Reactions between Peroxidizing Lipids and Histidyl Residue Analogues: Enhancement of Lipid Oxidation and Browning by 4-Methylimidazole[1]

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ABSTRACT

As a part of our study on the interactions between peroxidizing lipids and the histidyl imidazole side-chain in simple, low-moisture model systems, 4-methylimidazole (4MI) was reacted with methyl linoleate (ML). This analogue was chosen to avoid interference from other functional groups in histidine (free base) or in proteins. Changes in the concentrations of lipid hydroperoxides, carbonyls, 4MI, and brown pigments were followed over a period of 24 days. The results indicate that 4MI exhibits significant prooxidative activity by reducing the induction period as well as by enhancing the formation of brown pigments. These effects are more pronounced at high 4MI/ML molar ratios and under basic pH's. Upon interactions with peroxidizing ML, as much as 44% of initially present 4MI was destroyed by the sixth day of incubation.

INTRODUCTION

Amino acids are often observed to affect the course of lipid autoxidation in foods and in model systems (1-7), but very little is known about their modes of action. Of the amino acids that have been investigated, histidine commands a particular interest because it exhibits strong antioxidative as well as prooxidative activities, depending on reaction conditions.

In aqueous dispersions and emulsions of linoleic acid, Marcuse (1) found that histidine exhibits strong antioxidative activity at basic pH's. This antioxidative effect was almost completely inhibitory under favorable conditions, and extremely low concentrations of histidine were effective. An inversion to prooxidative activity was observed when histidine concentrations were increased beyond 10^{-1} mole of histidine per mole of linoleic acid. Coleman et al. (2) observed that ionic emulsifiers promote histidine-catalyzed autoxidation of emulsified methyl linoleate (ML), while nonionic emulsifiers have no effect. Karel et al. (3) observed that histidine exhibited concentration-dependent antioxidative activity when it was reacted with ML at acidic pH's in a low-moisture model system. Again, some prooxidative effects were observed when histidine concentrations were increased beyond 10^{-2} mole histidine per mole ML.

The ability of histidyl functional groups to form complexes with transition metal ions (8,9) partly accounts for the anti- and prooxidative effects of histidine. In emulsions (10) as well as in low-moisture systems (4), histidine completely eliminated the catalytic activity of cobalt ions due to the formation of a noncatalyzing 6-coordinated complex. On the other hand, histidine promoted the catalytic activity of manganese ions, probably through the formation of a transient 7-coordinated complex, which would have strong magnetic properties to attract polar hydroperoxides and hold them long enough for electron transfer, subsequently initiating free radical reactions (11). In addition to these chelating effects, there are also indications that the α-carbonyl and imidazole groups of histidine are capable of forming a hydrogen-bonded intramolecular complex (12) that may "fit" a hydroperoxide group in a manner suitable for stabilization.

Aside from the stated effects, which involve the participation of histidyl α-carbon functional groups as well as the imidazole side-chain, the imidazole group by itself may exert some effects on lipid autoxidation. There is some evidence for side chains of proteins affecting lipid oxidation. Lin and Olcott (13) found that the antioxidative effect of proline results from the formation of a stable nitroxide radical due to the oxidation of its secondary amino group. Tannenbaum et al. (14) observed that the methyl sulfide side-chain of methionine may decompose linoleate hydroperoxides and form carbonyl compounds which, in turn, would lead to nonenzymatic browning. Besides having a secondary amino group which may be oxidized to stable nitroxide radicals, the imidazole ring of histidine has been observed to exhibit high reactivities with hydroxyl radicals, which are the most damaging species generated during the high-energy irradiation of aqueous systems (15-17) and which may have reaction character-

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istics similar to those of alkoxy radicals generated during lipid autoxidation.

This paper reports the effects of a histidine analogue, 4-methylimidazole (4MI), on the autoxidation of ML in a low-moisture model system that simulates the structure of freeze-dried foods. The use of 4MI avoids interference from other functional groups in histidine (free base) or in proteins, thus permitting us to study the specific effects of the imidazole side-chain on lipid autoxidation.

EXPERIMENTAL PROCEDURES

Materials

Except for 4MI (Research Organic Chemical Co., Belleville, NJ), which was distilled twice under vacuum (0.02 mm Hg) at ca. 120 C, reagent-grade chemicals were used in this work without further purification. ML was greater than 99% pure as obtained from Nu-Chek Prep. Inc., Elysian, MN.

Model System Preparation

The reaction system consisted of an 11-cm diameter filter paper (Whatman No. 42, Ashless Grade) on which the reactants were dispersed in the following manner: 4MI, dissolved in deionized water at different concentrations and adjusted to pH's of 5.6, 8.6, or 10.6 with 10% HCl and NaOH, was applied first, using a 2-ml graduated pipette so that each filter paper would contain 0.075, 0.0075, or 0.000075 g of 4MI. Following lyophilization at room temperature, 0.265 g of ML, dissolved in hexane, was applied to each filter paper to achieve 1:1, 10^{-1}:1, and 10^{-3}:1 molar ratios of reactants. After the evaporation of hexane, the filter papers were incubated in the dark at 37 C from 0 to 24 days over $CaSO_4$. Instead of the pH's (e.g., 4, 7, 9) used in previous investigations of the effects of histidine on lipid autoxidation, pH's of 5.6, 8.6, and 10.6 were used in our study to account for the difference in pKa values between the imidazole group in histidine (pKa ≅ 6) and that of 4MI (pKa = 7.6). The use of buffers was avoided in our system because their component chemicals can affect lipid oxidation and add complexities to the interpretation of the results (18).

Analytical Methods

At the end of an incubation period, each filter paper was cut into small pieces and extracted with 10 ml of carbonyl-free methanol by shaking vigorously under nitrogen for 30 min in 20-ml Wheaton vials. Carbonyl-free methanol was prepared according to the method of Lappin and Clark (19). After filtration to remove cellulose fines, the resulting extract was made up to 10 ml with carbonyl-free methanol, and lipid hydroperoxide, total carbonyl, and 4MI concentrations as well as browning were measured. The concentration of lipid hydroperoxides was determined by using a slight modification of the AOAC iodometric titration method (20). One ml of the methanol extract was mixed with 9 ml of chloroform-acetic acid (1:3, v/v) solution and iodometrically titrated with a standardized 10^{-3} N solution of $Na_2S_2O_3$. Total carbonyl concentrations were determined by the 2,4-dinitrophenylhydrazine method (19,21). Since E_{max} and A_{max} of the hydrazones formed from different carbonyls have been reported to remain relatively constant (19,21), acetophenone (Eastman Kodak, Co., Rochester, NY) was used to provide a standard curve for calibration at each sampling period. Depending on carbonyl concentrations in the methanol extracts, 10 μl to 0.8 ml of the extract was made up to 1 ml with carbonyl-free methanol and subjected to the procedures described by Lappin and Clark (19).

Browning was determined by measuring the absorbance of the methanol extracts at 400 nm, using a Beckman DU Quartz Spectrophotometer. The amount of 4MI in methanol extracts from samples containing 1 mole 4MI per mole ML was determined by modifying the Pauly's reagent test as described by Macpherson (22); 0.5 ml of 0.9% sulfanilic acid in 1 N HCl and 0.5 ml of 5% $NaNO_2$ in water were added to 5 μl of methanol extracts. With gentle swirling, 1.5 ml of 20% Na_2CO_3 in water was added to produce red/orange Pauly's pigment. Finally, 10 ml 20% ethanol solution in water was added for color stabilization. The absorbance of the resulting solution was determined at 550 nm and calibrated against a standard curve of 4MI.

RESULTS AND DISCUSSION

Changes in the concentrations of lipid hydroperoxides and carbonyls in our model system at different sampling periods are presented in Figures 1 and 2, respectively. From these figures, one can observe that 4MI reduces the induction period and enhances the disappearance of carbonyls formed from lipid hydroperoxide decomposition. The effects are pronounced at high 4MI/ML molar ratios (1 mole 4MI/mole ML) and under basic pH's, while they are almost negligible at low molar ratios (10^{-3} mole 4MI/mole ML). In samples where 4MI was reacted with ML at 10^{-1} mole

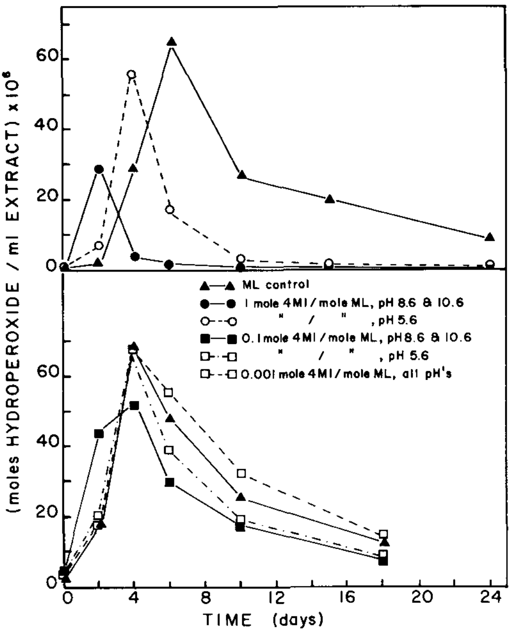

FIG. 1. Effects of 4MI on the formation and decomposition of lipid hydroperoxides.

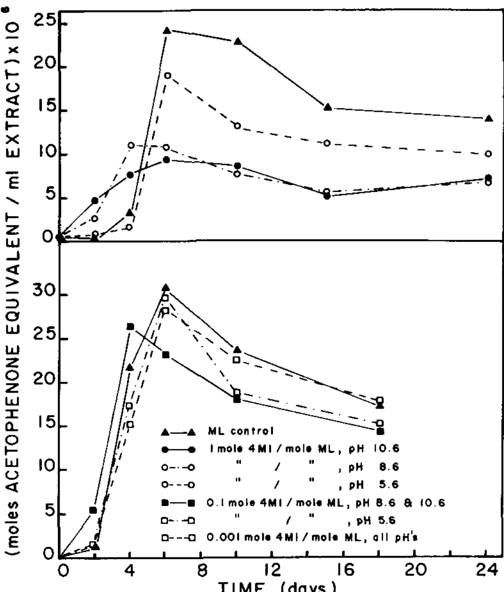

FIG. 2. Effects of 4MI on the formation and disappearance of carbonyls.

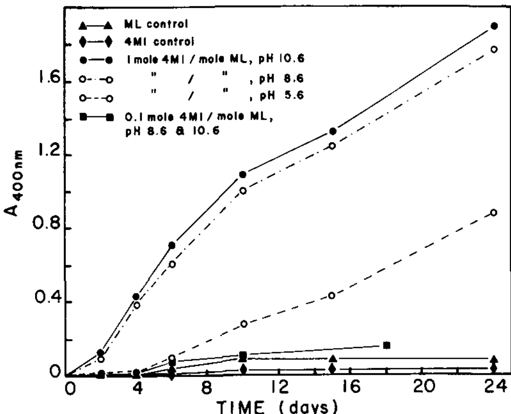

FIG. 3. Browning from the interactions of 4MI with peroxidizing ML.

4MI per mole ML, the stated effects were observed only under basic pH's. Although Mizuno and Chipault (23) have reported that lipid hydroperoxides may decompose to carbonyls during analysis and interfere with the determination of carbonyls in autoxidized fats by the 2,4-DNPH method, such interference does not appear to be a serious problem in our study. In addition to the finding that only a small fraction (12.5%) of methyl linoleate hydroperoxide is decomposed to carbonyls during analysis (23), we have observed that carbonyl concentrations in our samples remained relatively low when lipid hydroperoxide concentrations began to reach high levels after the induction period.

Figure 3 shows that the formation of brown pigments is also enhanced at high 4 MI/ML molar ratios and under basic pH's. In samples containing 10^{-1} mole 4MI per mole ML, browning was slightly accelerated under basic pH's but not at pH 5.6. At a lower molar ratio (10^{-3} mole 4MI/mole ML), 4MI had no effects on browning at any pH. The brown pigments from our samples were insoluble in water, 5% HCl, or 5% NaOH; partly soluble in ethyl ether or CCl_4; and soluble in alcohols or dimethyl sulfoxide. No nitrogen could be detected when these pigments were analyzed for their nitrogen content by the sodium fusion method (24) after repeated removal of 4MI by chromatography on a 1 x 50-cm silica gel 60 (EM Reagents) column, using 2-propanol as the mobile phase.

In Table I, one can observe that, upon exposure to peroxidizing ML, 4MI is rendered progressively unreactive to Pauly's reagent with increasing incubation time until a plateau is reached on the sixth day. This indicates a disruption of the imidazole ring because Pauly's reagent reacts only with imidazoles that do not have a substituted imino nitrogen or a carboxyalkyl substituent and that have at least one unsubstituted ring carbon atom (25). Again, degradation of 4MI was enhanced under basic

TABLE I

Loss of 4MI upon Interactions with Peroxidizing ML

Sample	% Loss				
	1 day	2 days	4 days	6 days	10 days
4 MI Controls (all pHs)	0	0	0	0	0
1 mole 4MI/mole ML (pH 5.6)	0	0	3	22	23
1 mole 4MI/mole ML (pHs 8.6 and 10.6)	0	22	36	44	44

pH's, and as much as 44% of initially present 4MI was lost after 6 days of incubation when it was reacted with ML at 1:1 molar ratio. It is interesting to note that 4MI degradation under either acidic or basic pH's did not proceed until high concentrations of lipid hydroperoxides began to accumulate in our samples (Fig. 1).

In a previous experiment where histidine was reacted with peroxidizing ML under reaction conditions similar to those used in the current investigation, Tjhio and Karel (4) found that low histidine/ML molar ratios (10^{-2}-10^{-3} mole histidine per mole ML) show early antioxidative activity followed by slightly prooxidative activity, while higher molar ratios (10^{-1} mole/mole ML) show an earlier and more powerful prooxidative effect. Our results indicate that the imidazole group of histidine is at least partly responsible for the observed prooxidative activity. As shortening of the induction period and browning were more pronounced at basic pH's, the free base form of the imidazole group is probably the active species. Since 4MI at 10^{-3} mole per mole ML showed no significant antioxidative activity, it can be postulated that the antioxidative activity of histidine, which is observed only at low concentrations of this amino acid (1,3,4), results from the formation of noncatalyzing complexes with transition metal ions (8,9,11) and/or hydrogen-bonded intramolecular complexes with an ability to stabilize lipid hydroperoxides (12). This antioxidative activity is probably lost when the reactive histidyl α-carbon functional groups are rapidly degraded upon interaction with peroxidizing ML (26). Although the imidazole group of histidine is reported to exhibit high reactivities with free radicals (15-17), our results do not indicate the formation of an antioxidative species, such as the stable nitroxide radical which is responsible for the antioxidative activity of proline (13).

The observation that no nitrogen could be detected in the brown pigments formed in our samples is consistent with the finding of Pokorny et al. (27), who studied the formation of brown pigments in mixtures of polyunsaturated fatty acid esters and casein or formaldehyde-treated casein. They found that the brown pigments contained very little nitrogen (1.1% by weight) and that browning occurred much faster in the casein-polyunsaturated fatty acid mixture than in the mixture containing formaldehyde-treated casein. Based on their observations that untreated casein also enhanced the decomposition of lipid hydroperoxides and the disappearance of carbonyls, Pokorny's group concluded that free amino groups in casein enhance the formation of brown pigments by catalyzing the decomposition of lipid hydroperoxides and the disappearance of carbonyls via Maillard-type polycondensation reactions. By an analogous mechanism, in which the basic imidazole nitrogen replaces the amino nitrogen, 4MI may enhance the formation of brown pigments from carbonyls formed during the decomposition of lipid hydroperoxides.

When histidine was reacted with peroxidizing ML in a low-moisture model system, degradative reactions were centered around the α-carbon functional groups, particularly the α-amino group, of histidine while the imidazole side-chain remained mostly intact, indicating the high relative stability of the imidazole ring (26). However, in this experiment, we have shown that as much as 44% of initially present 4MI becomes unreactive to Pauly's reagent due to the disruption of the imidazole ring upon interactions with peroxidizing ML. This observation may have nutritional as well as biological significance because histidyl residues in proteins are easily destroyed in the presence of peroxidizing lipids, and these residues, due to the acid-base characteristics of the imidazole group at physiological pH, often exist at active sites of enzymes and at metal-binding sites of metalloproteins. Our results indicate that extensive degradation of the imidazole ring takes place when 4MI, a histidine analogue which is more representative of the histidyl residues in proteins than free histidine, is reacted with peroxidizing ML. In proteins, α-carbon functional

groups of histidine participate in polypeptide linkages and they are expected to be much less reactive than their counterparts in free histidine.

Work is currently underway to characterize the reaction products resulting from the interactions between peroxidizing ML and histidyl residue analogues so that we can gain more insights on the reactions leading to the destruction of histidyl residues by peroxidizing lipids as well as the mechanisms leading to reduced induction periods and enhanced browning in the presence of an imidazole group.

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Linolenic Acid Deficiency: Changes in Fatty Acid Patterns in Female and Male Rats Raised on a Linolenic Acid-Deficient Diet for Two Generations

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ABSTRACT

Rats were fed for two generations a purified, linolenic acid-deficient diet in which the only source of lipid was purified methyl linoleate. This diet contained about 38 mg linolenic acid/kg diet. Control rats were given the same diet supplemented with methyl linolenate (2,500 mg/kg diet). Male and female rats ranged in age from weanling pups to adults. Lipids were extracted from liver, brain, kidney, spleen, heart, muscle, gastrointestinal tract, lung, ovary, testis, adrenal, plasma, erythrocytes, retina, and adipose tissue. Fatty acids of major phospholipid classes (choline phosphoglycerides, ethanolamine phosphoglycerides, and mixed serine phosphoglycerides plus inositol phosphoglycerides) or of total lipid extracts were measured by gas liquid chromatography. Growth rates and organ weights were similar in control and linolenic acid-deficient rats. The major effect of the deficiency was to lower the proportions of n-3 fatty acids, especially 22:6 n-3, in all the organs analyzed. Docosahexaenoic acid (22:6 n-3) was mainly replaced by 22:5 n-6 in deficient rats. The greatest changes in composition were found in brain, heart, muscle, retina, and liver.

INTRODUCTION

Linolenic acid (18:3 n-3) is a dietary essential fatty acid in the sense that it cannot be synthesized by vertebrates and must be obtained from the diet. It constitutes an important part of chloroplast membrane lipids and occurs also in other tissues of the plant, and is therefore present in many of our foods. Dietary linolenic acid is well absorbed during digestion and is incorporated into lipids or converted to higher homologs according to the classical scheme of desaturation and elongation:

$$18{:}3\ n\text{-}3 \rightarrow 18{:}4\ n\text{-}3 \rightarrow 20{:}4\ n\text{-}3 \rightarrow$$
$$20{:}5\ n\text{-}3 \rightarrow 22{:}5\ n\text{-}3 \rightarrow 22{:}6\ n\text{-}3 \rightarrow ?$$

Docosahexaenoic acid (22:6 n-3) is usually the most abundant member of the n-3 (linolenate) family of fatty acids in tissues of warm-blooded animals. It mainly occurs in membrane phospholipids, particularly in ethanolamine phosphoglycerides (EPG) and serine phosphoglycerides (SPG) and, in lesser proportions, in choline phosphoglycerides (CPG) (1). Docosahexaenoic acid in these phospholipids is selectively concentrated in certain tissues. For example, the retinas of pig, sheep, dog, rabbit, and human are rich in 22:6 n-3 (2), a fact which suggests that 22:6 n-3 may be a necessary constituent of the visual apparatus. Phospholipids of rat brain synaptosomal membranes are also rich in 22:6 n-3 (3), as are EPG in cerebral grey matter of human brain (4). These observations illustrate tissue specificity in incorporation of the higher homologs of linolenic acid into particular phospholipid molecules. This high degree of metabolic control suggests that 22:6 n-3 or a metabolite of 22:6 n-3 may serve a particular function in the animal.

So far, efforts to produce a linolenic acid deficiency in warm-blooded animals have not resulted in spectacular deficiency symptoms. It is difficult to make an experimental diet that is totally devoid of linolenic acid. Furthermore, linolenate homologs, especially 22:6 n-3, are tenaciously retained in tissue lipids, even when animals are fed fat-free diets (5). In an experiment designed to test the essentiality of 18:3 n-3, rats were raised for three generations on a diet in which purified methyl linoleate was the only source of lipid. There was little effect of linolenic acid deficiency on growth, reproduction, or behavior, although the content of 22:6 n-3 in tissue lipids declined greatly (6). More recently, Lamptey and Walker (7) have reported differences in physical activity and in learning ability, in rats given different levels of linolenic acid in the diet. This work will be reviewed more thoroughly in the Discussion.

A possible linolenic acid deficiency has been produced in Capuchin monkeys by keeping them for several months on a diet containing 5% corn oil as the source of essential fatty acid (8). The monkeys developed skin lesions, etc., which were cured by dietary linseed oil. This report is puzzling, because liver lipids in the deficient monkeys still contained considerable n-3 fatty acids.

