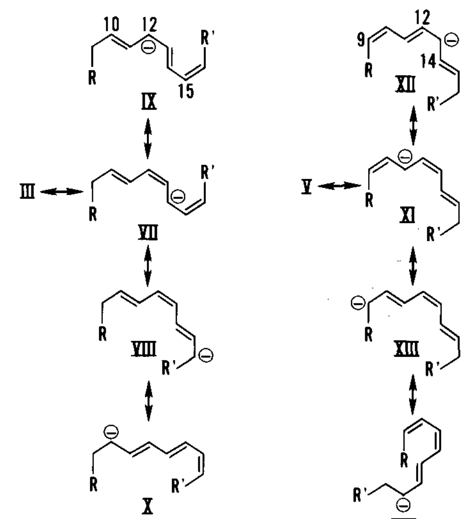
CT formed by the reactions at 60 and 90 C was characterized as being heterogeneous. The initial GLC analysis indicated two partially resolved peaks having relative retention times roughly corresponding to α- and β-eleostearate. The two triene constituents formed during the 90 C reaction were isolated, each in >90% purity. With the products of reaction at 140 C, the CT was isolated completely from CDT. GLC analysis indicated that the CT formed at 140 C was a more complex mixture of isomers than had been formed at the lower reaction temperatures. The triene material isolated from the 90 and 140 C reactions was analyzed for double bond location by ozonolysis-pyrolysis. The composition of the aldehyde ester fragments as determined by GLC is presented in Table V. The composition indicated at 90 C has been corrected for 10% CDT impurity. The two triene constituents (α and β) isolated from the products of reaction at this temperature were further characterized by GLC and IR. Relative retention times for the constituents were: α (1.850), β (1.961). Constituent α showed IR bands at 10.25 (strong) and 10.75 μ (weak), whereas constituent β showed IR bands at 10.10 and 10.84 μ. The IR bands for α are quite similar to those reported previously for 8-*trans*,10-*cis*,12-*trans* (23); the appearance of only one strong band (10.25 μ) was also noted by Mikolajczak et al. Wolff and Miwa (16) reported IR bands for 9,11,13 all-*trans*, which

TABLE V

GLC Analysis of the Fragments Produced by Ozonolysis-Pyrolysis of Conjugated Trienes

Temperature, C	Aldehyde esters, wt %					
	7	8	91	10	11	12
90	---	4.8	14.5	74.4	3.0	3.3
140	11.1	17.5	25.6	27.9	10.5	7.4

Scheme 1



Scheme 2

were quite similar to those shown by β. Based on results of ozonolysis-pyrolysis, GLC and IR the two main triene constituents are identified as (α) 10-*trans*,12-*cis*,14-*trans* and (β) 10-*trans*, 12-*trans*,14-*trans*.

As indicated by GLRC (Fig. 5) the fragment analysis shows the CT formed at 140 C is quite complex, precluding any estimation of geometric configuration. Appearance of initial double bonds in the 7, 8, and 12 position may be caused by the high energy of the reaction. Tritium exchange has occurred all along the chain and each fragment has been labeled. Mass spectrographic analysis of CT formed at 140 C with the deuterated reagent indicated that the deuterium content was 11 atoms per molecule. Deuteron magnetic resonance of this sample showed how the deuterium atoms were scattered: 20.0% were olefinic, 38.0% were α and 42.0% were β or farther to the double bond system.

A brief discussion of the mechanistic theory involved may be helpful in understanding these experimental observations. Expanding Nichols' (6) theory of carbanion formation during the conjugation of diene, it can be postulated that during the conjugation of triene, two carbanions exist as resonance hybrids as depicted in Scheme 1. The CDT, produced by protonation of II and VI, lacks an activated methylene group and, therefore, has little tendency to form a new carbanion required for the formation of CT. Rather, in relatively low energy reactions (60 and 90 C) these compounds accumulate and are the main constituents of the products of reaction at these temperatures. On the other hand, CDT produced by the protonation of III and V retains the activated methylene group. These compounds can then exist as second resonance hybrid carbanions as shown in Scheme 2. Protonation of VIII and XIII yields the CT system shown to be present mainly in the products of reactions at 60 and 90 C. The composition of the mixture of CT from each reaction shows 75-80% of the all-*trans* component. This ratio of components approaches the equilibrium ratio of *cis* to *trans* isomers reported during the isomerization of oleic acid to elaidic acid (24). As noted before, the CDT retaining an activated methylene group is highly susceptible to the extraction of a proton. It may be that such a carbanion, already possessing a conjugated system, is an energetic molecule which is favorable to a prototropic shift accompanied by an equilibrium elaidinization of *cis* bonds. Protonation of IX and XII yields CDT, which retains the activated methylene and provides a source for CT having double bonds in positions other than the 10,12,14 system determined to be the predominate CT in reactions at 60 and 90 C.