In contrast with these mammals, rainbow trout definitely require about 1% of either 18:3 n-3 or 22:6 n-3 in the diet for normal growth and behavior (9,10). Linoleic acid (18:2 n-6)

has little essential fatty acid activity in this species, but German carp seem to require both 18:2 n-6 and 18:3 n-3 in the diet (11). Both the red sea bream (12) and the turbot (13) require n-3 fatty acids, but these fish require the longer-chain homologs because they seem unable to elongate and desaturate 18:3 n-3. It is clear that in these species of fish, n-3 fatty acids perform some specific function.

The observations with fish strongly suggest that n-3 fatty acids may serve functions in warm-blooded animals. As a first step in the search for a possible function of n-3 fatty acids, we have raised rats for two generations on a diet very low in linolenic acid. This diet is known to reduce the concentrations of n-3 fatty acids in rats (6), but it was not known which tissues would be most severely affected. We have analyzed a large number of tissues in search of major changes caused by linolenic acid deficiency. The goal of these experiments was to find the tissues in which linolenic acid deficiency produced the most extreme changes in lipid composition. Changes in function should be expected in those tissues most greatly affected because alterations in membrane lipid composition can influence membrane permeabilities or the activities of membrane-bound enzymes.

EXPERIMENTAL DESIGN

Two generations of rats were used because depletion of n-3 fatty acids from rat tissue lipids is slow. Female weanling rats were started on experimental diets and bred when mature. Litters were raised, and animals of each sex were killed for analysis at 23 days (weaning age), 80-81, 92-97, 169-174, 190, and 235 days. Females of the first generation were killed at 145-297 days (controls) or 212-340 days (deficient). In 15 deficient and 11 control animals, the organs listed below were analyzed.

Animals and Diets

Female Long-Evans rats (about 45 g, Simonsens' Laboratories, Gilroy, CA) were housed individually in galvanized, screen-bottomed cages and were given food and water ad libitum. Room temperature was 21 C, and the laboratory was lighted from 8 a.m. to 8 p.m. Diets contained either 1.25% methyl linoleate (deficient diet) or 1.0% methyl linoleate plus 0.25% methyl linolenate (control). Methyl esters were obtained from Nu-Chek Prep., Elysian, MN, and we added 0.02% BHT (Sigma). Diets were prepared by adding methyl esters to premixed dry ingredients (Table I). Fresh diet was offered to the rats every 2 or 3 days.

TABLE I

Composition of Diets

Ingredient		Amount
		% by weight
Sucrose		75
Casein[a]		20
Minerals[b]		3.5
Choline		0.25
Methyl esters		1.25
		mg/kg diet
Thiamine HCl		5.0
Riboflavin		10
Pyridoxine HCl		9.6
Ca-pantothenate		30
meso-Inositol		100
Biotin		1.0
Folic acid		2.0
Nicotinamide		60
B_{12}		0.020
Menadione		0.40
Vitamin A palmitate (Units)		10,000
Vitamin D_3 (Units)		1,000
Vitamin E (Units)		220
	Control	Deficient
Methyl Linoleate	10,000	12,500
Methyl linolenate	2,500	~ 38

[a]Teklad, Grand Island Biol. Co.

[b]Reference 14.

Analysis of diet stored in air at room temperature or in a freezer showed that the methyl esters were stable for 3 days or more at room temperature and for more than a week in a freezer. There were small amounts of linolenic acid in the methyl linoleate and in the casein, which contributed about 38 mg 18:3 n-3/kg diet to the linolenate-deficient diet.

When the female rats weighed 200 g or more (about 8 weeks), they were mated with Long-Evans male rats. Litters were reduced to 6/dam, and pups were weaned at 22-23 days to their dam's diets. The original females are referred to as first generation and the offspring as second generation rats.

Extraction and Fractionation of Lipids

Unfasted rats were anesthetized with sodium pentobarbital, and blood was withdrawn from the heart into a heparinized syringe. Liver, heart, kidney, spleen, lung, gastrointestinal tract, ovary or testis, brain, adipose tissue, adrenal, retina, and muscle were homogenized in chloroform-methanol (2:1, v/v), to which 0.1 mg of hydroquinone had been added. Plasma and erythrocytes were separated and extracted. Gastrointestinal tract samples included the stomach, intestine, cecum, and colon. Muscle was taken from the hind leg and included parts

TABLE II

Concentrations and Distributions of Phospholipids in Tissues from Linolenic Acid-Deficient and Control Rats (Mean values[a])

Tissue	No.[b] in group	Age (days)	Phospholipid[c] (mg/g fresh wt)	Percentages of lipid phosphorus				
				O-Sph	CPG	SPG + IPG	EPG	"NL"
Brain	4	23	43.7[d]	8.3	39.4	11.2	39.0	2.1
	14	80-235	55.0	8.4	34.5	11.4	40.8	4.8
Liver	14	23-235	33.1	8.2	51.9	10.3	23.8	5.9
Lung	16	23-235	29.4	12.4	49.6	13.6	22.4	1.9
Kidney	4	23	27.6[d]	19.2	36.2	11.3	27.1	6.2
	14	80-235	34.2	16.7	35.8	11.8	28.8	6.8
Heart	18	23-235	25.9	6.5	40.1	7.6	33.2	12.7
Spleen	14	80-235	21.2	18.5	41.7	13.1	24.4	2.4
Testis	6	81-235	17.0	18.5	41.0	11.7	24.0	4.2
Digestive tract	16	23-235	12.8	16.2	42.0	11.4	27.0	3.4
Muscle	4	23	14.1[d]	6.1	50.4	10.1	29.6	3.8
	14	81-235	11.3	4.7	55.0	8.7	25.5	6.2

[a]Standard errors of mean less than 5% of mean for mg phospholipid, and less than 10% of mean for nearly all values of lipid phosphorus.

[b]Equal numbers of control and deficient rats, paired for age and sex.

[c]Phospholipid = lipid phosphorus x 25.

[d]Significant different between 23-day-old and older rats, $p < 0.025$ or less.

of the gluteus maximus, biceps femoris, and semitendinosus muscles. Subcutaneous adipose tissue was taken from the flank. All tissues except adrenal, retina, RBC, and plasma were dispersed with a Polytron PCU-2 homogenizer (Brinkmann). Each tissue was extracted twice. Combined extracts were reduced to dryness on a rotary evaporator, and lipids were taken up in petroleum ether (B.R. 30-45 C).

Reagent-grade chemicals and solvents were used in all analytical procedures. Lipid extracts were analyzed for phosphorus (15). Extracts of ovaries, adrenals, adipose tissue, retina, plasma, and erythrocytes were analyzed for total fatty acids. The other extracts were separated by thin layer chromatography (TLC) (16) into five fractions: "Neutral Lipids" ("NL"), ethanolamine phosphoglycerides (EPG), serine phosphoglycerides plus inositol phosphoglycerides (SPG + IPG), choline phosphoglycerides (CPG), and a fraction containing sphingomyelins, lysophosphatidylcholines, and the origin (0-Sph). The major components (cholesterol, triglycerides, EPG, IPG, SPG, CPG, Sph, and lysolecithins) were identified by comparison with authentic samples. EPG and SPG were identified with ninhydrin spray. There was no attempt to obtain absolutely pure fractions. The "Neutral Lipid" fraction contained phosphorus, which was tentatively attributed to phosphatidyl glycerol and/or phosphatidic acid. This fraction also contained cholesterol, triglycerides, etc.

Fatty acids of CPG, EPG, and SPG + IPG were converted to methyl esters and analyzed by gas liquid chromatography (GLC). Preliminary experiments had shown that the other fractions ("NL" and 0-Sph) contained little polyunsaturated fatty acid, so these fractions were not analyzed for fatty acids.

Methyl esters of EPG, CPG, and SPG + IPG were prepared by scraping TLC bands into screw-cap tubes. Methanol, 6 ml, a measured amount of internal standard (heptadecanoic acid, Nu-Chek Prep.), and 1 drop H_2SO_4 were added, and tubes were flushed with nitrogen and sealed. Tubes were heated 16 hr at 80 C. Tubes were cooled, and the silica gel was eluted three times with anhydrous methanol and once with 5% water in methanol. Eluates were combined, and their volumes were reduced by boiling to about 0.5-1.0 ml. An equal volume of water and 5 vol of petroleum ether were added. Methyl esters were recovered in the petroleum ether phase, and the aqueous phase was analyzed for phosphorus (15). This procedure forms methyl esters from fatty acid acyl esters, and it will produce dimethyl acetals when plasmalogens are present. In certain samples, GLC analysis showed components which did not correspond to the major fatty acid methyl esters or their homologs. These components were tentatively identified as dimethyl acetals, and they were excluded from the calculations of fatty acid patterns.

Gas Liquid Chromatography

Analyses of methyl esters were performed on a Varian 2100 chromatograph with hydro-

gen flame detectors and columns of 10% SP 216 PS on Supelcoport 100/120 (Supelco, Inc., Bellefont, PA). Retention times and relative responses of 16:0, 18:0, 18:1, 18:2 n-6, 18:3 n-3, 20:4 n-6, and 22:6 n-3 were determined by use of a standard mixture containing known amounts of these methyl esters. Individual methyl esters were obtained from Nu-Check Prep. Components other than those mentioned above were tentatively identified by use of log plots or by comparison of relative retention times with literature data (17). Data are reported as weight percentages of total fatty acid methyl esters.

Enzyme Assays

The essential fatty acid status of an animal is known to influence, in certain cases, the activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, malic enzyme, fatty acid synthetase (18), 5′-nucleotidase (19,20), and phosphodiesterase (Williams, Wehenmeyer, and Yang, unpublished). Therefore, liver homogenates were prepared from two control and two deficient rats of the same age and sex, and these enzymes were assayed. No consistent effects of diet were found. Likewise, no effects of diet were found on activities of 5′-nucleotidase in brain homogenate or liver plasma membranes, nor in phosphodiesterase of brain homogenate (Williams, Wehenmeyer, and Yang, unpublished).

Statistical Calculations

In all comparisons between diets, the control group of rats was compared with a set of deficient rats of the same ages and sexes. Statistical significance was calculated by use of the *t*-test 21). Standard errors (not shown) ranged from 1.8 to 37% of the mean values. Most of the standard errors were less than 10% of the mean.

RESULTS

Introduction

The diets had little effect on organ weights, lipid phosphorus concentrations, or phospholipid distributions, although some small effects might be demonstrated if larger numbers of animals were used. Therefore, these data for both diets were combined. Phospholipid concentrations did depend upon the ages of the animals in some cases, and the most extreme examples are shown.

Fatty acid compositions were profoundly affected by diet, and smaller effects of sex and age were visible also. Only in the deficient group were there enough animals for statistical evaluations of sex differences. For the sake of brevity, the relatively small effects of sex and age on fatty acid compositions are not shown in the tables, but are mentioned in the text.

Influence of Diet, Age, and Sex upon Organ Weights

Body weights of control rats were slightly greater, on the average, than deficient rats of the same age and sex, but the difference was not significant. Weights of organs were not influenced by linolenic acid deficiency (data not shown). Our values for male rats were similar to those found in laboratory chow-fed male rats of different ages (22).

Effects of Diet, Age, and Sex upon Phospholipid Concentrations and Distributions in Tissues

Table II shows concentrations and percentage distributions of phospholipids in tissues of control and linolenic acid-deficient rats. Linolenic acid deficiency had no significant influence upon phospholipid concentrations or distribution of lipid phosphorus in any tissue measured. The data for both diets are combined.

The age of the rat had no significant effect upon phospholipid concentrations in liver, lung, heart, spleen, testis, or gastrointestinal tract, at least at the ages measured (Table II). Brain and kidney phospholipid concentrations were significantly lower in weanlings (23 days) than in adults. Muscle phospholipid concentrations were greater in weanlings than in adults. The sex of the animal had no significant influence upon phospholipid concentration or distribution.

Influences of Diet, Age, and Sex upon Fatty Acid Patterns in Phospholipids of Rat Tissues

Brain: Polyunsaturated fatty acids of brain phospholipids were profoundly affected by the linolenic acid-deficient diet (Table III). In EPG, 22:6 n-3 and 22:5 n-3 declined, and 22:5 n-6, 22:4 n-6, and 20:4 n-6 were increased by the deficiency. In SPG + IPG, 22:6 n-3 decreased and 22:5 n-6 increased, each by more than tenfold. In CPG, 22:4 n-6 and 22:5 n-6 increased, and 22:6 n-3 decreased.

Brain CPG, SPG + IPG, and EPG in deficient females of the first generation retained about 1%, 6% and 9%, respectively, of 22:6 n-3 (data not shown), despite the fact that they had been given the deficient diet from weaning to ages of 212-340 days.

Of all the tissues analyzed, brain phospholipids contained the highest proportions of 18:1, and the lowest proportions of 18:2 n-6,

TABLE III

Fatty Acids in Brain Phospholipids of Linolenic Acid-Deficient and Control Rats (Weight percentages of total methyl esters[a])

	EPG		SPG + IPG		CPG	
Methyl ester	Control[b]	Deficient	Control[b]	Deficient	Control[b]	Deficient
16:0	7.7	8.0	5.3	4.8	39.5	40.9
18:0	22.5	23.9	36.5	39.6	16.1	15.6
18:1	22.6	19.9	23.1	23.5	29.6	28.4
18:2 n-6	0.27	0.23	0.27	0.15	0.43	0.43
20:4 n-6	11.8	13.8[c]	11.1	10.1	6.4	7.4
22:4 n-6	5.5	7.7[e]	2.8	3.4	0.83	1.2[d]
22:5 n-6	0.97	20.4[e]	0.90	14.6[e]	0.43	3.3[e]
22:5 n-3	0.33	0.02[e]	0.18	0.07	0.13	<0.01
22:6 n-3	23.7	1.8[e]	16.6	1.2[e]	4.1	0.50[e]

[a]Some minor components omitted.

[b]Six controls paired with six deficient rats of same age and sex.

[c,d,e]Underlined values significantly different from control values, $p < 0.01$, < 0.005, < 0.001, respectively.

TABLE IV

Fatty Acids in Liver Phospholipids of Linolenic Acid-Deficient and Control Rats (Weight percentages of total methyl esters[a])

	EPG		SPG + IPG		CPG	
Methyl ester	Control[b]	Deficient	Control[b]	Deficient	Control[b]	Deficient
16:0	18.4	16.6	7.9	7.5	22.1	21.6
18:0	28.1	25.4	43.3	40.2	27.5	25.2
18:1	5.9	6.2	4.3	4.3	12.0	10.9
18:2 n-6	2.6	2.6	1.8	1.9	7.8	6.7
20:4 n-6	25.0	31.8[c]	28.2	35.0[d]	19.2	26.5[f]
22:4 n-6	0.58	1.6[c]	0.69	1.2[e]	0.31	0.63[c]
22:5 n-6	1.2	14.1[c]	1.1	8.8[f]	0.66	5.6[c]
22:5 n-3	1.4	0.04[c]	1.0	0.02[c]	0.64	0.03[c]
22:6 n-3	15.6	0.71[c]	10.7	0.51[c]	7.1	0.34[c]

[a]Some minor components omitted.

[b]Nine controls paired with nine deficient rats of same age and sex.

[c,d,e,f]Underlined values significantly different from control values, $p < 0.001$, < 0.025, < 0.01, < 0.005, respectively.

with either diet. No significant differences between sexes were seen.

Liver: Fatty acids of liver phospholipids are shown in Table IV. In EPG, linolenic acid deficiency produced a large decrease in 22:6 n-3 and large increases in 22:5 n-6 and 20:4 n-6, plus smaller changes in 22:5 n-3 and 22:4 n-6. In CPG, 22:6 n-3 and 22:5 n-3 were reduced, while 22:5 n-6, 22:4 n-6, and 20:4 n-6 were increased. Similar changes were seen in SPG + IPG. No pronounced effects of age were seen in fatty acids of liver phospholipids.

In livers of deficient female rats, proportions of 22:5 n-6 were higher than in males, in CPG, SPG + IPG, and EPG ($p < 0.005$, < 0.40, and < 0.025). In CPG, deficient females had more 18:0 and less 16:0 than males did ($p < 0.01$, < 0.05; data not shown).

Lung: Linolenic acid deficiency produced smaller changes in fatty acid patterns of rat lung phospholipids (Table V). In EPG, 22:6 n-3 and 22:5 n-3 decreased, while 22:5 n-6, 22:4 n-6, and 20:4 n-6 increased. In SPG + IPG, 22:6 n-3 and 22-5 n-3 decreased, 22:5 n-6 and 22:4 n-6 increased, and 20:4 n-6 was unchanged. In CPG, 22:6 n-3 and 22:5 n-3 were reduced, while 22:5 n-6, 22:4 n-6, and 20:4 n-6 were increased.

No significant effects due to age or sex were seen in fatty acids of lung phospholipids.

Kidney: In control rats, kidney phospholipid contained relatively little n-3 fatty acid, and in deficient rats the levels were even lower (Table VI). Linolenic acid deficiency reduced 22:6 n-3 and 22:5 n-3, and increased 22:5 n-6 and 22:4

TABLE V

Fatty Acids in Lung Phospholipids in Linolenic Acid-Deficient and Control Rats
(Weight percentages of total methyl esters[a])

Methyl ester	EPG Control[b]	EPG Deficient	SPG + IPG Control[b]	SPG + IPG Deficient	CPG Control[b]	CPG Deficient
16:0	11.5	10.1	14.2	15.5	54.9	53.7
16:1	1.5	1.5	2.4	2.9	8.7	8.3
18:0	13.7	13.7	32.6	31.2	7.3	7.4
18:1	17.5	15.6	27.0	25.9	15.0	13.4
18:2 n-6	2.0	2.1	1.9	2.3	3.7	3.9
20:4 n-6	33.3	36.2[c]	13.0	13.1	7.6	9.8[f]
22:4 n-6	8.6	11.8[d]	3.2	4.3[d]	0.75	1.1[g]
22:5 n-6	1.2	7.1[e]	0.69	3.4[e]	0.36	1.2[e]
22:5 n-3	3.8	0.11[e]	1.4	0.08[e]	0.40	0.01[e]
22:6 n-3	6.1	0.26[e]	2.3	0.21[e]	0.75	0.04[e]

[a]Some minor components omitted.

[b]Eight controls paired with eight deficient rats of same age and sex.

[c,d,e,f,g]Underlined values significantly different from control values, $p < 0.05$, < 0.01, < 0.001, <0.025, < 0.005, respectively.

TABLE VI

Fatty Acids in Kidney Phospholipids of Linolenic Acid-Deficient and Control Rats
(Weight percentages of total methyl esters[a])

Methyl ester	EPG Control[b]	EPG Deficient	SPG + IPG Control[b]	SPG + IPG Deficient	CPG Control[b]	CPG Deficient
16:0	12.6	10.4	8.8	7.7	34.9	32.5
18:0	21.3	23.0	41.6	42.8	15.2	17.0
18:1	12.1	11.2	8.2	7.5	16.3	14.3
18:2 n-6	3.6	2.8	2.2	1.8	8.0	5.6[c]
20:4 n-6	42.8	45.2[d]	34.4	35.2	19.1	25.7[e]
22:4 n-6	0.92	1.5[e]	1.0	1.4[f]	0.40	0.66[c]
22:5 n-6	0.59	4.5[e]	0.52	2.5[e]	0.59	1.9[e]
22:5 n-3	0.50	0.03[e]	0.32	0.02[e]	0.37	<0.01[e]
22:6 n-3	4.4	0.37[e]	1.9	0.33[e]	2.4	0.24[e]

[a]Some minor components omitted.

[b]Nine controls paired with nine deficient rats of same age and sex.

[c,d,e,f]Underlined values significantly different from controls, $p < 0.01$, < 0.005, <0.001, < 0.05, respectively.

n-6, in all phospholipid classes. In EPG and CPG, 20:4 n-6 was increased by the deficient diet. The deficiency tended to reduce the proportions of 18:2 n-6, and this difference was significant in CPG. There was no significant influence of age on fatty acid patterns in kidneys.

In kidneys of deficient rats, percentages of 22:5 n-6 were higher in females than in males, in all phospholipid classes ($p < .025$ to $< .001$), and females had higher proportions of 18:1 in CPG and SPG + IPG ($p < .001$, $< .01$). Females also had lower proportions of 18:0 in EPG than males did ($p < .025$) (data not shown).

Heart: In rat hearts (Table VII), linolenic acid deficiency severely reduced 22:6 n-3 and 22:5 n-3 in all phospholipid classes and increased the proportions of 22:5 n-6 and 22:4 n-6. 20:4 n-6 was increased significantly in CPG and EPG but not in SPG + IPG. We saw no pronounced effects of age upon fatty acids in heart phospholipids.

Proportions of 22:5 n-6 were higher in deficient females than in males, in all classes of heart phospholipids ($p < .05$ to $< .001$). Deficient females also had levels of 18:2 n-6 lower than those of males, in CPG and EPG ($p < .05$, $< .005$). In SPG + IPG, deficient male rats had higher 18:0 and lower 16:0 than females did ($p < .025$, $< .05$) (data not shown).

Spleen: In rat spleen (Table VIII), linolenic acid deficiency reduced 22:6 n-3 and 22:5 n-3, and increased 22:5 n-6 and 22:4 n-6 in all phospholipid classes. In EPG, the deficiency increased the proportion of 20:4 n-6. There appeared to be no influences of age or sex upon phospholipid fatty acids in spleen.

TABLE VII

Fatty Acids in Heart Phospholipids in Linolenic Acid-Deficient and Control Rats
(Weight percentages of total methyl esters[a])

	EPG		SPG + IPG		CPG	
Methyl ester	Control[b]	Deficient	Control[b]	Deficient	Control[b]	Deficient
16:0	10.9	9.7	10.6	11.4	20.9	19.2
18:0	26.1	26.9	37.0	35.4	27.2	28.7
18:1	6.0	6.0	12.8	13.2	12.2	10.5
18:2 n-6	3.6	3.7	3.5	4.1	9.0	7.8
20:4 n-6	23.3	27.3[c]	18.7	17.2	23.8	28.0[d]
22:4 n-6	1.8	3.8[e]	1.7	3.5[e]	0.69	1.4[f]
22:5 n-6	2.1	21.1[e]	1.2	13.6[e]	0.47	3.0[e]
22:5 n-3	2.7	0.04[e]	2.0	0.06[e]	1.3	0.11[e]
22:6 n-3	23.3	0.72[e]	11.8	0.69[e]	4.1	0.22[e]

[a]Some minor components omitted.

[b]Nine controls paired with nine deficient rats of same age and sex.

[c,d,e,f]Underlined values significantly different from controls, $p < 0.025$, < 0.01, < 0.001, < 0.005, respectively.

TABLE VIII

Fatty Acids in Spleen Phospholipids of Linolenic Acid-Deficient and Control Rats
(Weight percentages of total methyl esters[a])

	EPG		SPG + IPG		CPG	
Methyl ester	Control[b]	Deficient	Control[b]	Deficient	Control[b]	Deficient
16:0	8.4	8.6	5.8	5.4	38.6	42.2
18:0	19.5	18.5	42.1	42.2	13.9	12.8
18:1	12.3	11.0	18.5	17.0	17.4	15.3
18:2 n-6	2.3	2.2	2.1	2.1	5.5	4.8
20:4 n-6	38.7	41.3[c]	22.1	24.0	17.9	19.1
22:4 n-6	6.5	9.7[d]	2.8	4.0[d]	1.0	1.3[d]
22:5 n-6	1.2	6.9[d]	0.63	3.7[d]	0.38	1.3[d]
22:5 n-3	3.6	0.08[d]	1.5	0.02[d]	0.52	< 0.01[d]
22:6 n-3	6.0	0.25[d]	2.9	0.30[d]	1.3	0.27[e]

[a]Some minor components omitted.

[b]Six controls paired with six deficient rats of same age and sex.

[c,d,e]Underlined values significantly different from control values, $p < 0.05$, < 0.001, < 0.005, respectively.

Testis: Fatty acids of phospholipids in testis are shown in Table IX. Linolenic acid deficiency had little influence on fatty acid patterns in this tissue, although it did reduce the already low proportions of 22:6 n-3 in all phospholipid classes. We saw no influence of age upon fatty acid patterns in testis.

Gastrointestinal tract: In phospholipids of gastrointestinal tract (Table X), linolenic acid deficiency reduced proportions of 22:6 n-3 and 22:5 n-3, and increased 22:5 n-6 in all phospholipid classes. In EPG and SPG + IPG, 20:4 n-6 was increased. In CPG and EPG, 22:4 n-6 was increased by the deficiency. Proportions of 22:5 n-6 were significantly higher in deficient females than in males, in SPG + IPG and in EPG ($p < .005$, $< .05$) (data not shown). There appeared to be no effect of age upon fatty acid patterns in phospholipids of gastrointestinal tract.

Muscle: Fatty acids of muscle phospholipids are shown in Table XI. Linolenic acid deficiency greatly reduced the proportions of 22:6 n-3 and 22:5 n-3 in all phospholipid classes, and increased those of 22:5 n-6 and 22:4 n-6. The deficiency significantly increased the proportions of 20:4 n-6 in CPG and EPG. In muscle phospholipids of control rats, the proportions of 22:6 n-3 increased somewhat with increasing age of the rat (data not shown). The three deficient females of the first generation had slightly higher proportions of 22:6 n-3 in phospholipids than were found in second-generation rats (data not shown). In deficient rats, proportions of 22:5 n-6 were significantly higher in females than in males, in CPG and EPG (p <

TABLE IX

Fatty Acids in Testis Phospholipids of Linolenic Acid-Deficient and Control Rats
(Weight percentages of total methyl esters[a])

	EPG		SPG + IPG		CPG	
Methyl ester	Control[b]	Deficient	Control[b]	Deficient	Control[b]	Deficient
16:0	23.0	21.2	25.3	30.8	41.9	42.4
18:0	7.2	7.7	22.7	21.4	5.6	6.1
18:1	9.6	8.2	8.5	8.7	18.5	17.1
18:2 n-6	2.7	1.5[c]	1.1	0.63	4.4	3.1[d]
20:4 n-6	23.0	24.7	22.0	19.0	14.4	15.5
22:4 n-6	3.2	3.7	2.0	2.3	1.2	1.2
22:5 n-6	27.9	31.9	16.5	16.6	11.8	13.4
22:5 n-3	0.23	0.10[d]	0.20	0.10	0.17	< 0.01
22:6 n-3	2.5	0.13[c]	1.2	0.17[e]	0.90	< 0.01[f]

[a]Some minor components omitted.

[b]Three controls paired with three deficient rats of same age.

[c,d,e,f]Underlined values significantly different from controls, $p < 0.005$, < 0.025, < 0.01, < 0.001, respectively.

TABLE X

Fatty Acids of Phospholipids in Gastrointestinal Tract of Linolenic Acid-Deficient and Control Rats
(Weight percentages of total methyl esters[a])

	EPG		SPG + IPG		CPG	
Methyl ester	Control[b]	Deficient	Control[b]	Deficient	Control[b]	Deficient
16:0	8.1	7.8	7.3	6.2	28.7	29.9
18:0	26.8	25.8	42.5	39.1	17.3	16.3
18:1	16.4	14.4	18.0	17.0	19.4	17.6
18:2 n-6	8.0	8.3	4.9	5.6	14.6	15.1
20:4 n-6	26.7	30.0[c]	17.3	21.6[c]	13.5	14.0
22:4 n-6	4.0	6.0[d]	3.3	4.6	0.96	1.4[e]
22:5 n-6	0.83	5.4[d]	0.71	3.7[d]	0.44	1.2[d]
22:5 n-3	1.7	< 0.01[d]	0.96	0.02[d]	0.39	0.01[d]
22:6 n-3	5.5	0.34[d]	2.7	0.23[d]	0.99	0.01[d]

[a]Some minor components omitted.

[b]Eight controls paired with eight deficient rats of same age and sex.

[c,d,e]Underlined values significantly different from controls, $p < 0.025$, < 0.001, < 0.01, respectively.

.01, < 0.25) (data not shown).

Other tissues: Fatty acids of total lipids from erythrocytes, plasma, ovary, retina, adrenal, and adipose tissue are shown in Table XII. Linolenic acid deficiency significantly reduced proportions of 22:6 n-3 and 22:5 n-3 in all samples except adipose tissue. In adipose tissue, these components occurred in such minute proportions that precise measurements were not possible. The deficiency increased the percentages of 22:5 n-6 in erythrocytes, plasma, ovary, retina, and adrenal and produced smaller increases in 22:4 n-6 in erythrocytes, plasma, ovary, and retina. Adrenals of both control and deficient rats had similar proportions of 22:4 n-6, but in deficient rats, higher proportions of 22:4 n-6 were found in females than in males ($p < .05$) (data not shown). First-generation deficient rats had higher proportions of 22:6 n-3 in retina than were found in second-generation rats (about 13% vs. 5%; data not shown) even though the first-generation rats had eaten the deficient diet for a longer time.

Summary of Results

The main effect of linolenic acid deficiency was to reduce the proportions of n-3 fatty acids, especially 22:6 n-3, in all tissues analyzed. The reduction in 22:6 n-3 was balanced mainly by increases in 22:5 n-6 and smaller increases in 22:4 n-6. In some tissues, 20:4 n-6 was increased in linolenic acid-deficient rats (muscle, liver). In several tissues of deficient rats, the proportions of 22:5 n-6 were higher in females than in males (liver, kidney, heart, muscle, and gastrointestinal tract).

TABLE XI

Fatty Acids in Muscle Phospholipids of Linolenic Acid-Deficient and Control Rats
(Weight percentages of total methyl esters[a])

	EPG		SPG + IPG		CPG	
Methyl ester	Control[b]	Deficient	Control[b]	Deficient	Control[b]	Deficient
16:0	7.9	7.5	7.4	7.4	36.9	35.4
18:0	23.5	25.8	39.9	40.0	8.2	8.7
18:1	5.7	6.4	10.6	10.6	13.0	11.0
18:2 n-6	5.1	6.0	2.7	3.6	14.5	14.8
20:4 n-6	13.0	<u>21.6</u>[c]	15.7	18.7	17.6	<u>24.4</u>[d]
22:4 n-6	1.3	<u>5.2</u>[e]	1.9	<u>4.0</u>[e]	0.46	<u>0.83</u>[c]
22:5 n-6	1.6	<u>24.6</u>[e]	1.2	<u>13.6</u>[e]	0.44	<u>2.3</u>[e]
22:5 n-3	4.9	<u>0.23</u>[e]	3.4	<u>0.07</u>[e]	1.2	<u>0.01</u>[e]
22:6 n-3	36.1	<u>1.5</u>[e]	15.9	<u>0.84</u>[e]	5.2	<u>0.14</u>[e]

[a]Some minor components omitted.

[b]Seven controls paired with seven deficient rats of same age and sex.

[c,d,e]Underlined values significantly different from control values, $p < 0.005$, < 0.01, < 0.001, respectively.

Phospholipids of muscle, brain, heart, and liver contained much higher percentages of 22:6 n-3 than were found in other tissues. The percentage of 22:6 n-3 was also very high in total fatty acids of retina.

Retina, brain, and muscle seemed to retain 22:6 n-3 more strongly than the other tissues, because first-generation deficient rats had higher proportions of 22:6 n-3 in lipids of these tissues than were found in second-generation deficient rats. In other tissues, there was little difference between the generations.

DISCUSSION

The main purpose of this work was to identify tissues in which linolenic acid deficiency produces large changes in lipid composition as a guide to future studies of function in such tissues. A few preliminary assays of enzyme activities in brain and liver were made (see Experimental Design), but no consistent effects of diet were found. Bernsohn and Spitz (20) studied 5'-nucleotidase activity in brain microsomes of EFA-deficient and chow-fed rats. They found that dietary linolenic acid could restore the 5'-nucleotidase activity in EFA-deficient rats to that found in chow-fed rats, but that dietary linoleic acid did not have this effect. In our rats, dietary linolenic acid did not influence 5'-nucleotidase activities in brain homogenate or liver plasma membranes. These few experiments obviously do not exclude the possibility of changes in enzyme activity, and future work should include studies of retina, heart, and muscle, in larger groups of animals.

One of the more interesting results of linolenic acid deficiency was the change that *did not occur* in fatty acid patterns. The deficiency caused practically no alterations in the proportions of 16:0, 18:0, or 18:1 in any lipid class or in any tissue, despite the great decline in 22:6 n-3 and increases in 22:5 n-6, 22:4 n-6, and in some cases, 20:4 n-6. The rats did not adjust their proportions of 16:0, 18:0, and 18:1 in response to 18:3 n-3 deficiency, although they are able to change these acids under other dietary conditions. This fact suggests that linolenic acid deficiency did not influence significantly any of the metabolic reactions in the formations of these acids or in their incorporations into lipid classes. This was true for all tissues analyzed.

It is significant that the linolenic acid-deficient rats replaced 22:6 n-3 very largely with 22:5 n-6, rather than with 20:4 n-6, which is normally the most abundant n-6 fatty acid in phospholipids. Such a specific replacement may indicate a structural requirements for a C_{22}-polyunsaturated fatty acid. Normally, the testis is the only organ that contains high proportions of 22:5 n-6 and little 22:6 n-3, regardless of diet. In this tissue, the rat foregoes his usual preference for C_{22}-polyunsaturated fatty acids of the n-3 family.

The proportions of 22:5 n-6 in deficient rats were higher in females than in males for liver, kidney, heart, gastrointestinal tract, and muscle. Peifer et al. (23) also have reported higher proportions of 22:5 n-6 in plasma and liver of female rats, when linoleic acid was the only source of dietary lipid for 64 days.

Each lipid class consists of molecular species differing in the structures of the fatty acyl chains. Thus there are 16:0-18:1 CPG, 16:0-18:2 n-6 CPG, 18:0-22:6 n-3 EPG, and so on. The distribution of molecular species within each lipid class is metabolically controlled. This

TABLE XII

Fatty Acids in Total Lipids of Erythrocyte, Plasma, Ovary, Retina, Adrenal, and Adipose Tissue of Linolenic Acid-Deficient and Control Rats

Tissue	Diet	No.[b]	Weight percentages of total methyl esters[a]								
			16:0	18:0	18:1	18:2 n-6	20:4 n-6	22:4 n-6	22:5 n-6	22:5 n-3	22:6 n-3
Erythrocyte	C	9	33.5	17.0	19.0	5.9	15.3	0.82	0.79	1.1	2.7
	D	9	31.5	15.9	16.9	6.3	21.3	2.0[c]	2.6[d]	0.06[c]	0.22[c]
Plasma	C	8	23.8	11.3	26.9	10.5	15.8	0.25	0.30	0.35	3.2
	D	8	22.9	10.4	23.3	11.6	22.0	0.44[d]	2.5[e]	0.02[c]	0.18[e]
Ovary	C	3	24.7	6.2	44.7	4.6	4.5	2.2	0.27	0.70	1.6
	D	3	25.5	5.7	41.9	6.4	4.7	3.6[f]	2.0[g]	0.03[d]	0.17[c]
Retina	C	6	17.9	23.3	11.1	0.82	9.6	1.5	0.70	0.45	33.7
	D	6	18.3	23.2	11.7	0.77	10.3	2.8[e]	26.4[e]	< 0.01[e]	5.1[e]
Adrenal	C	7	17.5	16.6	27.1	3.0	19.5	6.4	0.76	1.2	1.9
	D	7	18.6	14.9	30.0	3.7	17.0	5.9	2.7[c]	0.07[e]	0.10[e]
Adipose	C	6	27.7	5.7	50.5	5.4	0.43	0.08	< 0.01	< 0.01	0.07
	D	6	29.1	4.5	47.7	6.9	0.50	0.08	0.25	< 0.01	< 0.01

[a]Some minor components omitted.

[b]Controls paired with deficient rats of same age and sex.

[c,d,e,f,g]Underlined values significantly different from control values, $p < 0.005$, < 0.025, < 0.001, < 0.05, < 0.01, respectively.

is shown, for example, by the fact that two similar rats (same diet, age, sex) have very similar fatty acid compositions in their individual liver CPG, but this pattern is distinctly different from the pattern in the EPG from the same liver.

Phospholipids are believed to function structurally in protein-phospholipid complexes, in membranes, or in lipoproteins, and a particular structure is assumed to possess a specific function. The presence of a particular molecular species of phospholipid, therefore, should reflect the specific function of that phospholipid in that tissue. Unfortunately, the assignment of a particular function to each molecular lipid species seems to be a very distant goal because it is difficult, if not impossible, at present to isolate a functional unit for analysis.

In the tissues studied, n-3 fatty acids, especially 22:6 n-3, were located preferentially in muscle, brain, heart, liver, and retina of control rats. Much lower proportions were found in lung, gastrointestinal tract, kidney, testis, erythrocytes, plasma, ovary, or adrenal. The greater influence of linolenic acid deficiency upon fatty acid patterns in muscle, brain, heart, liver, and retina suggests that changes in function should be most clearly demonstrable in these tissues.

In muscle, the sarcoplasmic reticulum may be the source of high proportions of 22:6 n-3 because large percentages of this acid have been found in phospholipids of sarcoplasmic reticulum membranes (24,25). Changes in the fatty acids of sarcoplasmic membrane phospholipids should influence the behavior of these membranes. Yu et al. (26) measured calcium uptake in "sarcotubular membranes" and found little difference between membranes from rats fed a fat-free diet and from those fed the same diet plus corn oil. Seiler (27) found greater accumulation of calcium in "sarcoplasmic vesicles" from rats fed a fat-free diet in comparison with rats fed a chow diet. This apparent contradiction is probably due to differences in experimental procedure. These observations suggest that muscle function could be influenced by a decline in 22:6 n-3.

In retinas, linolenic acid deficiency reduced the 22:6 n-3 content to about 15% of the control level, and 22:5 n-6 was increased accordingly. Other workers (5,28) have altered retinal fatty acid patterns by giving rats essential fatty acid-deficient diets. In rats given fat-free diets for 10 weeks to 11 months, 22:6 n-3 levels were still 80-90% of normal levels, a result which was traced to disturbance in the normal renewal (29) of retinal rod outer segments (30,31). Benolken et al. (32) achieved a more severe depletion of 22:6 n-3 in retinas of rats raised for two generations on a diet that was fat-free except during gestation, when linoleic acid was added. In retinas of these second-generation rats, 22:6 n-3 was reduced to about 40% of the normal level, and electroretinograms showed changes in the a and b waves. Unfortunately, these rats and also those in which retinal renewal was impaired (30,31) were compared with rats fed a laboratory chow diet. Lab chow differs in several respects from fat-free diets. Thus, the observed differences in visual function cannot be attributed conclusively to essential fatty acid deficiency or to changes in retinal fatty acid pattern. Yet, the observed change in visual function under these conditions does suggest that it would be valuable to measure visual function in retinas abnormally low in 22:6 n-3.

Brains of control rats contained high proportions of 22:6 n-3, especially in EPG. In first-generation deficient females, the level of 22:6 n-3 in EPG was about 9%, i.e., about 40% of the control value (data not shown). In our second-generation deficient rats, 22:6 n-3 in EPG had fallen to 1.8%, or about 8% of the control value. Lamptey and Walker (7) observed impaired learning ability (maze test) in rats whose levels of 22:6 n-3 in brain phospholipids had been reduced by a low-linolenic acid diet. In their experiments, rats were raised for two generations with a soy oil diet (8,400 mg 18:3 n-3/kg diet) or a safflower oil diet (300 mg 18:3 n-3/kg diet). Growth rates, organ weights, gestation, etc., were normal and equal in both groups of rats. Male rats of the second generation were trained in a Y-maze discrimination test, and their performances were measured daily for 7 days. For the first 3 days, both groups of rats performed equally, but on the 4th and following days, the soy oil-fed rats increased their percentages of correct responses while those of the safflower oil-fed group did not increase. Analysis of brain phospholipids in these rats showed that the safflower oil diet had reduced the 22:6 n-3 to only 10-20% of the amount present in the soy oil-fed rats. The authors noted that the learning impairment in the safflower oil-fed rats may not have been due solely to changes in brain composition because visual function may also have been influenced by changes in retinal lipid composition. The retinas of these rats were not analyzed. The learning impairment phenomenon should be investigated more thoroughly, perhaps in severely depleted rats such as ours, so that the effect can be more precisely located. It seems likely that the profound changes in other tissues, especially retina, muscle, and heart,

could contribute to an impairment in maze performance.

Is there a dietary requirement for n-3 fatty acids in the rat? If there is, the requirement for survival under our conditions is no greater than about 40 mg/kg diet. This amount is much lower than might have been expected in comparison with the requirement for n-6 fatty acids. This level is closer to the amounts required for nutrients whose functions are catalytic in nature. The n-6 fatty acids, which fulfill all known requirements for essential fatty acids in warm-blooded animals, serve as precursors for endoperoxides, thromboxanes, and prostaglandins, in addition to their roles as membrane constituents. It is possible that n-3 fatty acids also may function through formation of such products (33,34). If n-3 fatty acids have no structural function, but serve only as precursors for materials of powerful biological activity, the dietary requirement for n-3 fatty acids might be very low. Although n-3 fatty acids occur in high concentrations in certain mammalian membranes, there is no direct evidence for a structural function. Indeed, the fact that our rats survived a replacement of 80-90% of their 22:6 n-3 with 22:5 n-6 suggests that a structural function may be performed adequately if not optimally by 22:5 n-6.

In future work, it should be possible to measure functional changes in partially deficient rats such as ours, particularly in muscle, brain, heart, liver, or retina. More pronounced changes in behavior might occur if the diet contained less linolenic acid, that is, if more highly purified methyl linoleate and casein were used. The deficiency of n-3 fatty acids might also be intensified if coprophagy were prevented (35).