The experimental evidence indicated that the initial removal of a proton from an activated methylene group proceeds fairly easily and complete isomerization of nonconjugated material is accomplished at 90 C. At these lower temperatures the CDT formed is mainly 9,11,15 and 9,13,15 (80-95%), whereas the CT is mainly 10,12,14 (74%). The small amount of CDT retained with the initial double bond in positions other than Δ9 indicates that the second deprotonation from molecules retaining the activated methylene group proceeds quite rapidly and yields the CT configuration. While

the shifting of a single bond to form CT is favored, the 26% of CT with initial double bond in positions Δ11 and Δ9 (X and XIV) may indicate a migration of the conjugated diene system to form CT. The CT with the initial double bonds in the Δ8 and Δ12 positions may indicate a migration of the conjugated triene system. Under different conditions intact conjugated systems have been shown to migrate (25). Whereas, in the reactions at 60 and 90 C some selectivity towards the shifting of the Δ12 bond was observed, at 140 C the amount of CDT retained indicates that there was less selectivity as to which bond in the triene system was shifted. As mentioned, when double bonds, other than Δ12, are shifted the CDT formed retains the activated methylene group and rapidly forms CT. The energy supplied at this temperature results in a greater migration of the intact conjugated system producing, in turn, a larger amount of CT with the initial double bond in positions other than Δ10. That 34% CDT was retained seems to substantiate the conclusion that an activated methylene group is necessary to provide for the formation of a carbanion, which leads to the conjugated triene. To test this hypothesis CDT isolated from the 90 C reaction and possessing double bond configurations 9,11,15 and 9,13,15, without an activated methylene group, was heated with 10 ml potassium *t*-butoxide for 4 hr at 90 C. There was no increase in UV absorption in the triene region of the spectra, apparently confirming the need for an activated methylene group for successful deprotonation.

ACKNOWLEDGMENT

W.K. Rohwedder obtained mass spectrometric analysis and C.A. Glass, nuclear magnetic resonance spectra.

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The Acyl and Alk-1-enyl Groups of the Major Phosphoglycerides From Ox Brain Myelin and Mouse Brain Microsomal, Mitochondrial and Myelin Fractions[1]

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ABSTRACT

The major phosphoglycerides from ox brain myelin and mouse brain microsomal, mitochondrial and myelin fractions were separated by preparative thin layer chromatography. Alk-1-enyl groups from the alk-1-enyl acyl glycerophosphorylethanolamines were reacted with 1,3-propanediol to form the 1,3-dioxolane derivatives. Acyl groups were converted to the methyl ester derivatives and the acyl groups from alk-1-enyl acyl glycerophosphorylethanolamines and diacyl glycerophosphorylethanolamines were also determined separately. The acyl and alk-1-enyl group compositions of the phosphoglycerides from microsomal and mitochondrial fractions were quite similar. The ethanolamine and serine phosphoglycerides contained large amounts of 18:0, 18:1, 20:4 and 22:6 acyl groups. The choline phosphoglycerides had small amounts of polyunsaturated acyl groups and large amounts of 16:0, 18:1 and 18:0 acyl groups. The mitochondrial cardiolipins contained unusual amounts of several acyl groups including 18:1, 52%; 18:2, 6%; and 16.1, 4%. A large portion of the mouse brain 18:2 is in that fraction. The myelin phosphoglycerides were deficient in saturated and 22:6 groups and markedly enriched in 18:1 and 20:1 groups when compared with the corresponding microsomal or mitochondrial phosphoglycerides.

INTRODUCTION

The lipid compositions and the acyl group compositions of brain lipids are distinctly different from the lipids of other organs. In addition, the comparison of mammalian brain myelin with other membranous fractions of the nervous system has revealed that myelin has a characteristic lipid and protein composition. Although the lipid composition of the myelin membrance has been reported for a number of mammalian species (1-7), very little information is available on the side chain compositions of the individual phosphoglycerides (8-10). The acyl group composition of the alk-1-enyl acyl glycerophosphorylethanolamine (GPE) (ethanolamine plasmalogen) has not been determined separately although it accounts for one third of the myelin phospholipids (11). A detailed knowledge of the lipid and the acyl and alk-1-enyl group compositions is required for the interpretation of metabolic, functional and structural relationships. For example, if the alk-1-enyl group compositions are different for the alk-1-enyl acyl GPE from myelin and microsomes, then the comparison of turnover rates is complicated and the site of biosynthesis cannot be determined with certainty.

The present investigation describes the lipid compositions and the alk-1-enyl and acyl side chain compositions of the major phosphoglycerides from ox brain myelin and mouse brain myelin, mitochondria and microsomes. The acyl groups were converted to fatty acid methyl esters and the alk-1-enyl groups were converted to aldehyde cyclic acetal derivatives. The methyl esters and cyclic acetals were analyzed by gas liquid chromatography (GLC).

MATERIALS AND METHODS

Ox Brain Myelin

Three ox brains were obtained from the slaughter house within 30 min of death of the animals and stored on ice for less than 1 hr before processing. The preparation of myelin from the ox medulla oblongata by method C and the procedure for lipid extraction were described previously (4).