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Lipids of the Antarctic Sei Whale, *Balaenoptera borealis*

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ABSTRACT

The blubber, liver, and muscle of the Antarctic sei whale were analyzed for total lipid content, composition of lipid by classes and positional distribution of fatty acids in individual lipids. The major glycerolipids (triglycerides, phosphatidylcholine, and phosphatidylethanolamine) were fractionated by silver nitrate thin layer chromatography. The phospholipid fractions were analyzed for fatty acid positional distribution. The whale stomach contained almost exclusively the amphipod *Parathemisto gaudichaudi.* Its lipids were also studied and compared with the lipids of the body tissues. The results indicate that the stomach content lipids are subjected to modifications before being deposited in the blubber, liver, and muscle. According to the silver nitrate thin layer chromatographic studies, liver and blubber triglycerides resemble each other in their patterns of positional distribution of fatty acids and in molecular species composition. The phospholipids of liver and blubber also exhibited closely related fatty acid distribution patterns. In general, while the proportions of lipid classes and their predominant fatty acids varied from tissue to tissue, the patterns according to which the lipids had been synthesized seemed to be common.

". . . And beneath the effulgent Antarctic skies I have boarded the Argo-Navis, and joined the chase against the starry Cetus far beyond the utmost stretch of Hydrus and the Flying Fish." (1).

INTRODUCTION

The sei whale belongs to the group of baleen whales, i.e., those that take their food with their baleen plates or whalebone. Most baleen whales feed predominantly on euphausiids (almost exclusively *Euphausia superba)* while they are in Antarctic waters. The sei whale, however, consumes large proportions of the amphipod, *Parathemisto gaudichaudi* when feeding in the Antarctic or in the area between the Antarctic and the Subantarctic convergence (2,3). In more northern waters, the sei whale also eats copepods such as *Drepanopus pectinatus* (4). Whereas both *E. superba* and *D. pectinatus* are herbivorous, *P. gaudichaudi* feeds on zooplankton, especially copepods (3). On these bases, Nemoto (2,3) has proposed the following food chain: Phytoplankton → small zooplankton, especially copepods → *P. gaudichaudi* → sei whale.

In previous studied (5.6), the lipids of two important species of Antarctic krill, *Euphausia superba* and *E. chrystallorophias,* were compared with the lipids of the phytoplankton they presumably eat. This report compares the lipids of the upper level of the Antarctic food chain proposed by Nemoto, i.e., the Antarctic sei whale, with those of the food encountered in its stomach. Various tissues of the whale are examined in an effort to establish the effect of diet on the tissue lipid composition. Furthermore, a combination of silver nitrate thin layer chromatography (Ag-TLC) and gas liquid chromatography (GLC) is applied to the study of structural characteristics of the predominant whale blubber and liver triglycerides, phosphatidylcholine (PC), and phosphatidylethanolamine (PE). Preliminary reports of this work have been presented (7,8).

EXPERIMENTAL PROCEDURES

A male sei whale, 52 feet in length, was captured on February 27, 1967, in the Indian sector of the Antarctic Ocean (45°54′ S; 49°17′ E) by a Japanese whaler. The blubber thickness was 5.5 cm. The stomach was partially filled with the amphipod, *P. gaudichaudi,* in a rather fresh state. Small amounts of copepods were also present. Two to three kg of stomach contents were washed with seawater. The water was drained off through gauze and the solid stomach contents frozen at -20 C. Upon arrival at the first port of call in Japan, the samples were deprived of their outer surface (skin in the case of blubber), cut into 1-3 cm cubes and extracted for lipids according to Folch et al. (9). The extracts were flushed with nitrogen and sent air-mail to the laboratory at Texas A&M where the rest of the analyses were performed during 1969-1971, except for the fractionation of liver and muscle lipids which was performed in 1974. The extracted lipids were kept in chloroform-methanol (2:1, v/v) solution at -20 C except for the air trip, during which the solution was not refrigerated.

Fractionation and quantitation of the lipid classes were done by TLC according to Freeman and West (10) with the modifications

TABLE I

The Lipids of the Antarctic Sei Whale

	Stomach contents	Blubber	Liver	Muscle
		(g/100 g wet weight)		
Total lipids	8.9	75.2	7.3	3.2
Lipid classes		(g/100 g dry weight)		
Phospholipids	12.1	3.3	30.8	14.6
Triglycerides	45.1	59.8	26.1	66.3
Free fatty acids	17.7	10.6	22.2	3.5
Diglycerides	1.4	1.2	4.0	3.5
Monoglycerides	3.4	10.6	7.0	2.4
Steroid esters	1.7	13.4[a]	2.0[a]	4.2[a]
Steroids	0.5	0.8	3.7	3.5
Hydrocarbons	18.1	0.3	1.6	1.3
Glycerol ethers			2.6	0.7

[a]May include true waxes.

indicated previously (6), or by silicic acid column chromatography. Pancreatic lipase hydrolyses were done by the procedure of Luddy et al. (11), with the precaution that the fatty acids of all the reaction products were analyzed (12). Ag-TLC fractionation of triglycerides was carried out as described previously (13). PC and PE were purified and fractionated by Ag-TLC as described by Shaw and Bottino (14). Ag-TLC fractions were hydrolyzed with king cobra (*Ophiophagus hannah*) venom (Ross Allen Reptile Institute, Silver Springs, FL) according to Nutter and Privett (15). Fatty acid methyl esters were obtained by refluxing with methanol in the presence of BF_3 (16). GLC analyses were performed in a Beckman GC-5 apparatus (Fullerton, CA) equipped with a dual flame ionization detector. Columns (6 ft x 1/8 in. ID) were packed with 10% silicone-treated diethyleneglycol succinate polyester (EGSS-X) on 100-200 mesh Gas Chrom P (Applied Science Labs, State College, PA). The temperature of the column was 175 C. Quantitative estimation was based on an Infotronics Model CRS-208 Digital Integrator (Columbia Scientific, Ind., Austin, TX) and a Hewlett-Packard Model 9810-A Desk Programmable Calculator (Hewlett-Packard, Cupertino, CA). Results are expressed as weight percent. Fatty acid methyl esters were identified by co-chromatography with known standards, by plotting relative retention times against chain length, and by GLC before and after hydrogenation.

RESULTS AND DISCUSSION

Lipid Compositions

As expected, the blubber was the richest in lipids of the tissues examined (Table I). About 60% of the blubber lipids were triglycerides, and only 3% phospholipids. Triglycerides were also the predominant lipids in the stomach contents and muscle, but the liver contained similar proportions of triglycerides and phospholipids.

Trophic Relations

The finding of the amphipod, *P. gaudichaudi*, as the almost exclusive component of the stomach contents, is in agreement with previous reports on the sei whale feeding habits (2,3). Table II includes the fatty acid compositions of lipids isolated from various whale tissues, stomach contents and, for comparison, the fatty acid composition of a sample of *P. gaudichaudi* collected in January of 1972 in the Pacific sector of the Antarctic Ocean, midway between New Zealand and the Antarctic Continent. The degree of similarity between pairs of fatty acid compositions was measured by determining the "distance" or D value between them, as has been done and discussed previously (5). The distance between the fatty acids of the total lipids of *P. gaudichaudi* and those of the whale stomach contents (not shown) was D = 12, which indicates a reasonable agreement, considering the different origins of the samples, and the slight copepod contamination in the stomach contents. Thus, to a certain degree, the fatty acid composition of the stomach contents can be taken to represent the composition of the food for a major part of the feeding period.

The triglycerides of the stomach contents were quite different from the organ triglycerides (D = 26 to 30). Of the body triglycerides, only those of muscle and blubber were close to each other (D = 6) which suggests that both may

TABLE II

Major Fatty Acids of Antarctic Sei Whale Stomach Contents, Blubber, Liver and Muscle[a]

		Sei Whale											
	Para.	Stomach contents[c]			Blubber			Liver				Muscle	
Fatty acids	gaudi.[b]	TG[d]	PL[d]	FFA[d]	TG	PL	FFA	TG	PC[d]	PE[d]	FFA	TG	PL
						weight %							
14:0	3.3	3.5	3.5	5.1	9.2	7.0	14.5	15.0	1.4	0.7	8.0	10.5	3.9
15:0	0.3	2.2	2.1	13.2	0.3	0.9	0.4	0.8	0.2	ND	0.7	ND	ND
16:0	14.6	12.7	24.2	5.2	7.5	12.7	9.9	19.9	14.0	11.4	16.7	9.9	12.4
18:0	1.6	1.2	4.2	0.6	1.7	8.3	2.5	6.5	32.3	36.7	4.6	2.0	9.9
16:1(n-7)[e]	6.8	6.2	6.6	8.2	5.1	2.2	4.8	4.1	1.7	1.3	5.1	3.6	2.2
18:1(n-9)[e]	16.7	12.2	11.6	13.1	13.3	9.1	14.9	13.3	17.1	10.1	8.6	11.2	30.3
20:1(n-9)[e]	3.8	9.9	1.2	16.1	28.5	19.8	24.7	3.4	0.3	0.4	7.2	24.6	0.5
22:1(n-11)[e]	4.6	0.5	1.2	1.1	10.5	ND[f]	1.4	5.1	1.7	3.8	10.3	10.5	7.9
18:2(n-3)	0.9	1.3	0.5	2.2	1.4	1.9	0.6	0.8	0.9	0.7	0.1	2.0	2.5
22:2(n-?)	ND	0.2	7.8	0.1	ND	1.2	ND	0.9	1.4	0.6	1.7	ND	0.5
18:4(n-3)	1.2	ND	4.8	ND	1.6	4.2	ND	3.9	0.1	0.1	ND	1.8	6.2
20:4(n-3)	0.8	0.6	0.4	0.8	3.6	5.8	11.0	1.3	3.4	ND	ND	4.1	ND
20:5(n-3)	16.7	23.6	9.7	6.9	2.8	8.3	1.8	4.8	12.4	13.4	15.6	3.4	11.3
22:5(n-3)	0.8	7.1	6.6	3.0	1.7	3.5	1.1	2.9	1.3	1.5	4.7	1.7	0.5
22:6(n-3)	17.3	9.6	8.9	9.8	7.0	5.1	3.8	11.7	7.3	13.0	7.9	9.3	4.1

[a]Only those fatty acids present at a level of 2% or more are included.

[b]*Para. gaudi.* = *Parathemisto gaudichaudi,* collected separately from stomach contents.

[c]Primarily *P. gaudichaudi.*

[d]TG = triglycerides; PL = phospholipids; FFA = free fatty acids; PC = phosphatidylcholine; PE = phosphatidylethanolamine.

[e]Other isomers might be present.

[f]ND = Not detected.

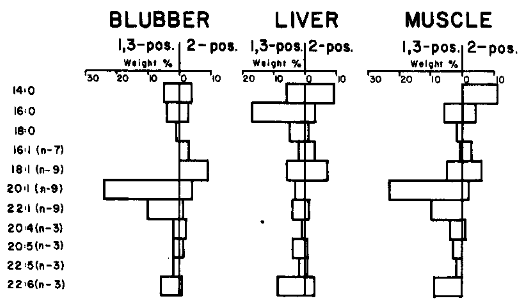

FIG. 1. Positional distribution of fatty acids in triglycerides of the Antarctic sei whale. The amounts of each acid in the various positions were calculated as follows: 2-position = MG/3; 1,3-positions = TG – (MG/3), where TG is the concentration of the fatty acid in the original triglycerides (Table II) and MG is that in the monoglyceride products of lipase hydrolysis. Thus, the bars to the left of the zero line indicate the fraction of the percentage of a given fatty acid which is present in the 1,3-positions. The bars at the right denote the fraction of the percentage present in the 2-position. For example, the blubber contained 28.5% 20:1 distributed as follows: 24.4% in the 1,3-positions and 4.1% in the 2-position. Only major fatty acids are included.

have a common origin. The phospholipids of the stomach contents were also quite different from those of the body tissues (D = 25 to 30). Phospholipids from different tissues differed markedly from each other (D = 31 to 35) but within the liver the distance between PC and PE was only D = 11, suggesting a close metabolic relationship as is true in land mammals. Considered together, these data suggest that the fatty acid composition of the food may have relatively little influence on the composition of the whale tissue lipids. Hansen and Cheah (17) studied the fatty acids of the blubber triglycerides of a sperm whale, *Physeter catodon*, and those of a squid found in its stomach. The distance between the two, calculated from the published data, is D = 17, which is somewhat below the range found in the present studies.

One of the reasons for the difference between the fatty acids of the sei whale stomach contents and those of the body tissues is that the high levels of polyunsaturated fatty acids in the stomach are not reflected in the organ lipids. Thus, the "biological magnification" of polyunsaturated acids detected in other trophic levels of the Antarctic Ocean (5) does not seem to occur in the step *P. gaudichaudi* → sei whale.

Triglyceride Fatty Acid Distribution

The fatty acid distribution in the triglycerides of sei whale blubber, liver, and muscle is depicted in Figure 1. These data were obtained by pancreatic lipase hydrolysis. Since this enzyme hydrolyzes with difficulty some of the

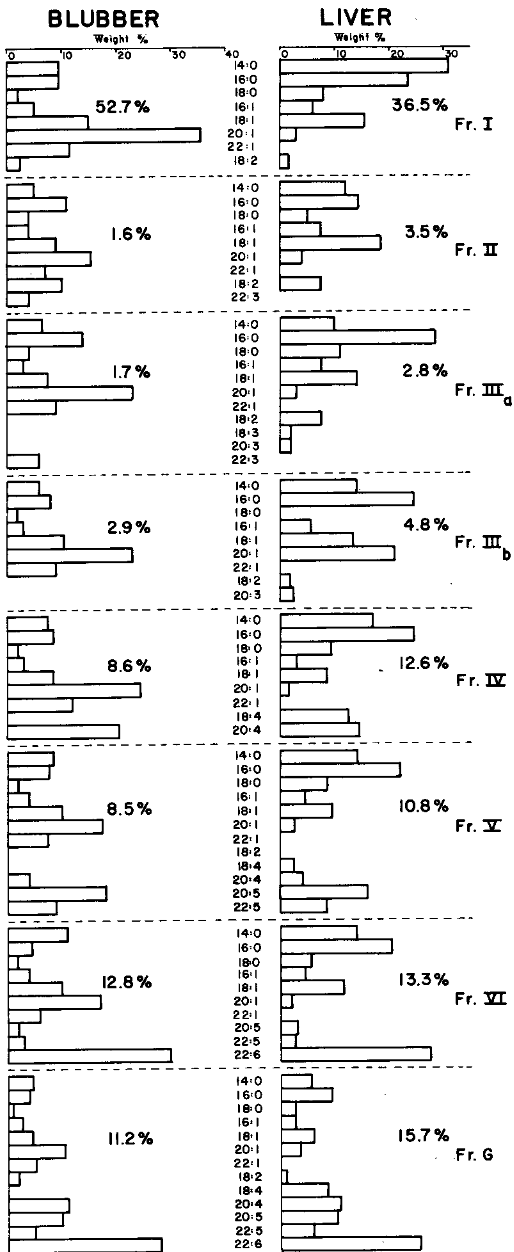

FIG. 2. Fatty acid composition of the Ag-TLC fractions of Antarctic sei whale triglycerides. Only those acids present at a level of 2% or more are included. The positions of the double bonds in the unsaturated fatty acids were as follows: 16:1(n-7); 18:1, and 20:1, all (n-9), although in these cases some isomers can be present; 22:1(n-11); 20:3(n-6); 18:2, 18:3, 18:4, 20:4, 20:5, 22:3, 22:5, and 22:6, all (n-3). Fr = Ag-TLC fractions. The Roman number of each fraction corresponds to the number of double bonds of its most abundant unsaturated fatty acids. Fraction G was a highly unsaturated fraction that remained at the origin of the Ag-TLC plates. Proportions of the fractions are in weight percent.

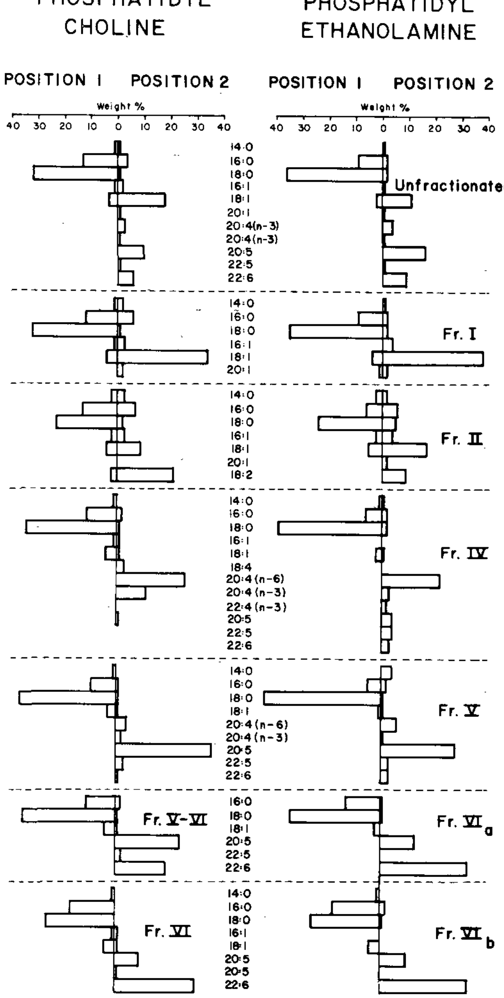

FIG. 3. Fatty acid distribution in Antarctic sei whale liver phosphatidylcholine and phosphatidylethanolamine, and in their Ag-TLC fractions. Fr = Ag-TLC fraction. The Roman number of the fractions denotes the number of double bonds of the unsaturated fatty acid predominating in the fraction. Only major fatty acids are shown. Except as indicated, the position of the double bonds in the fatty acids is as denoted in Table II and Figure 1. The positional distribution of the fatty acids was determined by phospholipase A_2 hydrolysis. The bars to the left of the zero line indicate the fraction of the percentage of a given fatty acid which is present in the 1-position. The bars to the right denote the fraction of the percentage present at the 2-position. For example, the unfractionated phosphatidylcholine contained 32.3% 18:0, distributed as follows: 31.7% in the 1-position and 0.6% in the 2-position.

polyunsaturated fatty acids (12), the data presented in the figure should be considered only as an approximation (13). However, the fatty acid distribution found in the blubber is in general agreement with the stereospecific analysis of sei whale blubber reported by Brockerhoff et al. (18), so that the data can be accepted with some degree of confidence. They indicate that the fatty acid distribution in the triglycerides of blubber, liver, and muscle is approximately the same: the C_{16} and C_{18} monoenoic fatty acids predominate in the 2-position whereas the C_{14}, C_{16}, and C_{18} saturated fatty acids and the C_{20} and C_{22} polyunsaturated acids occupy primarily the 1,3-positions.

Ag-TLC of Triglycerides

Figure 2 shows the results of Ag-TLC fractionation of three sei whale triglycerides. In a previous analysis of commercial whale oil, two patterns of distribution of fatty acids were found among Ag-TLC fractions. These patterns were based on the degree of unsaturation of the acids: one was that saturated and monoenoic fatty acids were present in significant amounts in all fractions. The second pattern was that highly unsaturated fatty acids appeared at high levels in only one fraction, except in the fraction which remained at the origin (fraction G) (13). Analogous patterns are seen now in the blubber triglycerides of the sei whale, in which saturated fatty acids (16:0>14:0>18:0) constitute about 20% of most fractions, and monoenoic fatty acids (20:1 >18:1>22:1>16:1) about 50%. The triglycerides of the liver are more saturated than those of the blubber. Consequently, the proportion of saturated to monoenoic fatty acids in the liver is the reverse of what is seen in the blubber. Another difference between blubber and liver triglycerides lies in the proportion of fraction I, which is about 53% in the former, but only 37% in the latter.

Ag-TLC of Liver PC and PE

Figure 3 shows the fatty acid composition and positional distribution of the Ag-TLC fractions of sei whale liver PC and PE. As in the triglycerides, two trends can be observed in the distribution of the fatty acids among the Ag-TLC fractions: saturated and monoenoic acids are found in significant amounts in all fractions whereas each polyunsaturated acid predominates in only one fraction. This analogous distribution in the whale triglycerides and phospholipids suggests that in the whale, as in land animals, these two lipid classes have a common origin.

Another characteristic of the whale liver PC, and to some extent also PE, is that in all fractions except in fraction I, the sum of saturated and monoenoic fatty acids is about 50% of the total acids. These proportions agree well with

the positional distribution of the acids. Palmitic and stearic acids predominate in the 1-position. Myristic acid and the C_{16} and C_{18} monoenes occupy the 2-position when associated with saturated acids but they are found in the 1-position when associated with polyunsaturated acids. Polyunsaturated acids predominate always in the 2-position. We have found this type of distribution in the phospholipids of shrimp (unpublished experiments) as well as in the hepatic phospholipids of pigs fed a fat-free diet (14). These findings suggest that the phospholipids of terrestrial and aquatic animals are synthesized according to similar patterns.

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The 6,9,12,15,18-Heneicosapentaenoic Acid of Seal Oil

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ABSTRACT

A heneicosapentaenoic acid isolated from seal oil has been identified as all-*cis*-heneicosa-6,9,12,15,18-pentaenoic acid (21:5ω3). The gas liquid chromatographic retention times of the methyl ester on four different liquid phases were identical to those of synthetic 21:5ω3, and the mass spectra of the pyrrolidide derivatives were very similar. The ω3 structure, unusual in an odd-chain polyethylenic acid, suggests as the origin α-oxidation of 22:5ω3, but ω-oxidation of the 21:6 hydrocarbon common in marine algae is also discussed.