The tissue was dispersed in buffered 0.32 M sucrose and centrifuged for 15 min at 13,500 *g*. The pellet was suspended in 0.8 M sucrose and

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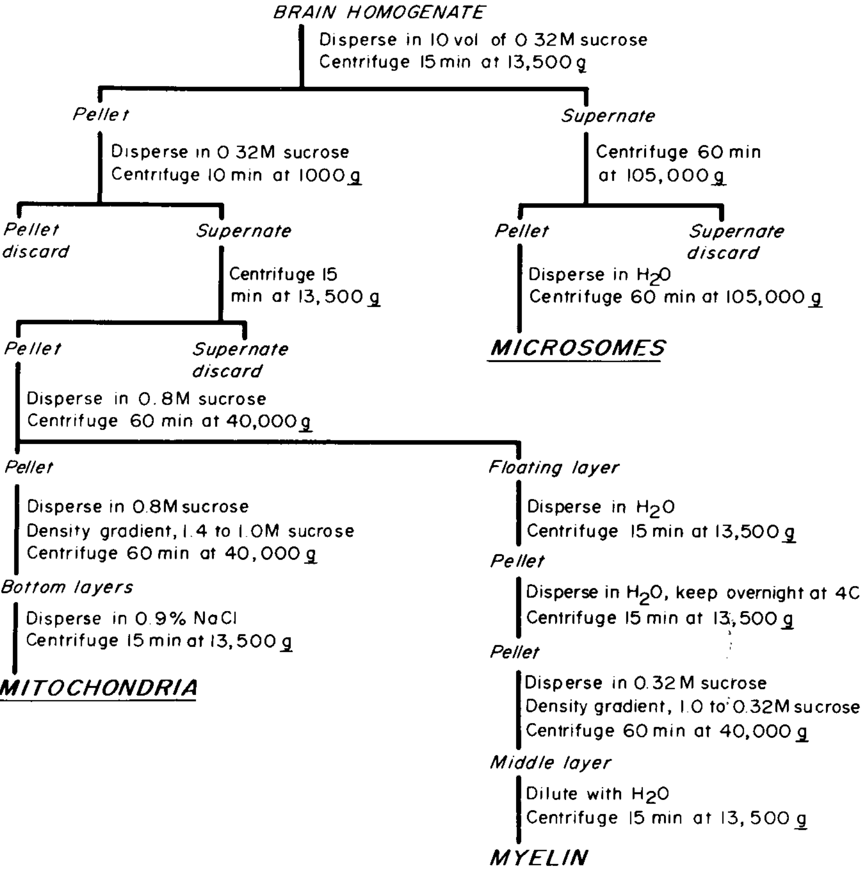

FIG. 1. An outline of the isolation procedure for mouse brain myelin, mitochondrial and microsomal fractions.

centrifuged for 60 min at 40,000 *g*. The floating layer (crude myelin) was purified by osmotic shock, reflotation and water washing for the removal of sucrose.

Mouse Brain Subcellular Fractions

Genetically homogenous female C57BL/10J mice were used at approximately three months of age in groups of nine. The brains were removed and dispersed in 10 vol. of cold 0.32 M sucrose-0.001 M Na_4EDTA-0.003 M Na_2HPO_4. The isolation procedure is outlined in Figure 1. The SW 25.1 swinging bucket rotor was used in a Spinco model L ultracentrifuge. These procedures for myelin and microsomal fractions are similar to those described previously (4,5). The mitochondrial fraction was purified by further centrifugation through a continuous sucrose gradient. One or two brown homogenous layers were found at or near the bottom of the centrifuge tube. Samples of several fractions were examined by electron microscopy (5).

Lipid Extraction and Analysis

The isolated fractions were washed twice by centrifugation and dispersed in 10 ml water. A small portion was taken for the determination of protein content. The remainder was mixed with 20 vol. of chloroform-methanol (2:1 v/v) and filtered through paper. The filtrate was taken to dryness with a rotary evaporator with several additions of ethanol. The residue was dissolved in 35 ml of chloroform-methanol (2:1 v/v). Seven milliliters of 0.58% NaCl was added (12) and the mixture was kept at 4 C overnight. The upper phase was discarded. The lower phase was washed twice with upper phase mixture, taken to dryness, and the residue was stored in chloroform at 4 C.

The analytical methods for protein, cholesterol and lipid phosphorus contents (5) and for the determination of phospholipid compositions by two dimensional thin layer chromatography (TLC) (11) have been described.

TABLE I

Lipid Compositions of Myelin, Mitochondria and Microsomes

Component	Ox myelin	Mouse myelin	Mouse microsomes	Mouse mitochondria
Protein[a]	18.5	31.9 31.8	44.4 , 44.1	71.6 , 72.8
Cholesterol[b]	1.26	1.35, 1.29	0.64, 0.79	0.36, 0.28
Acid-labile EPG[b]	0.33	0.32	0.19	0.12
Acid-stable EPG[b]	0.09	0.14	0.18	0.21
CPG[b]	0.21	0.25	0.34	0.38
Sph[b]	0.17	0.06	0.06	0.05
SPG + IPG[b]	0.20	0.24	0.23	0.20
CL[b]	---	---	---	0.04

[a]Per cent of protein plus lipid.
[b]Mole ratio, component to lipid P.

Acyl and Alk-1-enyl Group Compositions

The major phosphoglyceride classes of microsomal and myelin lipids were separated by preparative TLC using chloroform-methanol-15 M NH_4OH (65:25:4 v/v/v) as the solvent for development. For the mitochondrial lipids, the developing solvent was chloroform-methanol-water (65:25:4 v/v/v). Derivatives of the alk-1-enyl and acyl groups of the ethanolamine phosphoglycerides (EPG) were prepared by a procedure that gives a separate fraction for the acyl groups from the alk-1-enyl acyl GPE (14,15). The Silica Gel G (Brinkmann Instruments, Des Plaines, Ill.) that contained the EPG was placed in a column of Unisil silicic acid (Clarkson Chemical Co., Williamsport, Pa.) (5 g). The column was eluted with 100 ml methanol. Cyclic acetal derivatives of the alk-1-enyl groups were prepared by reacting the EPG with 1,3-propanediol in the presence of *p*-toluenesulfonic acid. After removal of the excess propanediol, the reaction mixture was separated by TLC to give bands containing cyclic acetals, acid stable EPG (diacyl and alkyl acyl GPE) and monoacyl GPE. The cyclic acetals were eluted and applied to another TLC plate that was developed with toluene. The purified cyclic acetals were eluted, taken to dryness, and dissolved in hexane for GLC.