INTRODUCTION

A heneicosapentaenoic acid, tentatively identified from gas liquid chromatographic studies, appeared to be widely distributed in marine lipids. It has been reported in almost every phylum of marine animals, for example in crustacea such as copepods (1,2), or euphausiids and shrimps (3-5), molluscs (6), in fishes (5,7,10), and in higher animals such as marine turtles, seals, dolphins, and large whales (11-14). Most of these identifications were carried out using a semilog plot between carbon number and retention time, which could only tentatively identify the structure of the fatty acid (15). Supplemental information such as nitromethane enrichment (16), resistance to urea complexing (15), and particularly a usually near-quantitative conversion to heneicosanoic acid by hydrogenation (9,10) had been used to support the heneicosaenoic skelton, but the position of the double bonds in the molecule had never been established. Polyethylenic fatty acids with structures 21:4ω2, 21:5ω3, 21:5ω2, or even 21:5ω1,[1] have been postulated, and other reports left the problem open. A positive identification as all-*cis*-heneicosa-6,9,12,15,18-pentaenoic acid (21:5ω3) has now been carried out, and we discuss possible biosynthetic pathway schemes for this acid.

EXPERIMENTAL PROCEDURES

A sample of commercial seal oil (code H-6, VIII-163A, mostly from blubber of harp seals, *Phoca [pagophilus] groenlandicus*) was used for this study. After saponification according to the AOCS method Ca-6b-53, and removal of the unsaponifiable fraction, the fatty acids were warmed with urea (5 parts) and methanol (10 parts) to give a clear solution. The solution was cooled slowly in a domestic refrigerator, and the non-urea-complexing fatty acids (NUCF) were recovered and converted to methyl esters by refluxing for 10 min with 7% BF_3 in methanol. A nitrogen atmosphere was maintained at all times. Gas liquid chromatography (GLC) of methyl esters was carried out with a Perkin-Elmer model 900 chromatograph equipped with a flame ionization detector. The wall-coated open-tubular (capillary) columns used were of stainless steel, 46 m in length x 0.25 mm ID, coated with SILAR 5CP or SILAR 7CP (Applied Science Laboratories, State College, PA), butanediol succinate polyester (BDS), or Apiezon-L (ApL). The columns were operated isothermally at 170 C (SILAR 7CP and BDS), 180 C (SILAR 5CP), and 200 C (Ap-L). Helium was used as the carrier gas (17) at 60 psig (BDS, SILAR 5CP, SILAR 7CP) and 80 psig (ApL). Injector, detector, and manifold temperatures were maintained at 250 C.

The NUCF methyl esters (50 μl) were treated to yield pyrrolidide derivatives by the

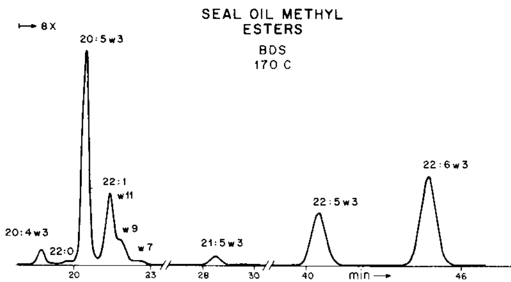

FIG. 1. Part of open-tubular gas liquid chromatographic analysis of methyl esters of fatty acids from seal oil. No attenuation changes.

[1]A shorthand notation of chain length: number of ethylenic bonds can be supplemented for position of single double bonds or methylene-interrupted polyethylenic bonds by an "ωn" where n is the number of carbon atoms between the center of the final double to and including the terminal methyl group.

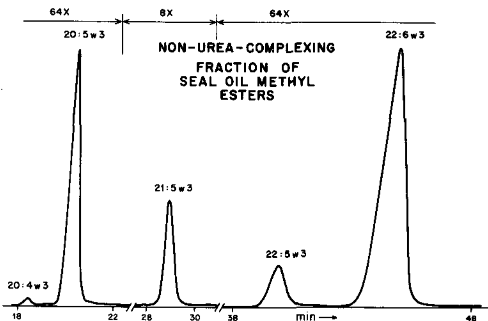

FIG. 2. Part of open-tubular gas liquid chromatographic analysis of methyl esters of fatty acids resistent to urea complex formation. Note attenuation changes.

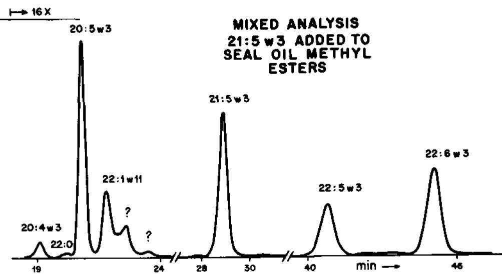

FIG. 3. Part of open-tubular gas liquid chromatographic analysis of mixed sample injection of methyl esters of fatty acids from seal oil (Fig. 1) and C_{21} material prepared from authentic 20:5ω3. An artifact falls in 22:1ω10 position and another after 22:1ω7.

method of Andersson and Holman (18). Mass spectra of both methyl esters and pyrrolidide derivatives of fatty acids were recorded with a DuPont 21-491 mass spectrometer, coupled with a Hewlett-Packard 5750 gas chromatograph, at an ionization potential of 80 eV. The source and stainless-steel jet-separator were maintained at 270 C. The 1% OV-1 glass column (2 m x 4 mm ID) was operated isothermally at 200 C and 15 ml/min of helium with a split to divide the carrier gas between a flame ionization detector and the jet-separator.

Synthesis of reference 21:5ω3 methyl ester by elongation of authentic 20:5ω3 (Hormel Institute) was carried out by the Arndt-Eistert procedure (19) as modified by Struijk et al. (20). Recovery and purity were monitored by open-tubular analytical GLC. The pyrrolidide derivative was prepared as described above, and mass spectra of both methyl ester and derivative were obtained as for the 21:5 component in the seal oil NUCF fraction.

RESULTS

The fatty acid composition of commercial seal oils is fairly well established (21). The heneicosapentaenoic is more obvious than in most fish oils but even so rarely represents more than 0.5% in weight. Of the two major pentaenoic acid components (Fig. 1), the 22:5ω3 is usually also a more prominent component than in fish oils. As expected, the non-urea-complexing fatty acid fraction was greatly enriched in the two even-chain pentaenoic components, but more especially in 22:6ω3 (Fig. 2). The enrichment of the component presumed to be 21:5 was in proportion to that of 20:5ω3 and 22:5ω3.

Hydrogenation of the NUCF fraction methyl esters quantitatively confirmed that there was essentially only a single C_{21} polyunsaturated fatty acid. Mixed samples with the synthetic 21:5ω3 added to the total methyl esters of seal oil (Fig. 1) or to the NUCF fraction, showed perfect matches on all four liquid phases in open-tubular columns (e.g., Fig. 3) strongly indicating an ω3 methylene-interrupted structure. The small modifications in parts of the analysis depicted in Figure 3 compared to Figure 1 are caused by certain impurities left in the synthetic sample. The retention behavior of the synthetic 21:5ω3 is reported for the four wall-coated open-tubular columns of varying polarities (Table I). Mass spectra of both natural and synthetic pyrrolidide derivatives of 21:5 are presented in Figure 4. According to Andersson and Holman (18), "if an interval of 12 atomic mass units, instead of 14, is observed between the most intense peaks of clusters of fragments

TABLE I
Retention Characteristics of the Methyl Ester of the Synthetic Heneicosapentaenoic Acid on Columns of Varying Polarity.

Stationary phase	Operating conditions: Column temperature (C)	Operating conditions: Helium pressure, gauge (lb/in.2)	Equivalent chain length
BDS	170	60	23.06
SILAR 5CP	180	60	23.09
SILAR 7CP	170	60	23.67
Apiezon-L	200	80	19.91

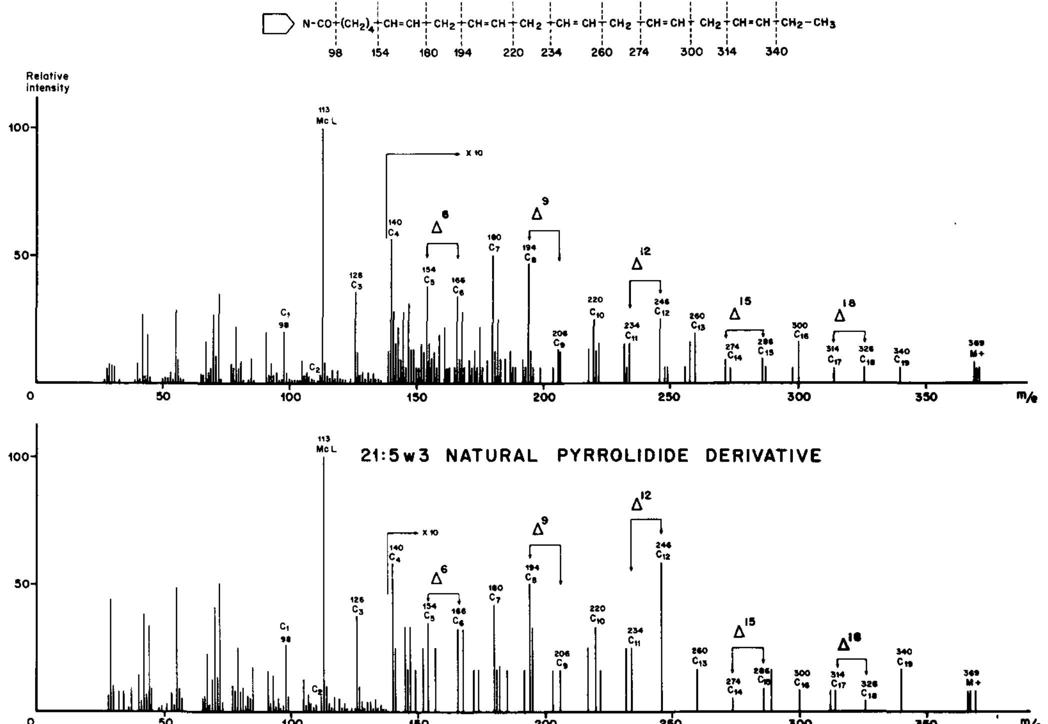

FIG. 4. A comparison of the spectra of pyrrolidide derivatives of 21:5ω3 isolated from seal oil and synthesized from authentic 20:5ω3. The spectra were run on peaks emerging from packed-column gas chromatography. Intervals corresponding to double bond positions are marked Δ^6, Δ^9, Δ^{12}, Δ^{15} and Δ^{18}.

containing n and n-1 carbon atoms of the acid moiety, a double bond occurs between carbon n and n+1 in the molecule." This rule was established from analyses of monoenoic fatty acid, and in a later report (22) they added that in the case of polyunsaturated acids "the presence of a peak relatively more intense than the peak clusters which flank it and which are involved in probable intervals of 12 amu, indicates the presence of a methylene-interrupted system. If the prominent peak contains m carbons of the fatty acid residue, the methylene carbon in the molecule was at position m+1." The spectrum of the synthetic derivative (Fig. 4) shows the base peak at m/e 113 corresponding to a McLafferty rearrangement and the other major fragment at m/e 98 and 126. As noted by Joseph (23), interpretation by the first rule stated above is difficult because of fragments of odd m/e in the region of C_9 (m/e = 206) and C_{11} (m/e = 234). Using the second rule, it becomes possible to confirm that the positions of the double bonds in the 21:5 synthetic are in positions 6, 9, 12, 15, and 18 through prominent fragments at m/e 180, 220, 260, 300, and 340. The similarity of the fragmentation patterns of the mass spectra of the synthetic and seal oil 21:5 derivatives (Fig. 4), combined with the GLC behavior, indicates that we are dealing with the same ethylenic structures in the positional sense. The total coincidences in GLC on several liquid phases indicate that only *cis* ethylenic unsaturation is involved (24).

DISCUSSION

The ω3 structure found for the 21:5 from seal oil is somewhat surprising. Indeed, from the work of Schlenk (25), one would have expected an ω2 structure corresponding to the ω3 family of the common fatty acids with even chain lengths. We did not prepare a 21:5ω2 fatty acid for mass spectral comparison, but it would be unlikely that 21:5ω2 would both show the same mass spectrum as synthetic 21:5ω3 and have an identical gas liquid

TABLE II

Occurrence of Heneicosapentaenoic Acid in Various Types of Marine Organisms.

Source of fatty acids	% of Total fatty acid (or range) in weight %	References
Natural particulate matter	0.1-0.5	Mayzaud, unpublished data
Diatom *Skeletonema costatum*	0.2-0.4	(39)
Copepods *Calanus + Centropages sp.*	0.1-0.2	(1)
Copepods *Temora longicornia*	<0.1	(2)
Euphausid *Meganyctiphanes norvegica*	0.2-0.7	(3,4)
Euphausid *Euphausia sp.*	0.1-1.1	(5)
Herring oil *Clupea harengus*	0.1-0.3	(7)
Sturgeon oil *Acipenser oxyrhynchus*	0.19	(8)
Mackerel oil *Scomber scrombrus*	0.2-0.6	(9)
Fin whale *(Balaenopterus physalus)* blubber	0.1-0.9	(13)
Dolphin *(Tursiops truncatus)* milk	0.2	(11)

chromatographic behavior on four liquid phases. Various possible biosynthetic pathways can be considered. The most obvious would be an α-oxidation of 22:5ω3 which would proceed through an α-hydroxy acid intermediary (26-28). Although 22:5ω3 is a normal constituent of marine lipids, α-oxidation in animals usually affects specifically the free fatty acid fraction, generally emphasizing acids of C_{20} and longer chain lengths (29). It would be difficult to envision the natural occurrence of 22:5ω3 in the free fatty acid pool without accompanying 20:5ω3, 22:6ω3, etc., and formation of parallel odd-chain products. Of course, it is always possible that the α-oxidation enzyme system has a greater activity towards 22:5ω3 (Δ^7 structure) than for 20:5ω3 or 22:6ω3 (Δ^5,$\Delta^{4'}$ structures). Alternatively, 21:5ω3 could be more resistent to β-oxidation than the corresponding 19:5ω3 and 21:6ω3 odd-chain products of α-oxidation. This is an area requiring further selective study of odd-chain polyunsaturated fatty acids in marine lipids.

At first glance, the 21:6 hydrocarbon (heneicosa-3,6,9,12,15,18-hexaene), discovered independently in unicellular marine plants by several groups at more or less the same time (30-32), is an attractive point of origin since α-oxidation of hydrocarbon chains is a well-known process by which hydrocarbons are broken down by microorganisms (33,34). Attack on either end of the 21:6 hydrocarbon chain could yield the same 21:5ω3 acid product. Some hydrocarbon chains such as those in phytanic (3,7,11,15-tetramethylhexadecanoic) acid can be broken down by higher animals through both α- and ω-oxidation, which is necessary in this specific biochemical case because the β-position is blocked by the 3-methyl substitute (27,28,34,35). One could even suppose that in the 21:6 hydrocarbon the biochemical ω-oxidation could concurrently lead to reduction of the proximal ethylenic bond by hydrogen transfer. There exists an alternative 21:6 hydrocarbon (heneicosa-1,6,9,12,15,18-hexaene) produced by the brown macrophyte *Fucus vesiculosus* (36). As this is a common macrophyte on the North Atlantic littoral (37), one would expect a product of facile oxidation of the terminal carbon of the vinyl group to be most noticeable in the marine lipids examined by this laboratory. It must be noted that freshwater fish oils also apparently contain the 21:5 fatty acid (38), and it is known that freshwater plants produce heneicosa-3,6,9,12,15,18-hexaene (32).

Assuming that most of the 21:5 or 21:4 components reported in the literature are indeed 21:5ω3, it seems to occur in similar proportions in almost all trophic levels of the marine food web, from the particulate matter to the sea mammals (Table II). No obvious trends can be shown which would suggest a particular trophic origin. One report (39) suggests that 21:5ω3 could be present in the diatom *Skeletonema costatum*, and its presence in particulate matter (Mayzaud, unpublished data) may support a basic algal origin. This would not rule out the presence of a minor biosynthetic pathway, probably that of α-oxidation common to all marine plant or animal lipid metabolism, and the wide occurrence could result from the extensive recycling of fatty acids in the marine food web. The final consideration is the relatively uniform degree of accumulation in diverse marine lipids (Table II). It has recently been suggested that in the marine milieu, animals as diverse as marine invertebrates (40) and mammals (21) have species-peculiar lipid com-

positions when total or depot fatty acids are examined in sufficient detail. At the same time, studies on odd-chain fatty acids in smelt *Osmerus mordax* show that any changes from the species-peculiar fatty acid composition induced by a radical fatty acid dietary input are rapidly offset once the input ceases (41). This process is also documented (25) for the mullet *(Mugil cephalus)*. In higher animals, individual primates *(Macaca fascicularis)* on a pure vegetable oil diet, a lard: corn oil (3:1) diet, or a partially hydrogenated marine oil diet (42) showed that depot fats of the animal examined from each group had respectively, 0.2, 0.3, 1%, of 17:0 in total fatty acids (43). It was concluded that 1% 17:0 was tolerated by the primate adipose tissue as a proportion normal for the species. We propose that the ubiquitous 21:5ω3 is tolerated by most marine organisms as a minor lipid component at ≤1% but prevented from accumulating at higher proportions of fatty acids.

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Measurement In Vitro of the Esterification of Yeast Sterols

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ABSTRACT

A highly reproducible and rapid procedure is described for isolating free sterols and sterol esters from cell-free extracts of yeast. Using this technique, the sterol esterifying enzyme has been studied. Sterol ester hydrolase has also been demonstrated. In agreement with whole cell experiments, the enzyme activity for sterol ester synthesis has been found to increase on entrance of the culture into the stationary phase of growth.

INTRODUCTION

The presence of fatty acid esters of sterols in yeast has been known for many years (1). Although little is certain about the function of sterols in cells, virtually nothing is known about the physiological role of sterol esters. While the sterols are of importance in membrane structure and function, it seems unlikely that the sterol esters are involved there, since the esters pack very poorly in phospholipid bilayers (2). Work from this laboratory has established that the fatty acid moieties of sterol esters are predominantly unsaturated C-16 and C-18 acids (3). The degree of esterification of the yeast sterols was found to vary with the growth medium. In all media, however, a sharp increase in esterification was observed immediately prior to entry of the culture into the stationary phase of growth (4).

Virtually no data are available on the mechanism of sterol esterification in the cell. Work with in vitro systems is complicated because the usual extraction procedures either fail to recover the total sterol fractions, or else hydrolysis of the ester occurs during preparation for extraction. Methods that involve successive solvent extractions are cumbersome and awkward because of the formation of emulsions refractory to separation. Reproducible quantitation becomes difficult. For these reasons, a simple reproducible isolation procedure for total sterols and sterol esters was perfected. In this communication are described that procedure, its use in defining some aspects of the esterification reaction, and the relationship of the esterifying enzyme to the culture phase of the organism.

MATERIALS AND METHODS

Organisms and Cultural Conditions

A haploid uracil auxotroph of *Saccharomyces cerevisiae* (stock 3701B) was used in this study. Growth measurements were as described previously (4). The organism was routinely cultured in a 1% tryptone, 0.5% yeast extract, 0.1% glucose broth to which 2% ethanol was added following sterilization of the medium. All cultures were incubated at 28 C on a New Brunswick rotary shaker for 60 hr. The cells were harvested by centrifugation at 1,500 x G, washed with distilled water, and resuspended in 0.1 M phosphate buffer at pH 7.0. The thick cell suspension (1 g wet weight per ml of buffer) was then broken with 45 sec disintegration in a Bronwill MSK cell homogenizer using 0.25 mm glass beads and cooling with liquid CO_2. Unbroken cells and other debris were removed by centrifugation at 1,500 x G for 15 min. The supernatant was then centrifuged at 5,000 x G for 15 min and the pellet (designated P2) was resuspended in phosphate buffer. The supernatant (S1) was centrifuged at 20,000 x G for 20 min and the pellet (P3) was collected. The supernatant (S2) was centrifuged at 104,000 x G for 45 min and the resulting pellet (P4) collected, and the supernatant (S3) was saved. The P3 and P4 pellets were resuspended in 0.1 M phosphate buffer at pH 7.0. In several experiments, the enzyme source was 3,000 x G supernatant which was obtained by centrifuging the cell extract twice at that force. Proteins were determined by the method of Lowry (5).

Extraction of Sterols and Sterol Esters

The enzyme assays were performed in Kimax ground glass-stoppered test tubes (19 x 150 mm with standard taper No. 19 glass stopper). The reaction mixture consisted of 0.3 mg coenzyme A, 1.0 mg of ATP, 0.5 ml of enzyme preparation, (^{3}H)-zymosterol, and 0.1 M phosphate buffer to bring the reaction mixture to 1.0 ml. Incubation was for 5 hr at 27 C on a reciprocating shaker. The reaction was terminated by the addition of 1.0 g of diatomaceous earth previously coated with lanosterol. When endogenous substrates and colorimetric analyses were employed, the lanosterol was omitted. The protein was caused to adhere and precipitate onto the solid support by the addition

TABLE I

Sterol Recovery from Yeast Cell Extracts [a]

	Extraction procedure	μg Sterol recovered	%Saponification
A	Direct solvent extraction	31	41
B	Diatomaceous earth	84.2	112
C	Cold alkalie	9.5	13
D	Alkaline pyrogallol	75.	100
E	DMSO treatment (cold)	8.5	11
F	DMSO treatment (hot)	19.	25

[a]The recovery procedures are described in Results. The sterols were estimated using the sulfuric acid-acetic anhydride colorimetric procedure.

of 5.0 ml of acetone. The acetone was added while agitating the tube on a Vortex mixer. The solvent was recovered by centrifugation for 5 min in an International explosion-proof centrifuge at 500 x G. The solvent was decanted with a syringe and placed in a flask. The diatomaceous earth was reextracted by vigorous shaking in 10.0 ml of chloroform-methanol (4:1, v/v). The solvent was collected as was the acetone and the solid support extracted again with 5.0 ml of diethyl ether. The three solvent extracts were pooled and evaporated under negative pressure on a rotary evaporator. The dried residue was stored in a vacuum dessicator over P_2O_5 overnight.

The free sterols and the sterol-esters were separated on short activated alumina columns as described (4). For these experiments, we increased the amount of the ether-hexane wash to 15.0 ml.

Enzyme activity units are defined as the percent substrate esterified during the experiment per milligram of protein present in the reaction mixture. This value is used since our procedure recovers both the free substrate and the esterified product and compensates for any slight variations in recoveries.

Preparation of Substrates

Zymosterol was isolated from a yeast mutant (designated 8R1) using our previously published procedure (6). 2,2,4,4,(^{3}H) Zymosterol was prepared by tritium exchange with 3-ketozymosterone and reduction with sodium borohydride according to the procedure of Klein and Knight (7) as modified (8). Sterol fatty acid esters were prepared by the method of Knapps and Nicholas (9) and treated as described in our earlier work (4). For in vitro ester synthesis, the zymosterol substrate was dispersed in lecithin as follows: 10 mg of lecithin was dissolved in chloroform and evaporated to dryness, cold zymosterol dissolved in benzene was mixed with tritiated zymosterol to give a final activity of 430,000 counts/min/mg sterol and added to the lecithin. The benzene was evaporated, and 5 ml of 30 mM NaCl was added. The lecithin-zymosterol-saline mixture was sonicated for 10 min with cooling in ice at setting 4 on a Branson sonifier. The final solution was centrifuged at 12,000 x G for 1 hr and the supernatant used as substrate. In later experiments, the centrifugation step was eliminated without noticeable effect on the esterase activity.

Identification and Quantitation of Sterol Substrates and Products

Preliminary separations by thin layer chromatography (TLC) and acetylation were performed by our published procedures (10). Quantitative gas liquid chromatography (GLC) employed a Varian 2740 gas chromatograph coupled with a CDS-1111 data processor. TLC was done on silica gel plates (Merck HF-254) of 0.25 mm thickness.

Materials

All solvents, except as noted, were purchased from Mallinckrodt (St. Louis, MO) and were redistilled prior to use. Gas chromatographic supplies were from Supelco, Inc. (Bellefonte, PA). Dimethyl sulfoxide was from J.T. Baker (Phillipsburg, NJ). Precoated silica gel plates were products of E-M Laboratories, Co. (Elmsford, NY). Diatomaceous earth was from Johns Manville (Denver, CO) and was sieved on standard mesh screens to retain the 60 to 80 mesh fractions.

RESULTS

Sterol Extraction

Six procedures were used to assess the value of the diatomaceous earth method for sterol extraction from yeast cell-free extracts (11). The endogenous sterols from the samples were assayed colorimetrically. One ml of extract was used in each case. The techniques used were: A. the yeast preparation was extracted twice with

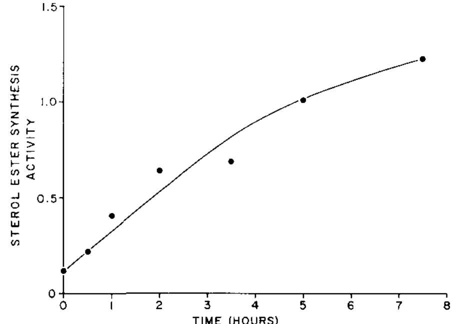

FIG. 1. Enzyme activity as a function of the time of the reaction. The 5,000 x G resuspended pellet was used as enzyme source. Each reaction consisted of 2.72 mg protein, 1 mg ATP, 0.3 mg CoA, and [^{3}H] zymosterol in a total volume of 1.0 ml in 0.1 M phosphate buffer at pH 7.0. Activity is as described in the text.

3 volumes of hexane followed by three volumes of diethyl ether. The extracts were pooled and analyzed; B. the diatomaceous earth procedure as described in Methods; C. the extract was adjusted to 3N NaOH and extracted three times with three volumes of hexane; D. the alkaline pyrogallol saponification was performed (12) and the sterols extracted three times with hexane; E. to the extract was added 1 ml of DMSO, and the sterols extracted with hexane; and F. the procedure as in E was followed except the mixture was heated for 30 min at 100 C and then the sterols extracted into hexane. In each case, the solvents were removed by evaporation under reduced pressure. The dried residues were dissolved in chloroform and assayed by a modified Lieberman-Burchard color test (13). Table I gives the results of the extraction. The alkaline pyrogallol procedure was assigned as the optimal procedure for comparison based on its use in a wide variety of published experiments. Each procedure was performed in duplicate. These procedures were selected as representative of those used in many circumstances for sterol extractions. The diatomaceous earth procedure is superior to any of those tested. Other extractions involving methanol, ethanol, and/or acetone were tried, but caused precipitation of the cell extracts into globules from which sterols were poorly extracted. Because of the wide variability in results, those data are not presented here. The solvent extracts from B and D were separated by TLC in cyclohexane-ethyl acetate (85:15, v/v). Authentic reference samples of ergosterol and ergosteryl myristate were used to locate the free and sterol esters. From the saponification procedure, virtually all of the sterol was recovered in the free sterol band. Extraction procedure B using the diatomaceous

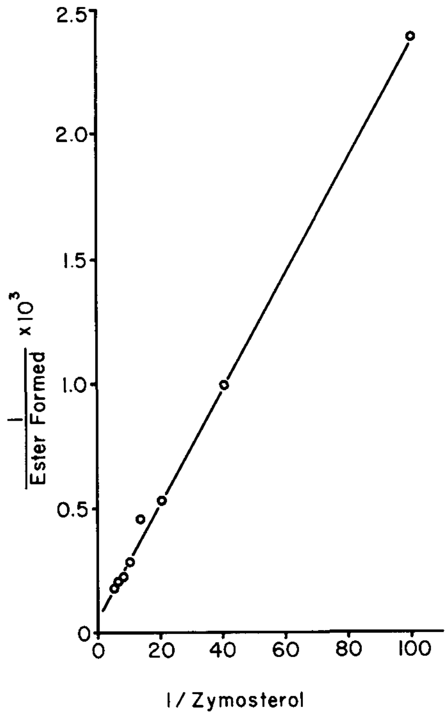

FIG. 2. Double reciprocal plot of sterol ester formed as a function of substrate concentration. Reaction conditions were identical to those of Figure 1 except for varying substrate concentration. The enzyme source was at 15 mg protein/ml.

earth gave in excess of 80% of the sterol esterified. The latter was the expected result, since under the growth conditions employed (60 hr), most of the yeast sterols are known to be esterified (4).

Labeled zymosterol and zymosteryl oleate can be recovered from cell extracts using the diatomaceous earth procedure. To estimate the reproducibility of the method, 20,000 cpm of the lecithin-emulsified (^{3}H)-zymosterol was added to each of twelve tubes containing either 0.5 or 1.0 ml of 3000 x G supernatant of broken yeast. Incubation was for 5 hr at 28 C. The experiment was performed in duplicate. The reaction mixtures were subjected to solvent-diatomaceous earth extraction, and the recovered counts determined in the free and esterified fractions. From these experiments, it was determined that at the 95% confidence interval the recovery of labeled sterol was within ± 2.3% of the mean.

It was observed that when the amount of extract was reduced considerably from that given above, the recovery of proffered sterol began to decline, unless multiple solvent extractions were performed. This loss in sterol re-

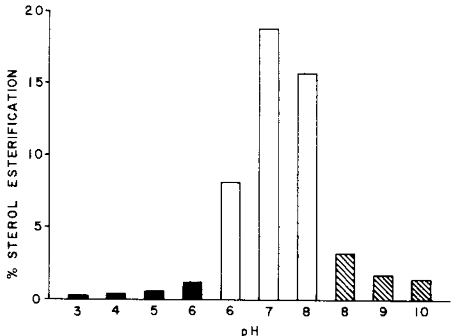

FIG. 3. Effect of pH and buffer on sterol esterification. The reaction contents were as described in Figure 1, and each vessel was allowed to react for 5 hr. All buffers were at 0.1 M and were as follows: pH 3-6, citrate/phosphate; pH 6-8, phosphate; and pH 8-10, tris chloride.

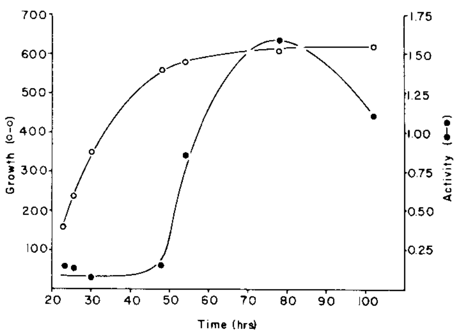

FIG. 4. Sterol esterifying activity as a function of age of the cell culture. (○-○) Open circles are culture turbidity as measured photometrically. (•-•) Closed circles are activities of sterol esterifying enzyme at the indicated points.

covery at low protein levels can be minimized by coating cold carrier sterol onto the diatomaceous earth prior to addition of the extract. To 18 g of sieved diatomaceous earth suspended to a slurry in methylene chloride was added 50 mg of lanosterol. The suspension was mixed thoroughly and the solvent evaporated to dryness under vacuum.

Esterification In Vitro

Sterol esterification in cell extracts was assayed using labeled free zymosterol as substrate. This sterol was selected as the test substrate, since it is easily labeled to high specific activity, and it is one of the sterols found to be most highly esterified under all growth conditions (4,10). The reaction mixtures are described in Materials and Methods. The dispersed sterol substrate was prepared immediately prior to use. Using the fractionation procedure described, esterification has been found to occur principally in the particulate fractions of the cell extracts. For a 5 hr incubation time, the activities for the various fractions demonstrating significant esterification are: 3,000 x G supernatant, 0.64; 5,000 x G pellet (P2), 1.03; and the 104,000 x G pellet (P4), 0.8. The supernatants designated S1, S2, and S3 gave low activity figures. The P2 component was used routinely for our assays. Figure 1 shows that the esterifying activity is proportional to time of reaction. Labeled sterol ester recovered is proportional to the concentration of substrate added. Figure 2 shows a double reciprocal plot of sterol ester recovered as a function of substrate zymosterol added. However, kinetic constants were not calculated because of the various endogenous sterols that are present in the enzyme sources. The effect of pH on the reaction mixture was measured using citrate-phosphate, phosphate, and tris buffers. Figure 3 shows the results of one such experiment. Optimal pH is at 7. It is interesting that both citrate and tris failed to give the stimulation to the reaction that was observed in phosphate alone.

Assay of Sterol Ester Hydrolase

The esterified sterol being measured could be the summation of two different enzymic processes, the formation of the sterol ester, and its degradation by a lower level of sterol ester hydrolysis. This assay procedure can be used equally as effectively to monitor hydrolysis of sterol esters as well as sterol synthesis. Cleavage of sterol esters was measured with zymosteryl oleate as substrate. Hydrolysis of ester does occur, but only to an average of 17% during the course of the experiment.

Effect of Stage of Growth on Sterol Esterification

Because we had demonstrated that esterification of yeast sterols increases on the culture's entrance into the stationary phase (Fig. 4), we assayed the enzyme during the culture cycle of the organism. Over a tenfold increase in sterol esterase was observed in the later phases of growth.

DISCUSSION

Several procedures for sterol ester and free sterol recoveries from enzymic reactions were tried in an effort to obtain an extraction method which would be highly reproducible and sufficiently easy to permit a large number of assays. The most generally encountered problems were poor recovery of the substrates and

products, appearance of severe emulsions which were difficult and time consuming to break, and impracticality of the method for small volumes of sample. It was observed that somewhat polar organic solvents were necessary for sterol extraction in quantity. However, these caused precipitation of the cell extracts giving hard globules of protein from which sterol yields were greatly reduced. The procedure we have developed is one of the easiest assays that we have observed for total sterol extraction. It takes advantage of the use of several organic solvents known to promote sterol extraction and precipitation of the cell extract. The solvent-diatomaceous earth procedure has now been employed in several different enzymic reactions with sterols and, without exception, has given consistent and high yields of product.

Using the diatomaceous earth extraction method, we have been able to get an accurate measure of sterol esterification in cell-free extracts of yeast. That esters of the sterol were being synthesized was established using thin layer chromatographic separation procedures with known reference compounds. The esterification is dependent on substrate concentration and time of reaction. It has an optimal pH of 7.0, and is stimulated by phosphate.

The esterification process appears to reflect the physiological state of the cell. We have observed a marked increase in the percentage of sterol esters upon entry of the culture into the stationary phase of growth (4). From our experiments here, we conclude that this results from a sharp rise in the esterifying enzymes. Although hydrolase enzyme is observed, the low activity would fail to account for the pool of free sterols present in the cells during the early growth phases. Yeast cultured under growth inhibitory conditions caused by trifluperidol also accumulate sterol esters (14). Thus, esterification cannot be caused simply by exhaustion of growth nutrients from the culture medium.

The physiological role for the enzyme remains obscure. Once esterified, the sterols may be removed effectively from participating in a variety of enzymic and structural interactions. Esterified sterols do not participate in the usual biosynthetic methyl transferase reactions (15). Maintenance of a precise pool of free sterols may be essential, and esterification could prevent exchange of membrane sterols with the minor sterols that tend to accumulate during late growth phase. It is particularly interesting that the culture is able to anticipate entry into the stationary growth phase as reflected by the increase in the esterification of sterols. The controls affecting production of the esterifying enzyme are intriguing since the esterification occurs without a substantial increase in the total sterols per cell (4). An understanding of the overall role of sterols in yeast cell physiology must now also include the esterification process.

ACKNOWLEDGMENTS

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Identification of the Free and Conjugated Sterol in a Non-Photosynthetic Diatom, *Nitzschia alba*, as 24-Methylene Cholesterol[1]

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ABSTRACT

Previous studies on the sterol fraction of the nonphotosynthetic marine diatom, *Nitzschia alba*, indicated the major sterol to be either brassicasterol (24*R*-methylcholesta-5,22-dien-3β-ol) or 22-dehydrocampesterol (24*S*-methylcholesta-5,22-dien-3β-ol) on the basis only of gas chromatography-mass spectral analysis. The present studies using nuclear magnetic resonance, infrared, and gas chromatography-mass spectrometry on the free and bound sterol fractions isolated by preparative thin layer chromatography showed the presence in both fractions of a single sterol, with spectral and chromatographic properties identical with those reported for 24-methylenecholesterol (ergosta-5,24(28)-dien-3β-ol). This sterol may be the precursor of 24-methyl sterols found in diatoms. The bound sterol fraction was found to consist of a single compound identified as 24-methylenecholesterol sulfate. No sterol esters or sterol glycosides were detected.

INTRODUCTION

In a previous study (1) on the sterols of the non-photosynthetic marine diatom, *Nitzschia alba*, we reported the major sterol to be either brassicasterol (24*R*-methylcholesta-5,22-dien-3β-ol) or 22-dehydrocampesterol (24*S*-methylcholesta-5,22-dien-3β-ol). This assignment was based only on gas chromatography (GC)-mass spectral analysis which is incapable of distinguishing between these two C-24 epimers (2,3) (see Fig, 1). Recent studies (2-5) have shown that C-24 epimers may be readily distinguished by their 100 or 200 MHz nuclear magnetic resonance (NMR) spectra, thus permitting the assignment of the major sterol in several species of photosynthetic diatoms (4,5) as the 24*S* (or 24-α) isomer, 22-dehydrocampesterol (see reviews [6,7]).

In view of these findings, we undertook a reexamination of free and conjugated sterols of *N. alba* by high resolution NMR as well as by infrared and GC-mass spectrometry. Surprisingly, both the free and bound sterol fractions yielded only a single sterol which was neither of the C-24 epimers, brassicasterol, or 22-dehydrocampesterol, nor was it the isomeric codisterol (Fig. 1) recently identified in the green alga *Codium fragile* (8). The present communication deals with the identification of the *N. alba* sterol as 24-methylenecholesterol (ergosta-5-24(28)-dien-3β-ol), a positional isomer of codisterol and the presumed precursor of the C-24 epimeric sterols (4,6).

MATERIALS AND METHODS

Cells of *N. alba* (Lewin and Lewin strain) were grown at 30 C in a synthetic seawater medium containing Na_2SiO_3 and glucose (9) as described previously (10) and harvested in the late logarithmic growth phase. Total lipids were extracted from the cells (1) and fractionated on a column of Biosil A silicic acid as described elsewhere (11). Neutral lipids (containing free sterols) were eluted with 10 column volumes of chloroform (Fraction I), glycolipids and sulfolipids with 3 column volumes of acetone followed by 3 column volumes of methanol-acetone (1:9, v/v) (Fraction II), and phospholipids with 10 column volumes of methanol (Fraction III). Fraction I (neutral lipids) was further fractionated (11) by chromatography on a column of Biosil A silicic acid using petroleum ether (bp 40-60 C) to elute hydrocarbons, petroleum ether-ethyl ether (9:1, v/v) to elute triglycerides and free fatty acids, petroleum ether-ethyl ether (1:1, v/v) for sterols, and finally ethyl ether and chloroform to remove unidentified polar species of neutral lipids. The pure free sterol component was isolated by preparative thin layer chromatography (TLC) of the petroleum ether-ethyl ether (1:1) fraction on 1 mm thick Silica Gel H layers developed twice in petroleum ether-ethyl ether-acetic acid (50:50:1, v/v); after visualization of the lipid spots with dichlorofluorescein (12), the sterol band was eluted from the plate with chloroform and finally purified by passage through a small column of Biosil A silicic acid eluted with

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BRASSICASTEROL

24R-methylcholesta-5, 22-dien-3β-ol

22-DEHYDROCAMPESTEROL

24 S - methylcholesta - 5, 22 - dien - 3β - ol

CODISTEROL

24 S-methylcholesta-5,25-dien-3β-ol

24-METHYLENECHOLESTEROL

ergosta - 5, 24 (28) - dien - 3β - ol

FIG. 1. Structures of isomeric diunsaturated C_{28}-sterols: brassicasterol, 22-dehydrocampesterol, codisterol, and 24-methylenecholesterol.

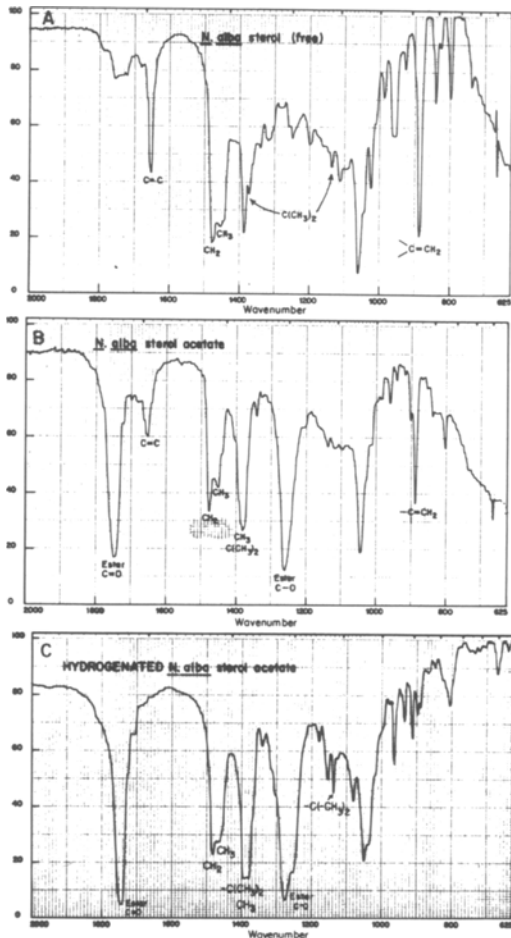

FIG. 2. Infrared spectra (in KBr) of: A, *N. alba* (free) sterol; B, *N. alba* sterol acetate; C, hydrogenated *N. alba* sterol acetate.

petroleum ether to remove extraneous lipid followed by petroleum ether-ethyl ether (1:1) to remove the sterol. The free sterol thus obtained was TLC pure (R_f 0.45) and gave a red color with the sterol spray reagent (12).