Methyl ester derivatives of phosphoglyceride acyl groups were prepared by alkaline methanolysis of the phosphoglycerides in the presence of Silica Gel G (13). The method was modified by adding 8 ml chloroform, 1 ml methanol and 3 ml water to the 3 ml of reaction mixture. The methyl esters were recovered from the lower phase, purified by TLC with toluene development, then eluted, taken to dryness, and dissolved in hexane for GLC. In one case, part of the methyl ester preparation was subjected to TLC on plates impregnated with silver nitrate (16).

The operating conditions for GLC with a Varian Aerograph Gas Chromatograph Model 204B (Varian Aerograph, Walnut Creek, Calif.) were described previously (15). In the present investigation, the column temperature was 210 C for the separation of cyclic acetals. Three gas chromatographic tracings were then averaged for each of the ox brain preparations or for each of the mouse brain preparations from nine pooled brains.

RESULTS

For the isolation of myelin and mitochondrial fractions, the emphasis was on purity of the fraction and not on quantitative yield. When these fractions were examined under the electron microscope, the purified myelin frac-

TABLE II

The Alk-1-enyl Group Compositions of Ethanolamine Phosphoglycerides

Chain length, unsaturation	Ox myelin, wt %	Mouse myelin, wt %	Mouse mitochondria, wt %	Mouse microsomes, wt %
16:0	24.3	16.6	22.2	22.1
16:1	---	0.9	---	0.6
17:0	1.7	---	1.0	---
18:0	22.5	31.5	50.1	48.9
18:1	51.3	51.0	26.7	29.5

TABLE III

The Acyl Group Compositions of the EPG, CPG and SPG from Ox and Mouse Brain Myelin

	Ox brain myelin, wt %					Mouse brain myelin, wt %				
	EPG					EPG				
Chain length, unsaturation	Total acyl	Alk-1-enyl acyl	Diacyl	Diacyl GPC	Diacyl GPS[a]	Total acyl	Alk-1-enyl acyl	Diacyl	Diacyl GPC	Diacyl GPS
16:0	4.2	2.0	5.8	36.6	1.8	7.1	2.4	8.4	30.0	3.6
16:1	---	0.8	---	---	---	---	0.9	---	0.6	---
18:0	7.3	1.0	19.8	9.9	37.8	12.0	0.9	25.6	18.5	41.3
18:1	46.2	48.3	39.1	47.0	43.9	33.0	42.8	35.3	42.8	37.7
18:2	0.6	---	0.7	0.7	---	1.0	---	0.9	0.8	---
20:0	---	---	---	---	0.7	1.1	2.6	2.8	1.2	1.2
20:1	13.2	16.4	10.4	3.4	6.4	14.5	20.6	7.2	3.8	4.7
20:2	0.8	0.9	0.6	---	---	---	---	---	---	---
20:3(*n*-3)	0.8	1.1	0.5	---	---	---	---	---	---	---
20:3(*n*-9)	0.8	0.9	0.7	---	0.8	1.0	1.2	---	---	1.6
20:4(*n*-6)[b]	7.5	8.3	7.4	1.0	3.8	12.9	11.6	9.7	2.5	5.6
22:2(*n*-6)	2.6	2.4	2.1	---	1.5	---	---	---	---	---
22:4(*n*-6)	12.4	15.6	8.5	---	2.3	9.9	11.8	3.2	---	2.9
22:5(*n*-6)	0.8	1.0	--	---	---	---	---	---	---	---
22:5(*n*-3)	---	---	---	---	---	---	---	3.5	---	---
22:6(*n*-3)	2.7	2.9	5.0	---	---	6.9	5.8	5.0	---	2.5

[a]GPS: *sn*-glycero-3-phosphorylserine.
[b]Including small amount of 24:1.

tion was free of other contaminants. The first microsomal pellet contained a few small mitochondria and nerve ending particles which may not have survived the osmotic shock with distilled water. In the mitochondrial fraction, part of the mitochondria were surrounded by a plasma membrane and some were swollen; no myelin or other recognizable contaminants were found. The lipid compositions of the myelin, microsomal and mitochondrial fractions are shown in Table I. Two dimensional TLC of the mitochondrial lipids showed very small amounts of material with the chromatographic behavior of galactolipids. With the solvent system containing chloroform-methanol-15 M NH_4OH (65:25:4 v/v/v) for two dimensional TLC of the mitochondrial lipids, the cardiolipin (CL) was found just above and overlapping the acid stable EPG. For estimation of the CL content, chloroform-methanol-water (65:25:4 v/v/v) was used in the first dimension. As obtained by preparative TLC, the EPG and choline phosphoglycerides (CPG) classes were not contaminated by any other glycerides. The serine phosphoglycerides (SPG) band from the TLC plates contained most of the inostol phosphoglycerides (IPG), but the latter accounts for a small amount of the total lipid phosphorus in the myelin and microsomal fractions. The SPG band is completely separated from sphingomyelin (Sph) and polyphosphoinositides. The CL band could contain some cerebrosides, but the latter do not release methyl esters during the alkaline methanolysis procedure.

The results of the GLC analyses are given in Tables II through IV. The values represent the means from several analyses on each of two to four preparations of the subcellular fractions. The standard errors of the mean were similar to those given previously (13,15). The acyl group compositions of the total EPG fractions can be calculated from the relative amounts and the compositions of the alk-1-enyl acyl GPE and diacyl GPE. When the calculated acyl group composition of the total EPG was compared with the experimentally determined composition (Tables III and IV), the agreement was quite good with a maximum absolute deviation of 21%. The deviations for 22:6 acyl groups were both positive and negative, indicating that the longer procedure for the separate determinations did not cause an appreciable loss of polyunsatured acyl groups.

The saturated and monoenoic acyl groups from one ox myelin EPG preparation were separated by TLC on $AgNO_3$ impregnated plates. In addition to those acyl groups reported in Table I, traces of 14:0, 17:0, 20:0, 22:0, 24:0, 19:1, 21:1 and 24:1 were detected. The 24:1 is coincident with the 20:4 under our conditions. On the basis of the amount of 24:1 in the monoene fraction, the ratio of 20:4 to 24:1 in the total EPG methyl esters from ox myelin was estimated as 3:1. Only trace amounts of alk-1-enyl acyl glycerophosphorylcholine (GPC) were present. The alk-1-enyl group composition of one ox myelin CPG sample was 16:0, 43%; 17:0, 2%; 18:0, 21%; and 18:1, 35%.

DISCUSSION

Purified myelin preparations from the medulla oblongatae of three ox brains were made by a previously published procedure (4,5,17). The protein contents and lipid compositions were in agreement with previous results for ox brain myelin (17,18), and squirrel monkey spinal cord myelin (4). The protein content of the mouse brain myelin is higher than found before (5), but the differences in protein content are probably due to differences in the washing procedure causing more or less extraction of proteins (17). The protein contents of the mouse brain microsomal fractions were the same as before (5).

By the careful examination of electron micrographs, we found that a part of the mitochondria were enclosed by a plasma membrane and therefore were nerve ending particles. The lipids of the mitochondrial fraction were predominantly from mitochondria but may also include a small amount of lipid from the plasma membranes of the nerve ending particles. Values for the lipid composition of brain mitochondrial fractions are available for the ox (19), guinea pig (20), and rat (21-26). There is general agreement on a protein-lipid weight ratio of 2:1 and cholesterol-lipid phosphorus molar ratio of 1:3. The alk-1-enyl group content of the mitochondrial phospholipids is less than that of microsomal or myelin fractions.

The CL from brain mitochondria have a distinctive composition. For the mouse, we found unusually large amounts of 16:1 (4.5%), 18:1 (51%) and 18:2 (5.5%). The corresponding values for the rat (21) are 5.0%, 18.6% and 10.3%, and for the ox (28) 8.4%,54% and 17.8%. The content of 18:2 in brain mitochondrial Cl represents a substantial portion of the 18:2 content in the brain.

The analytical scheme for the EPG (15) permits separate analyses of the alk-1-enyl acyl GPE and the diacyl GPE. These two types can be compared if the alk-1-enyl group and the acyl group compositions of the alk-1-enyl acyl GPE are added, then divided by two. As for

TABLE IV

The Acyl Group Compositions of the Major Phosphoglycerides From Mouse Brain Mitochondria and Microsomes

Chain length, unsaturation	Microsomes, wt %					Mitochondria, wt %				
	EPG					EPG				
	Total acyl	Alk-1-enyl acyl	Diacyl	Diacyl GPC	Diacyl GPS[a]	Total acyl	Alk-1-enyl acyl	Diacyl	Diacyl GPC	CL
16:0	6.2	1.1	9.4	42.7	2.3	6.2	2.0	6.2	40.5	7.2
16:1	--	0.4	--	--	--	--	--	--	--	4.5
18:0	21.5	1.3	32.9	13.7	38.2	32.2	4.5	36.0	15.5	6.4
18:1	16.3	21.7	16.0	30.7	16.7	14.8	10.1	13.7	26.5	51.5
18:2	0.6	--	--	0.7	0.4	--	--	--	--	5.5
20:0	0.7	0.6	--	0.4	1.0	--	--	--	--	--
20:1	3.4	9.0	1.6	1.9	2.0	0.9	2.6	0.5	1.4	0.6
20:3(*n*-9)	0.6	0.6	0.5	--	--	--	--	--	--	1.8
20:4(*n*-6)	13.6	14.2	11.7	4.7	9.3	13.9	18.4	13.7	8.4	12.4
22:4(*n*-6)	5.9	11.0	3.5	1.1	3.4	4.3	10.4	3.1	7.7	--
22:6(*n*-3)	32.0	39.6	23.3	4.0	26.4	27.6	51.6	27.0	--	10.6

[a]GPS: *sn*-glycero-3-phosphorylserine.

myelin EPG (Tables III and IV), it is apparent that the diacyl GPE have a higher content of 20:4 and 22:6 polyunsaturated acyl groups and a lower content of 18:1 side chains. Among the saturated acyl groups, the ratio of 18:0 to 16:0 in myelin diacyl GPE is about 3. For the saturated alk-1-enyl groups from the myelin alk-1-enyl acyl GPE, the ratio of 18:0 to 16:0 is about 2 for the mouse and 0.9 for the ox (Table II). These differences demonstrate the importance of separate analyses for the two types of EPG.