The "bound" sterol (sterol sulfate) was isolated from Fraction II (glycolipid + sulfolipid fraction described above) by preparative TLC on Silica Gel H in chloroform-methanol-28% ammonia (65:35:5, v/v) (R_f, 0.67) and precipitated from acetone as the ammonium salt, as described elsewhere (13). The sterol component of the sterol sulfate was obtained in two ways: (a) by hydrolysis with 2.5% methanolic-HCl under reflux for 5 hr (1,12), and (b) by solvolysis in 0.005M HCl in anhydrous tetrahydrofuran at room temperature for 2 hr (13,14). The sterol obtained was purified by preparative TLC as described above.

Authentic sterol samples (brassicasterol, campesterol, stigmasterol, and β-sitosterol) were purchased from Supelco Inc. (Bellefonte, PA); brassicasterol was also isolated from rapeseed oil unsaponifiable material by preparative TLC as described above. 24-Methylenecholesterol was a gift from Dr. D.R. Idler, Marine Sciences Laboratory, St. John's, Newfoundland.

Free sterols were converted to acetate esters by reaction with anhydrous pyridine-acetic anhydride (2:1, v/v) for 16 hr at room temperature as described eleswhere (12). Sterol acetates (10 mg) were hydrogenated in ethyl acetate solution (3 ml) with palladium on carbon catalyst (10 mg) for 4 hr at room temperature and a slightly positive pressure of hydrogen. After centrifugation of the catalyst, the reduced pro-

TABLE I

Gas Liquid and Thin Layer Chromatographic Properties of Sterols

Sterol	GLC relative retention[a]		TLC[b] R_f of acetate	Number of double bonds per molecule
	Free sterol	Acetate		
N. alba sterol	1.31	1.32	0.17	2
Brassicasterol	1.14	1.15	0.46	2
24-Methylenecholesterol[c]	---	1.31	0.17	2
Campesterol	1.36	1.38	0.58	1
Stigmasterol	1.48	1.51	---	2
β-Sitosterol	1.79	1.81	---	1
Cholesterol	1.00	1.00	0.52	1
Cholestanol	---	---	0.62	0
Hydrogenated *N. alba* sterol	---	1.39	0.64	0
Tetrahydrobrassicasterol	---	1.39	0.61	0
Dihydrocampesterol	---	1.39	0.61	0

[a]On a column (46 cm x 0.6 mm) of 3% SE-30 on Chromosorb W at 190C and flow rate of 110 ml/min; relative to cholesterol (ret. time, 20.9 min.) or cholesterol acetate (ret. time, 29.2 min.).
[b]On 10% $AgNO_3$-Silica Gel H plates in solvent system $CHCl_3$-MeOH; 99.6:0.4.
[c]Authentic sample from Dr. D. Idler.

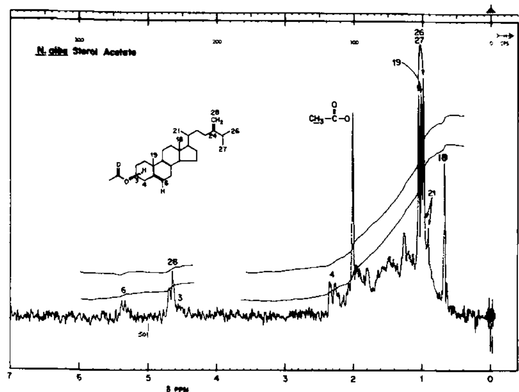

FIG. 3. PMR spectrum (in $CDCl_3$) of *N. alba* sterol acetate.

duct was obtained by evaporation of the solvent and was purified by preparative TLC on Silica Gel H in petroleum ether-ethyl ether-acetic acid (90:10:1, v/v).

Sterols were analyzed as acetates by TLC on 10% $AgNO_3$-Silica Gel H in the solvent system chloroform-methanol (99.6:0.4, v/v) and in both the free form and as acetates by gas liquid chromatography (GLC) on a column (46 cm x 0.6 mm) of 3% SE-30 on Chromosorb W at 190 C.

Infrared spectra were taken in KBr on a Pye-Unicam infrared spectrometer. Proton magnetic resonance spectra were measured with a Varian HA-100 spectrometer in $CDCl_3$ containing 1% tetramethylsilane as internal reference. Mass spectra of the sterol acetates were obtained with a Finnigan 3100D quadrupole mass spectrometer after GLC on a 1.5 m column (2 mm ID) of 3% OV-17 at 240 C; the TMS derivative of the *N. alba* sterol was analyzed on a Shimadzu-LKB 9000 GC-mass spectrometer by Dr. A. Hayashi, Kinki University, Japan.

RESULTS AND DISCUSSION

The free sterol isolated from *N. alba* showed a single component both on GLC and $AgNO_3$-SiO_2 TLC which was well resolved from brassicasterol but had similar chromatographic mobilities to those of 24-methylenecholesterol (Table I). The hydrogenated *N. alba* sterol, however, had GLC retention time and TLC mobility identical with those for tetrahydrobrassicasterol or dihydrocampesterol (Table I).

The acetate of the *N. alba* sterol showed a mass spectrum (Table II) with ion peaks at m/e 380 (M^+-acetate), 365 (M^+-CH_3-acetate), 255 (M^+-side-chain-acetate), and 213 (M^+-side-chain-42-acetate) indicating it was a disunsaturated C_{28} steryl acetate. In addition, an ion peak at m/e 296 (M^+-84-acetate), ascribed to loss of part of the side chain by MacClafferty rearrangement, is characteristic of 24-methylene sterols but not of 25-methylene sterols such as codisterol (8). Brassicasterol acetate had an almost identical mass spectrum (Table II), but that of codisterol differed in several respects (see Table II and Ref. 8). The TMS derivative of the *N. alba* sterol has a mass spectrum identical to that reported for the TMS derivative of 24-methylenecholesterol (15) (Table III). The hydrogenated *N. alba* sterol acetate showed a molecular ion peak at m/e 444 and a spectrum identical to that of tetrahydrobrassi-

TABLE II

Mass Spectral Data[a] for *N. alba* Sterol Acetate and Reference Compounds

Fragment	*N. alba* sterol acetate		24-Methylene-cholesterol acetate[b]		Codisterol acetate[c]		Brassicasterol acetate		Hydrogenated *N. alba* sterol acetate		Tetrahydro-brassicasterol acetate	
	m/e	R.I.	m/e	R.I.	m/e	R.I.	m/e	R.I.	m/e	R.I.	m/e	R.I.
M^+					440	3			444	0.4	444	0.1
M^+-15									439	0.3	439	0.1
M^+-60	380	2.5	380	100	380	100	380	13	384	2.5	384	0.4
M^+-75	365	0.9	365	24			365	3	369	3.8	369	0.7
M^+-60-30									354	0.1		
M^+-60-42	338	0.1					338	1	342	0.2		
M^+-60-43			337	6			337	2				
M^+-99-15									330	0.2		
M^+-127					313	4						
M^+-129									315	0.3		
M^+-60-83	297	1.2							301	0.2	301	0.1
M^+-60-84	296	4.8	296	63	296	5	296	2	300	0.1		
M^+-154									290	0.4	290	0.2
M^+-60-97	283	0.8					283	2	287	0.1		
M^+-60-98	282	1.1					282	4	286	0.1	286	0.1
M^+-60-99	281	3.3	281	26			281	2	285	0.6	285	0.2
M^+-168	272	0.6	272	10			272	1	276	5.0	276	2.6
M^+-169	271	0.2							275	5.0	275	2.7
M^+-181	259	1.9					259	3				
M^+-60-125	255	2.3			255	6	255	32	259	1.0	259	0.4
M^+-60-127	253	6.5	253	30			253	9	257	4.0	257	2.7
M^+-189	251	0.1					251	1	255	3.0	255	0.5
M^+-212	228	2.7	228	40	228	13	228	7	232	2.0		
M^+-214	226	1					226	2	230	9	230	8
M^+-60-125-42	213	9	213	25	213	13	213	16	217	15	217	13
M^+-60-127-42	211	5					211	6	215	72	215	53
Base peak	81	100					145	100	147	100	107	100

[a]Analyses of acetates carried out here were on a Finnigan 3100D quadrupole mass spectrometer.
[b]Data supplied by Dr. L.J. Goad.
[c]Data from Ref. 8.

TABLE III

Mass Spectral Data[a] for TMS Derivative of *N. alba* Sterol and 24-Methylenecholesterol[b]

Fragmentation	*N. alba* sterol-TMS		24-Methylene-cholesterol-TMS[b]	
	m/e	R.I.	m/e	R.I.
M^+	470	25	470	30
M^+ - 15(CH_3)	455	25	455	23
M^3 - 84(C_{23} to C_{28} + H)[c]	386	51	386	67
M^+ - 90(TMS-OH)	380	57	380	78
M^+ - 99(CH_3 + C_{23} to C_{28} + H)	371	14	371	14
M^+ - 105(CH_3 + TMS-OH)	365	38	365	52
M^+ - 125(side chain)	345	5	345	30
M^+ - 127(side chain + 2H)	343	32	343	42
M^+ - 129(C_1 to C_3 + TMS-O)	341	73	341	100
M^+ - 152(side chain + 27)	318	2	318	3
M^+ - 167(side chain + 42)	303	3	303	---
M^+ - 174(C_{23} to C_{28} + H + TMS-OH)[c]	296	45	296	67
M^+ - 189(CH_3 + C_{23} to C_{28} + H + TMS-OH)	281	25	281	45
M^+ - 211(TMS-OH + 121)	259	17	259	24
M^+ - 213(C_{23} to C_{28} + H + C_1 to C_3 + TMS-O)	257	39	257	60
M^+ - 215(side chain + TMS-OH)	255	17	255	48
M^+ - 217(side chain + 2H + TMS-OH)	253	25	253	52
M^+ - 241(side chain + 27 + TMS-O)	229	9	229	30
M^+ - 243	227	12	---	---
M^+ - 257(side chain + 42 + TMS-OH)	213	23	213	42
M^+ - 259(side chain + 2H + 42 + TMS-OH)	211	11	211	25
M^+ - 341(base peak)	129	100	129	---

[a]Analysis on Shimadzu-LKB 9000 GC-mass spectrometer on a 2% OV-1 column at 250 C.
[b]Data from Ref. 15.
[c]Characteristic peaks for 24-methylenecholesterol (15).

casterol or dihydrocampesterol acetates (Table II), confirming that the *N. alba* sterol contained two double bonds and was probably an isomer of brassicasterol in which the side-chain double bond was in a position other than at C_{22}.

The infrared spectrum of the free *N. alba* sterol (Fig. 2A) showed characteristic bands at 890 cm^{-1} and 1655 cm^{-1} indicative of a $R_1R_2C=CH_2$ group and bands at 1390-1375 cm^{-1} (doublet) and 1140 cm^{-1} corresponding to an isopropyl group. The spectrum of the acetate derivative (Fig. 2B), however, did not show the shoulder at 1375 cm^{-1} characteristic of an isopropyl group but only a broad peak at 1380 cm^{-1}. These spectra were identical with those of authentic 24-methylenecholesterol (see Ref. 16,17). In contrast, brassicasterol showed bands at 1680 cm^{-1} and 960 cm^{-1} indicative of a *trans*-double bond, apart from bands at 1395-1380 cm^{-1} (doublet) and 1140 cm^{-1} indicative of an isopropyl group, and codisterol showed bands for a terminal methylene at 890 and 1645 cm^{-1} but lacked any isopropyl bands (8). However, the infrared spectrum of the hydrogenated *N. alba* (Fig. 2C) sterol was identical with that of tetrahydrobrassicasterol or dihydrocampesterol. This evidence is consistent with the presence in the *N. alba* sterol of a methylene group located at C-24.

Confirmation of the terminal double bond at C-24 was obtained by the NMR spectrum (Fig. 3; Table IV) which showed a doublet at δ4.66 and 4.72 due to two protons of a terminal methylene group assigned as C-28, since C-26 methylene protons give a singlet at δ4.66 (see assignments for codisterol in Talbe IV); in brassicasterol, the C-22,23 protons of the Δ^{22} double bond gave a multiplet centered at δ5.2. The second double bond in the *N. alba* sterol was located at the Δ^5 position by the multiplet centered at δ5.36 due to the C-6 proton and by the presence of C-18 and C-19 methyl proton singlets at δ0.68 and 1.04, respectively. The signal at δ1.62 due to the C-27 methyl protons in the spectrum of codisterol (8) was absent from the spectrum of the *N. alba* sterol. The spectrum of the *N. alba* sterol acetate was, in fact, identical with that of an authentic sample of 24-methylenecholesterol acetate.

TABLE IV

NMR Assignments for *N. alba* Sterol (as Acetate)

Group assignment	Chemical shift[a] (δ), ppm: *N. alba* sterol acetate "Free"	*N. alba* sterol acetate From sulfate ester	24-Methylenecholesterol acetate[b]	Codisterol acetate (8)	Brassicasterol acetate (rapeseed)
=CH(C-6), multiplet	5.35 - 5.40	5.31 - 5.43	5.3 - 5.4	5.35	5.36 - 5.43
=CH(C-28), doublet	4.66 + 4.72	4.66 + 4.71	4.66 + 4.72	---	---
=CH_2(C-26), singlet	---	---	---	4.66	---
O-C-H(C-3α), multiplet	4.63	4.63	4.63	4.63	4.60
CH_3-CO-(C-3 acetate), singlet	2.02	2.03	2.02	2.02	2.03
CH_3-C=(C-27), singlet	---	---	---	1.62	---
CH_3(C-19), singlet	1.02	1.03	1.03	1.02	1.02
CH_3(C-28), doublet	---	---	---	0.93 + 1.00	0.89 + 0.95
CH_3(C-21), doublet	0.91 + 0.97	0.91 - 0.98	0.92 + 0.98	0.87 + 0.95	0.98 + 1.05
CH_3(C-18), singlet	0.68	0.68	0.69	0.66	0.69
HC=CH(C-22,23), multiplet	---	---	---	---	5.18 - 5.25
CH_3(C-26), doublet	1.00 + 1.07	1.00 + 1.07	1.00 + 1.07	---	0.88 + 0.81
CH_3(C-27), doublet					0.86 + 0.79

[a]In $CDCl_3$.
[b]Authentic sample from Dr. D. Idler.

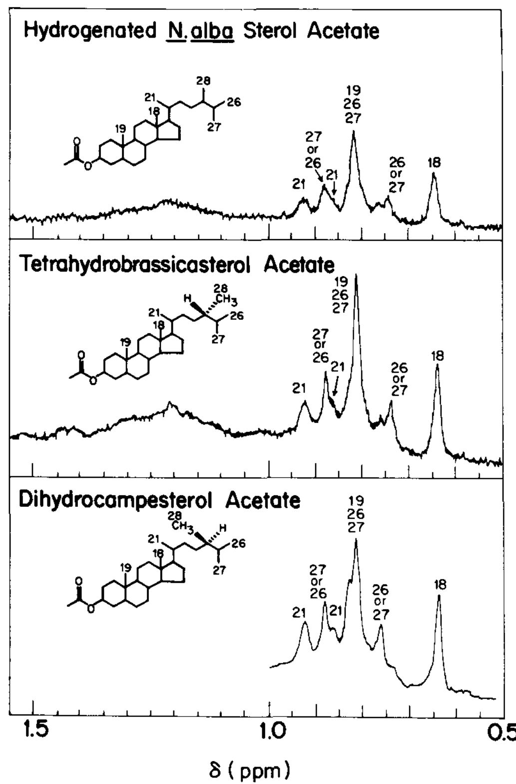

FIG. 4. PMR spectrum (in $CDCl_3$) of acetates of: A, hydrogenated *N. alba* sterol; B. tetrahydrobrassicasterol; and C, dihydrocampesterol.

The tetrahydro derivative of the *N. alba* sterol had an NMR spectrum (Fig. 4) similar to that of a mixture of tetrahydrobrassicasterol and dihydrocampesterol, the two epimeric sterols that would be expected on catalytic hydrogenation of the C-24 methylene group.

The sterol obtained from the sterol sulfate of *N. alba* by mild acid solvolysis (13) showed a single component on GLC and TLC with identical retention time and R_f value, respectively, and identical mass, infrared, and NMR spectra to those of the free sterol in *N. alba*. When strong acid-catalyzed methanolysis was used to cleave the sterol sulfate, a mixture of isomeric sterols and derivatives was obtained as described previously for cholesteryl esters (18).

It may thus be concluded that *N. alba* contains a single sterol, present in both free form and in conjugated form as the sulfate ester, having the structure 24-methylenecholesterol (Fig. 1). The presence in *N. alba* of a minor sterol reported to be clionasterol (1) is in error and is probably attributable to formation of artifacts during strong acid-catalyzed methanolysis of the sulfate ester (see Ref. 18). It should also be mentioned that contrary to our previous suggestion (1), no sterol esters or sterol glycosides have been detected (11,13) on TLC of the neutral lipids (Fraction I) or the glycolipids (Fraction II), respectively.

The finding of 24-methylenecholesterol as the only sterol in *N. alba* is unusual, since all diatoms so far examined appear to contain 22-dehydrocampesterol as principal sterol (4,6,7). However, there is good evidence that 24-methylenecholesterol is the precursor of 22-dehydrocampesterol in the diatom *Phaeodactylem tricornutum* (4,6). Thus, the absence of a 24-methyl sterol in *N. alba* may indicate that this non-photosynthetic diatom lacks the reductase necessary for its formation. 24-Methylenecholesterol occurs in significant amounts in marine invertebrates (16) and is believed to be the precursor of 24-methyl sterols in nature (6,16,17). On the basis of the present findings, 24-methylenecholesterol may have a dietary origin in marine invertebrates.

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Distribution of Bile Acids in Rats

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ABSTRACT

Distribution and biliary and fecal excretion of bile acids were examined in Wistar strain male rats of about 300 g body weight. The pool size of the rats on ordinary diet was 40 mg/rat, biliary secretion was 14 mg/hr, and fecal excretion was 10 mg/day. Bile acids were mainly located in the small and large intestinal contents, 87% and 10%, respectively; but a portion was found in the intestinal wall and the liver. Rats fed 2% cholesterol-supplemented diet for a week showed similar values for pool size and biliary secretion with the rats on ordinary diet, but higher values for fecal excretion and distribution ratio in the large intestinal contents. Cholic acid was a major component in the bile, small intestinal wall, small intestinal content and liver, while the bile acid composition ratios were roughly similar to each other, although a relatively large amount of α-muricholic acid was found in the intentinal wall and liver. Both the wall and content compositions of the large intestine were similar to that of the feces, in which lithocholic, deoxycholic, α- and β-muricholic acids were the main components, although the ratios of α- and β-muricholic acids in the large intestinal wall were larger than those in the intestinal contents or feces. The high concentrations of these bile acids may indicate a difference of transport velocity across the cell membrane, but the mechanism is not known.

INTRODUCTION

Bile acids secreted into the intestinal lumen through the bile duct are mostly absorbed from the intestine mainly at the distal part of the ileum and again secreted into the bile from the liver. The frequency of the enterohepatic circulation of bile acids is calculated by dividing secretion rate by pool size and is reported to be several times a day.

The pool size is determined by several techniques. Mok et al. (1) used the washout method with rats, but the value obtained was larger than that from Lindstedt's isotopic dilution method (2,3). Fisher et al. (4) measured the amount of bile acids in the intestine, liver, and serum, and reported a value of about 50 μmoles/100 g body weight for the pool size. This value is larger than that of Mok et al. (1).

On the other hand, rat bile is comprised of several kinds of bile acids: cholic, chenodeoxycholic, and their secondary bile acids. Cholesterol feeding increases the formation of bile acids (5,6), especially that of chenodeoxycholic acid (7,8). Since the biological significance of cholic and chenodeoxycholic acid is different (9) and the half-life of chenodeoxycholic acid is shorter than that of cholic acid (7), changes in the bile acid composition in the pool may affect the enterohepatic circulation.

Therefore, we determined the pool size, secretion, synthesis, turnover frequency, and composition ratio of bile acids in tissues and intestinal contents in rats fed ordinary and 2% cholesterol-supplemented diet, according to the method of Fisher et al. (4), and examined the effect of cholesterol feeding in rats.

MATERIALS AND METHODS

Wistar strain male rats, 3 to 4 months old, were kept in an air-conditioned room (25 ± 2 C, 50-60% humidity) lighted 12 hr a day (8:00 a.m. to 8:00 p.m.) and maintained on a balanced commercial stock diet (Japan CLEA, CA-1, Tokyo Japan). The diet was supplemented with 2% cholesterol and given for 2 weeks ad libitum.

Rats were individually caged, and feces were collected for 2 days before sacrifice as described previously (8). Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and the bile duct was cannulated with PE-10 polyethylene tubing to collect bile for 30 min. Next, blood was withdrawn by heart puncture, and the liver and the intestine were removed. The liver was homogenized with nine volumes of saline. The small intestine, from the entry of the bile duct to the end of the terminal ileum, and the large intestine, caecum and colon, were cut longitudinally, and the contents were washed out with saline. The intestinal tissues were homogenized with nine volumes of saline, and the contents were lyophilized to dryness.