The EPG from myelin are strikingly different from the microsomal and mitochondrial EPG. The alk-1-enyl groups from the alk-1-enyl acyl GPE are about 70% saturated in microsomes and mitochondria and only 50% saturated in the myelin. The corresponding values for the 1-acyl groups of the diacyl GPE are 84% and 68%, if all saturated acyl groups are assumed to be at the 1 position. The 2 position of both EPG types is apparently mostly occupied by polyunsaturated acyl groups in the microsomal and mitochondrial EPG. A significantly lower polyunsaturated acyl group content was found for both types of myelin EPG. Instead, both types of myelin EPG have an unusually high content of 18:1 and 20:1 acyl groups. The myelin CPG and SPG differ from the microsomal and mitochondrial CPG and SPG by a pronounced decrease in the polyunsaturated acyl group content and a corresponding increase in 18:1 acyl groups.

From the chemical compositions and the examinations of electron micrographs, we have concluded that these subcellular fractions are quite pure. In general, the myelin phosphoglycerides are very low in polyunsaturated acyl groups of the linolenate family (*n*-3), low in polyunsaturated acyl groups of the linoleate family (*n*-6), low in saturated acyl or alk-1-enyl groups, very high in 18:1 acyl or alk-1-enyl groups, and high in 20:1 acyl groups. These differences in composition could be due to a specificity for particular molecular species of these phosphoglycerides by the myelin membrane or the differences could be due to the origin of the myelin sheath from the oligodendroglial cells. A recent study has indicated that the oligodendroglial lipids are quite similar to the myelin lipids and important differences have been found in the lipid compositions of microsomes from white and gray matter (29,30). In liver and heart subcellular fractions, the lipid specificity seems to be only for the polar portions of the lipids (27). This concept has been supported by the demonstration of phospholipid exchange in vitro between microsomes and mitochondria (31). Fortunately, the

extent of phospholipid exchange in the brain is quite low under the conditions of subcellular fractionation (32). Additional analyses of specific types of brain cells are required before the unique acyl and alk-1-enyl group compositions and the high content of alk-1-enyl acyl GPE of myelin can be ascribed to an oligodendroglial cell specificity or to a myelin membrance specificity.

Obviously, the molecular species of the myelin phosphoglycerides have a composition that is quite different from the molecular species composition of the microsomal and mitochondrial phosphoglycerides. Since marked differences in the turnover rates of different molecular species of liver phosphoglycerides have been reported (33,34), these differences in composition must also be considered in the comparison of the lipid metabolism of subcellular fractions from brain tissue.

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SHORT COMMUNICATION

Comparative Effects of Docosa-4,7,10,13,16-Pentaenoic Acid and Docosa-4,7,10,13,16,19-Hexaenoic Acid on the Desaturation of Linoleic Acid and α-Linolenic Acid

ABSTRACT

Varying concentrations of free docosa-4,7,10,13,16-pentaenoic acid or its CoA ester were incubated with a given variable concentration of 1-^{14}C-linoleate or 1-^{14}C-α-linolenate as either the free fatty acid or the CoA ester, microsomal enzymes, and the appropriate cofactors for fatty acid desaturation. The results obtained were compared to the effects of docosa-4,7,10,13,16,19-hexaenoyl CoA when incubated in a similar manner in the presence of the labeled substrates. Both feedback and crossed inhibition effects were observed; these inhibition effects may play a role in the regulation of polyunsaturated fatty acid biosynthesis.

It has been postulated that the microsomal desaturation of linoleic acid and α-linolenic acid to γ -linolenic acid (1) and octadeca-6,9,12,15-tetraenoic acid (2), respectively, may be regulated by competitive reactions with other unsaturated fatty acids of 18 carbons and by product inhibition (3). Furthermore, it has been shown that intermediate members of the linoleic acid family, eicosa-8,11,14-trienoic acid and arachidonic acid, inhibit linoleate desaturation (4,5). Docosa-4,7,10,13,16,19-hexaenoic acid, the terminal member of the α-linolenic acid family, also produces a feedback inhibition of α-linolenate desaturation (6). In this paper, evidence is presented for the existence of a feedback inhibitory effect on the oxidative desaturation of linoleic acid to γ -linolenic acid whereby docosa-4,7,10,13,16-pentaenoic acid, the terminal member of the linoleic acid series, could evoke an inhibition of either linoleate or α-linolenate desaturation.

Docosa-4,7,10,13,16-pentaenoic acid was obtained from rat testicle. It was separated from the other fatty acids by thin layer chromatography (ethyl ether-petroleum ether, 9:1 v/v) on Silica Gel G impregnated with silver nitrate. Gas liquid chromatography (GLC) indicated that the final preparation contained less than 4% impurities. The chain length of the methyl ester derivative was verified by hydrogenation (7) and subsequent GLC. The positions of the double bonds were determined by ozonolysis (8).

To prove that inhibitory effects are produced at the desaturation step and not at the acylation step, two types of experiments were performed. In the first type, increasing concentrations of the free docosa-4,7,10,13,16-pentaenoic acid were incubated

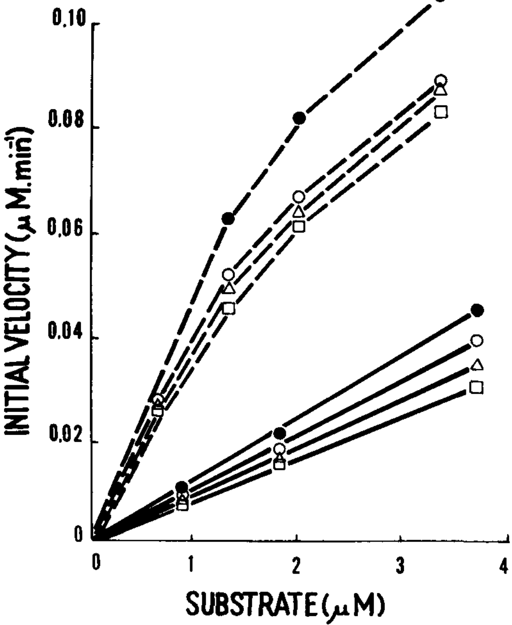


FIG. 1. Effect of docosa-4,7,10,13,16-pentaenoic acid on the oxidative desaturation of linoleic acid to γ-linolenic acid and α-linolenic acid to octadeca-6,9,12,15-tetraenoic acid. Increasing amounts of linoleic acid were incubated in the absence (●——●) and in the presence of 0.66 (○——○); 2.00 (Δ——Δ) and 3.33 (□——□) μM docosa-4,7,10,13,16-pentaenoic acid. Increasing amounts of α-linolenic acid were also incubated in the absence (●- - -●) and in the presence of 0.66 (○- - -○); 2.00 (Δ- - -Δ) and 3.33 (□- - -□) μM docosa-4,7,10,13,16-pentaenoic acid.

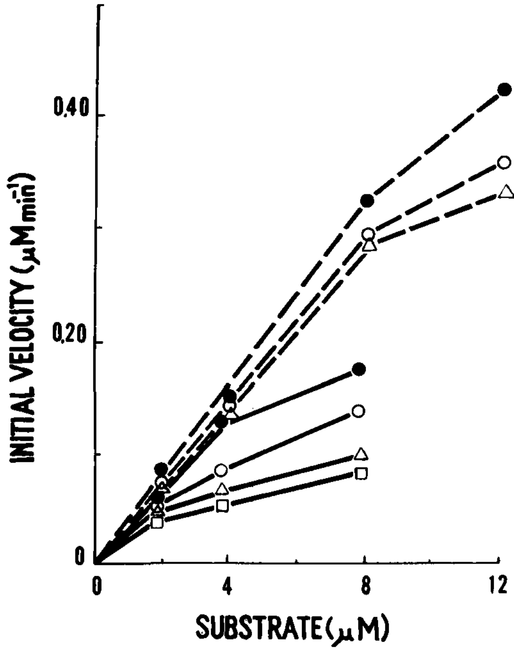


FIG. 2. Effect of docosa-4,7,10,13,16-pentaenoyl CoA on the oxidative desaturation of linoleyl CoA to γ-linolenyl CoA and α-linolenyl CoA into octadeca-6,9,12,15-tetraenoyl CoA. Increasing amounts of linoleyl CoA were incubated in the absence (●——●) and in the presence of 4.00 (○——○); 8.00 (Δ——Δ) and 16.00 (□——□) μM docosa-4,7,10,13,16-pentaenoyl CoA. Increasing amounts of α-linolenyl CoA were also incubated in the absence (●- - -●) and in the presence of 20.00 (○- - -○) and 40.00 (Δ- - -Δ) μM docosapentaenoyl CoA.

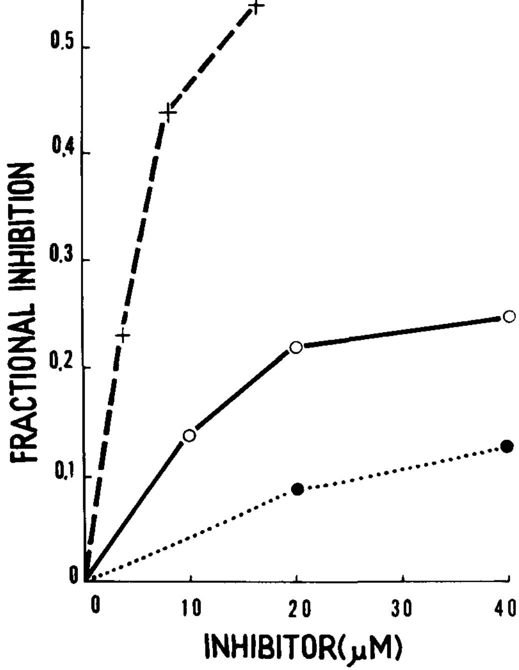
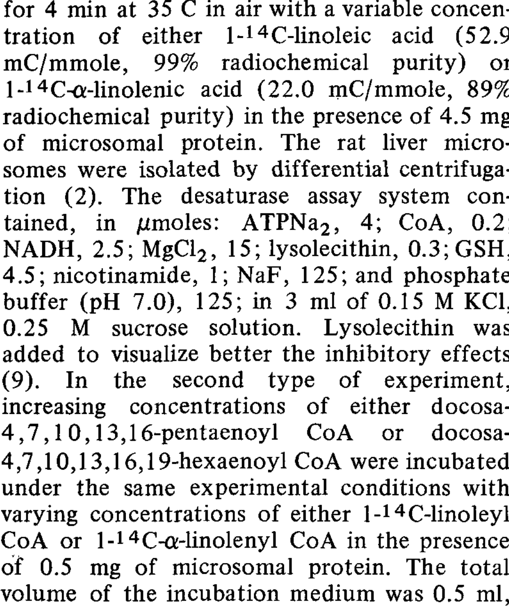


FIG. 3. Comparative inhibitory effects of docosapentaenoyl CoA and docosahexaenoyl CoA on linoleyl CoA and α-linolenyl CoA desaturation. Fractional inhibitions (fractional inhibition = 1 - velocity in the presence of inhibitor/velocity in the absence of inhibitor) are calculated for a substrate concentration 7 μM. (+- - -+) linoleyl CoA desaturation inhibited by docosapentaenoyl CoA. α-linolenyl CoA inhibited by (○——○) docosahexaenoyl CoA and (●···●) docosapentaenoyl CoA.


for 4 min at 35 C in air with a variable concentration of either 1-^{14}C-linoleic acid (52.9 mC/mmole, 99% radiochemical purity) or 1-^{14}C-α-linolenic acid (22.0 mC/mmole, 89% radiochemical purity) in the presence of 4.5 mg of microsomal protein. The rat liver microsomes were isolated by differential centrifugation (2). The desaturase assay system contained, in μmoles: $ATPNa_2$, 4; CoA, 0.2; NADH, 2.5; $MgCl_2$, 15; lysolecithin, 0.3; GSH, 4.5; nicotinamide, 1; NaF, 125; and phosphate buffer (pH 7.0), 125; in 3 ml of 0.15 M KCl, 0.25 M sucrose solution. Lysolecithin was added to visualize better the inhibitory effects (9). In the second type of experiment, increasing concentrations of either docosa-4,7,10,13,16-pentaenoyl CoA or docosa-4,7,10,13,16,19-hexaenoyl CoA were incubated under the same experimental conditions with varying concentrations of either 1-^{14}C-linoleyl CoA or 1-^{14}C-α-linolenyl CoA in the presence of 0.5 mg of microsomal protein. The total volume of the incubation medium was 0.5 ml, and it contained 0.2 μmoles of NADH and 0.03 μmoles of phosphate buffer (pH 7.2). The acyl CoA thioesters were synthesized by the procedure of Kornberg and Pricer (10). After a 4 min incubation, the desaturation reaction was stopped by the addition of 2 ml of 10% (w/v) KOH. The amounts of γ-linolenic or octadeca-6,9,12,15-tetraenoic acid synthesized from 1-^{14}C-linoleic or 1-^{14}C-α-linolenic acid, respectively, under the different experimental conditions were measured by gas liquid radiochromatography using a Pye apparatus equipped with a proportional counter, as described in a previous report (2). From these values the approximate initial velocities, expressed as μmole substrate converted per liter per minute, were calculated for duplicate samples, and the mean plotted versus substrate concentration. The velocities were referred to the aforementioned amounts of microsomal protein used.

The curves shown in Figure 1 demonstrate that free docosapentaenoic acid, the terminal

member of the linoleic acid family, inhibits the desaturation of either linoleic or α-linolenic acid; however, no proportional inhibition was seen in the presence of increasing concentrations of the inhibitor. For this reason, the Ki was not calculated. The approximate Km were 3.9×10^{-5} M and 3.2×10^{-5} M for linoleic and α-linolenic acid respectively. An inhibitory effect was also seen when the acyl-CoAs were tested (Fig. 2). The approximate Km were 2.4×10^{-5} M and 2.9×10^{-5} M for linoleyl CoA and α-linolenyl CoA respectively. Therefore the inhibition is produced at the desaturation step and not at the acylation. Docosapentaenoyl-CoA inhibited the desaturation of either linoleyl CoA or α-linolenyl CoA. However, the inhibitory effect of this acid was more pronounced on the desaturation of its own precursor. In a similar manner, docosahexaenoyl CoA produced an inhibitory effect on its own precursor, and this inhibition was greater than the inhibition evoked by docosapentaenoyl CoA on the same substrate (Fig. 3). Since both of these highly unsaturated acids are terminal members of the linoleic and α-linolenic acid families, they cannot be further desaturated in the liver. Hence, in this situation one is dealing with the phenomena of feedback inhibition and crossed inhibition. Because the only structural difference between both of these 22-carbon acids is the existence of a double bond at carbons 19 and 20, that is the specific double bond that distinguishes α-linolenic acid from linoleic acid series, the desaturating enzyme or enzymes for linoleic and α-linolenic acids must have a structure capable of detecting this small difference in the fatty acid effector molecule. The feedback and crossed inhibition found between both linoleic and α-linolenic acid families may have importance in the complicated regulation of the biosynthesis of polyunsaturated fatty acids as it was postulated in a previous work (3).

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