Lipid Determination in Serum and Tissues

Serum and tissue homogenates were extracted in ten volumes of ethanol by refluxing for 20 min at 90-95 C, and cholesterol levels were determined as reported previously (10,11). Phospholipids were determined by the method of Gomori (12). Serum bile acids were determined by the method of Sandberg et al. (13). Bile acids in the tissue homogenates were extracted with 95% ethanol containing 0.1%

ammonium hydroxide solution by refluxing for 30 min (14), isolated through an Amberlyst A-26 column according to the procedures of Sandberg et al. (13), and quantified by gas liquid chromatography (GLC).

Lipid Determination in Bile

Bile was extracted with 20 volumes of ethanol, brought to boiling once for several minutes, cooled to room temperature, then filtered through Toyo filter paper No. 2 (Toyo Roshi Co. Ltd., Tokyo, Japan). Biliary cholesterol and bile acids were determined as reported previously (15) and quantified by GLC. Biliary phospholipid was determined by the method of Gomori (12).

Lipid Determination in Feces and Intestinal Contents

Fecal sterols and bile acids were determined as reported previously (8). Dried and powdered feces were extracted with absolute ethanol, hydrolyzed in sodium hydroxide solution under pressure, then sterols and bile acids were extracted with ethyl ether and quantified by GLC. The lyophilized intestinal contents were similarly measured.

RESULTS

The mean values of the amounts of sterols, phospholipid, and bile acids in the serum, bile, liver, intestinal walls, intestinal contents, and feces of rats fed the ordinary diet are presented in Table 1.

Cholesterol was the sole sterol mainly found in the serum, liver, bile, small intestine and its contents. No detectable amount of coprostanol or other sterols was found; the amount would be 2% or less if present. Coprostanol increased in the large intestinal contents and further in the feces to about 40% of the total sterols.

During the initial 30 min after bile duct cannulation, 7.14 mg/rat of bile acids was secreted into the bile. This value was calculated to be 14.27 mg/hr and 342.7 mg/day. The main component of the bile acids was cholic acid, occurring at about 80%. Other components were chenodeoxycholic, α-muricholic, deoxycholic, hyodeoxycholic, and lithocholic acids. They were identified by thin layer chromatography (TLC), GLC, and gas chromatography-mass spectrometry (GC-MS). The configuration of hyodeoxycholic acid, 6α or 6β, remains unknown. In addition to these components, some unidentified peaks were found in the gas chromatograms, P8, P10, and P11. Other minor components, probably keto bile acids, were also found. They are presented as "others" in the present data.

The small intestinal contents had a large amount of bile acids, and the percentage was about 87% of the pool. The bile acid composition was similar to that of the bile, but with less chenodeoxycholic, hyodeoxycholic, and P8 acids. The small intestinal wall contained an appreciable amount of bile acids. The composition ratio was similar to those of the bile and small intestinal contents, but the amount of α-muricholic acid was markedly greater and those of lithocholic, deoxycholic, and chenodeoxycholic acids slightly less. A greater amount of α-muricholic acid was also found in the liver. α-Muricholic acid in the small intestinal wall was confirmed by GC-MS analysis.

The rats excreted daily about 10 mg/rat of bile acids into the feces. Lithocholic, deoxycholic, α-muricholic, β-muricholic, and P10 acids were major components at 14%, 37%, 7%, 11%, and 26%, respectively.

The large intestinal contents had about 3 mg/rat of bile acids, and the composition ratio was very similar to that of the feces, although the amount of β-muricholic acid was lower and that of P10 acid higher than in the feces. The large intestinal wall also contained bile acids but a very small amount. Although the ratios of α- and β-muricholic acids increased, they were not as reliable as in the other preparations because of the low quantities.

Table II shows the data for rats fed 2% cholesterol-supplemented diet for 1 week. No significant elevation was found in serum cholesterol and phospholipid levels, but the liver cholesterol concentration was markedly high. The sterol contents in the intestine increased about tenfold. Biliary secretion of cholesterol slightly decreased while those of phospholipid and bile acids did not significantly change. The compositional ratio of chenodeoxycholic acid increased and that of cholic acid decreased.

The total amount of sterols in the small intestinal contents was higher than that of the rats on ordinary diet, but the amount of bile acids was similar. The bile acid composition in the small intestinal contents resembled that in the bile, although the amounts of deoxycholic, chenodeoxycholic, and hyodeoxycholic acids were slightly lower and that of cholic acid higher.

The bile acid content in the small intestinal wall of the rats on cholesterol diet was slightly higher than that of the rats on ordinary diet. The composition ratio of α-muricholic acid was high, and those of lithocholic and deoxycholic acids were low compared with that in the bile and small intestinal contents. These changes were similar to those in the rats on ordinary diet (Table I). The bile acid content in the liver

TABLE I

Distribution of Sterols, Phospholipid, and Bile Acids in Rats on Ordinary Diet

	Serum (mg/rat)	Bile (mg/day)	Small intestine Contents (mg/rat)	Small intestine Wall (mg/rat)	Liver (mg/rat)	Feces (mg/day)	Large intestine Contents (mg/rat)	Large intestine Wall (mg/rat)
Total sterols	10.9 ± 0.98[a]	7.1 ± 0.27[a]	5.9 ± 1.07[a]	8.5 ± 0.82[a]	72.3 ± 3.73[a]	11.12 ± 0.475[a]	4.5 ± 0.29[a]	2.9 ± 0.19[a]
Coprostanol	nd[b]	nd	nd	nd	nd	4.32 ± 0.497	1.1 ± 0.20	nd
Cholesterol	10.9 ± 0.98	7.1 ± 0.27	5.9 ± 1.07	8.5 ± 0.82	72.3 ± 3.73	6.80 ± 0.775	3.4 ± 0.29	2.9 ± 0.19
Phospholipid	20.0 ± 0.84	76.6 ± 5.14	---[c]	63.0 ± 7.67	551 ± 36.5	---[c]	---[c]	25.9 ± 0.63
Bile acids	nd	343 ± 36.2	28.6 ± 2.52	1.02 ± 0.221	0.23 ± 0.055	9.85 ± 1.318	3.14 ± 0.743	0.04 ± 0.010
Bile acid composition (%)								
3α		0.5 ± 0.17	0.8 ± 0.15	0.2 ± 0.10	nd	13.7 ± 1.19	11.7 ± 1.21	1.0 ± 0.66
$3\alpha12\alpha$		2.9 ± 0.22	1.3 ± 0.22	0.5 ± 0.10	1.4 ± 0.38	36.7 ± 2.32	31.6 ± 3.80	13.4 ± 5.87
$3\alpha6\beta7\alpha$		3.1 ± 0.44	2.3 ± 0.30	7.4 ± 0.88	10.5 ± 0.95	5.6 ± 0.96	6.3 ± 1.11	29.7 ± 13.45
$3\alpha7\alpha$		5.0 ± 0.71	2.2 ± 0.45	1.7 ± 0.38	1.2 ± 0.50	nd	nd	nd
$3\alpha6\alpha$ (or $3\alpha6\beta$)		1.8 ± 1.30	nd	nd	nd	1.4 ± 0.41	0.7 ± 0.35	nd
$3\alpha7\alpha12\alpha$		79.4 ± 1.72	83.0 ± 0.98	76.5 ± 2.85	65.9 ± 1.15	---[d]	---[d]	---[d]
$3\alpha6\beta7\beta$		---[d]	---[d]	---[d]	---[d]	10.9 ± 2.92	1.1 ± 0.53	38.8 ± 4.24
P8		3.7 ± 0.32	0.6 ± 0.49	4.9 ± 1.01	8.6 ± 1.15	1.1 ± 0.82	nd	nd
P10		0.2 ± 0.07	nd	nd	nd	26.3 ± 2.01	44.7 ± 1.79	17.1 ± 9.45
P11		1.2 ± 0.12	5.1 ± 0.85	2.9 ± 0.95	5.8 ± 0.89	3.1 ± 0.71	1.5 ± 0.61	nd
Others		2.5 ± 0.73	4.2 ± 0.73	2.6 ± 0.55	5.4 ± 1.05	0.8 ± 0.43	2.4 ± 0.40	nd

[a]Mean ± SE in five rats (body weight: 298 ± 4.2 g).

[b]Not detectable.

[c]Not determined.

[d]Cholic and β-muricholic acids were not separable under our experimental conditions.

TABLE II

Distribution of Sterols, Phospholipid, and Bile Acids in Rats on 2% Cholesterol Diet

	Serum (mg/rat)	Bile (mg/day)	Small intestine Contents (mg/rat)	Small intestine Wall (mg/rat)	Liver (mg/rat)	Feces (mg/day)	Large intestine Contents (mg/rat)	Large intestine Wall (mg/rat)
Total sterols	10.5 ± 0.67[a]	8.1 ± 0.23[a]	40.6 ± 1.36[a]	8.7 ± 0.55[a]	118.6 ± 10.04[a]	218.4 ± 8.58[a]	58.1 ± 6.12[a]	3.0 ± 0.19[a]
Coprostanol	nd[b]	nd	nd	nd	nd	47.4 ± 3.56	0.8 ± 0.08	nd
Cholesterol	10.5 ± 0.67	8.1 ± 0.23	40.6 ± 1.36	8.7 ± 0.55	118.6 ± 10.04	168.4 ± 5.96	57.3 ± 6.09	3.0 ± 0.19
Phospholipid	13.2 ± 0.91	83.0 ± 5.00	---[c]	55.0 ± 4.03	419 ± 2.0	---[c]	---[c]	22.2 ± 0.84
Bile acids	nd	342 ± 59.3	25.2 ± 3.30	1.78 ± 0.398	0.12 ± 0.022	15.85 ± 0.582	5.74 ± 0.567	0.12 ± 0.031
Bile acid composition (%)								
3α		0.8 ± 0.24	1.3 ± 0.22	0.5 ± 0.17	nd	16.3 ± 1.95	11.8 ± 3.85	2.8 ± 1.62
3α12α		4.4 ± 1.00	1.7 ± 0.59	1.1 ± 0.29	1.4 ± 0.88	24.3 ± 1.72	20.1 ± 2.34	17.4 ± 4.09
3α6β7α		5.6 ± 0.76	4.7 ± 1.12	10.2 ± 1.26	13.1 ± 1.90	8.5 ± 1.14	9.4 ± 0.17	14.5 ± 4.05
3α7α		12.9 ± 1.94	6.6 ± 0.89	4.1 ± 0.53	1.5 ± 1.05	nd	nd	nd
3α6α (or 3α6β)		2.9 ± 1.39	nd	nd	nd	2.9 ± 0.52	1.3 ± 0.39	nd
3α7α12α		67.6 ± 2.69	77.1 ± 1.26	74.5 ± 2.61	73.1 ± 6.10	---[d]	---[d]	---[d]
3α6β7β		---[d]	---[d]	---[d]	---[d]	14.5 ± 2.35	5.1 ± 0.56	22.3 ± 7.62
P8		3.2 ± 0.72	0.1 ± 0.08	4.5 ± 0.66	3.8 ± 2.17	2.4 ± 0.61	nd	nd
P10		0.1 ± 0.10	nd	nd	nd	24.1 ± 4.55	40.1 ± 5.98	43.0 ± 8.44
P11		1.1 ± 0.25	2.9 ± 0.57	1.9 ± 0.23	2.4 ± 1.23	3.5 ± 0.68	3.1 ± 0.67	nd
Others		1.6 ± 0.56	3.9 ± 0.63	3.4 ± 0.14	2.2 ± 1.18	0.3 ± 0.22	0.6 ± 0.27	nd

[a]Mean ± SE in five rats (Body weight: 249 ± 3.2 g). 2% Cholesterol diet was given for 2 week ad libitum.

[b]nd = Not detectable.

[c]Not determined.

[d]Cholic and β-muricholic acids are not separable under our experimental conditions.

TABLE III

Pool Size, Secretion, Synthesis, and Turnover Frequency of Bile Acids in Rats Fed Ordinary and 2% Cholesterol Diet for a Week

	Ordinary diet	Cholesterol diet
Number of rats	5	5
Body weight (g)	298 ± 4.2[a]	249 ± 3.2[a]
Pool size (mg/rat)	40.2 ± 1.67	40.1 ± 3.37
Secretion (mg/day)	343 ± 36.2	342 ± 59.3
Synthesis (mg/day)[b]	9.9 ± 1.32	15.9 ± 0.58
Turnover frequency[c]	8.6 ± 1.07	8.6 ± 0.82

[a]Mean ± SE.

[b]The amount excreted into the feces was regarded as the amount of synthesis.

[c]Turnover frequency was calculated dividing the secretion by the pool size.

was low, but the composition ratio was similar to that in the small intestinal wall, and the increase of α-muricholic acid was very remarkable.

Fecal excretion of bile acids increased upon feeding of the cholesterol diet. The bile acid composition was similar to that in the ordinary-diet rats, but the secondary bile acids from chenodeoxycholic acid, such as lithocholic, α- and β-muricholic acids, had increased. The bile acid content in the large intestinal wall was low, and the composition ratio fluctuated greatly with each rat.

Pool size, secretion, synthesis, and turnover frequency were calculated from the present data and are shown in Table III. Cholesterol feeding increased the synthesis but caused almost no change in the pool size, secretion, and turnover frequency.

DISCUSSION

The present experiments have confirmed the previous reports (4,16,17) that bile acids are mainly located in the small intestinal contents in rats and further demonstrated that the composition ratios of bile acids differ with the site of the enterohepatic circulation.

The rats fed ordinary diet secreted 7.14 mg of bile acid during the first 30 min of bile duct cannulation and excreted an average of 9.85 mg daily into the feces. Another 33.03 mg of bile acids was found in the small and large intestinal contents (87% and 10%, respectively), the small and large intestinal walls (3% and 0.1%), and the liver (1%). Therefore, the pool size was calculated to be 40.17 mg/rat. This value agrees with that reported by Ho (17) but is slightly lower than that of Fisher et al. (4) and higher than that of Shefer et al. (3). In other experiments, we obtained pool size values of 40-45 mg/rat for rats of verious ages by the washout method (Uchida et al., unpublished data).

Similarly, the rats fed 2% cholesterol diet for a week secreted 7.13 mg of bile acids into the bile during the first 30 min, excreted 15.85 mg daily into the feces, and had 32.96 mg in the intestinal tracts and liver. The pool size was, therefore, 40.09 mg/rat, which was close to that of the rats on the ordinary diet. When the value was calculated for the function of body weight, a slight increase was found in rats on the cholesterol diet (39.8 μmoles/100 g body weight vs. 33.3 μmoles/100 g body weight).

Compared with rats on the ordinary diet, the distribution of bile acids in rats on the cholesterol diet was lower in the small intestinal contents (77% vs. 87%) and higher in the small intestinal wall (5% vs. 3%) and large intestinal contents (17% vs. 10%). The increase in the large intestinal contents was predominant and agreed with the fact that the fecal excretion of bile acids was about twofold higher in rats on the cholesterol diet. The increase in the large intestinal contents was mainly due to the increase of lithocholic, α-muricholic, and β-muricholic acids. α- and β-Muricholic acids were first isolated by Doisy's group in St. Louis (Matschiner et al., 18) from the bile of rats and found to be metabolites of chenodeoxycholic acid. Gustafsson et al. (19) have reported that cholesterol feeding increases β-muricholic acid in the intestinal contents, although they did not separate the small and large intestines. Since lithocholic, α- and β--muricholic acids are derived from chenodeoxycholic acid, their large amounts in the large intestinal tract and the feces are considered to reflect the increase of chenodeoxycholic acid formation.

Cholic and β-muricholic acid showed almost the same retention time with a relative retention time (RRT) to deoxycholate of 1.92-1.96 and were not separable from each other when subjected to analysis as methyl ester trifluoroacetate derivatives on a QF-1 column. Preliminary experiments using a combination of TLC

and GLC revealed that cholic acid was the major component in bile, liver, and small intestinal preparations, while β-muricholic acid was predominant in the feces and large intestinal contents. In the present experiments, therefore, the peak of RRT 1.92-1.96 was regarded as cholic acid in the bile, small intestine and liver, and as β-muricholic acid in the feces and large intestine. Thus, the cholic acid fraction in the former group may contain a little β-muricholic acid, and the β-muricholic acid fraction in the latter may contain cholic acid.

Although the composition ratios of bile acids in the small intestinal contents, wall, and liver were roughly similar to that of the bile, and the ratios in the large intestinal contents and wall resembled that in the feces, consistent differences were found among them. Chenodeoxycholic acid in the small intestinal contents was lower and cholic acid was higher than in the bile of rats on the ordinary and cholesterol diets. α-Muricholic acid in the small intestinal wall was significantly higher, but deoxycholic acid was lower than in the bile. The P8 acid was low in the intestinal contents but high in the intestinal tissue. A similar but more remarkable increase of α-muricholic acid was found in the liver. When the feces and large intestinal contents were compared, the increase of β-muricholic acid and the decrease of P10 in the feces were significant. Although P10 acid is not yet identified, the data suggest that P10 is an intermediate for the formation of β-muricholic acid.

Note that the ratio of α-muricholic acid is high in the small intestinal wall and liver. Since 6β-hydroxylation activity occurs in rat liver and increases with feeding of cholesterol (19), the high ratio of α-mucirholic acid in the liver may be attributable to the presence of the activity, but this does not explain the accumulation of this bile acid in the small intestinal wall. Small intestinal cells may have 6β-hydroxylation activity or different velocities for individual bile acids passing through the cells. The latter seems likely, since the composition ratios of bile acids are different not only between the intestinal contents and wall, but also between the liver and bile. Okishio and Nair (14) have also reported the difference of bile acid composition among portal blood plasma, peripheral blood plasma, and the liver.

Ho (17) has examined the circadian difference in the distribution of bile acids and demonstrated that the amount of bile acids in the intestinal tract changes with the movement of intestinal contents from the proximal to the distal, and from the small intestine to the colon. As our experiments were performed between 10:00 a.m. and noon, the distribution of bile acids may be different if the animals are sacrificed at a different time.

Our content of bile acids in the liver was lower than that reported by Fisher et al. (4). This is probably related to the fact that we determined the concentration in the liver after collecting the bile for 30 min.

As shown in Table III, cholesterol feeding increased the synthesis of bile acids but caused almost no change on the pool size, secretion, or turnover frequency. This may be related to the increase of chenodeoxycholic acid in the cholesterol-fed rats, since chenodeoxycholic acid has a shorter half-life than cholic acid (8) and is more effecitvely excreted into feces (9). Tyor et al. (20) have shown that intestinal absorption of tauro- or glycochenodeoxycholate is lower than that of the corresponding cholate.

Cholesterol was mainly located in the liver, and coprostanol was not found in the bile, liver, and small intestine but occurred in large amounts in the feces and large intestinal contents. The ratio of coprostanol was larger in the feces than in the large intestinal contents. The amount of sterol excreted into the feces was 11.12 mg/rat, and the ratio of coprostanol was about 39%. Kellog and Wostmann (21) reported the presence of about 10% of lathosterol in rat feces, but we did not find such a large amount.

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Nutritional and Metabolic Studies of Distillable Fractions from Fresh and Thermally Oxidized Corn Oil and Olive Oil

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ABSTRACT

A semisynthetic diet containing 15% by weight of dietary fat was fed to six groups of male Wistar rats for 28 days. Two groups received the distillable fraction of fresh corn oil (DCO) or fresh olive oil (DOO), two groups the distillable fraction of the thermally oxidized fats (OCO,OOO), and two groups received the respective fresh fats as controls (FCO, FOO). Substantial changes in the fatty acid composition occurred in the fats upon thermal oxidation. Only the rats that received OOO showed overt symptoms of heated fat toxicity. This was reflected in the histological scores of these animals, with the liver sustaining the most numerous and severe lesions. Tissue fatty acid changes of any significance were confined largely to the polar liver lipids of the rats that were fed OCO or OOO. The results of this study would suggest that the relatively greater toxicity of OOO, compared to OCO, may in part be due to the high oleic:linoleic acid ratio of the fresh olive oil and in part to a higher tocopherol content of the corn oil.

INTRODUCTION

Numerous studies have been conducted on the amount and types of toxic substances formed in fats used for cooking or deep drying and also on which of the commercial edible fats are most susceptible to degradation at high temperatures (1-4). It is known that nutritive properties of a fat can be impaired readily by thermal oxidation as indicated by subsequent nutritional evaluation of the oxidized fat using test animals. Several criteria for assessing heated fat toxicity have been employed (5-9).

Early work in this field involved the feeding of fats that had undergone rather severe damage by heat and oxidation. Animals fed these materials invariably exhibit both morphological and biochemical changes (10,11). Examination of fat depots of rats that had received oxidized soybean oil revealed relatively high levels of fat oxidation products (12). These authors also found that the tissue lipids of rats fed the oxidized soybean oil contained lower than normal levels of PUFA. This reflected a decrease in linoleate content of the heated soybean oil. Fat oxidation products were reported by Evarts and Bieri (13) in rats fed high levels of unsaturated fats with inadequate levels of vitamin E.

Heating conditions of the fats in this work, with respect to time, temperature, and aeration, were chosen to be similar to treatments that fats receive during commercial deep-frying operations. It is believed by most investigators that the higher the content of unsaturated fatty acids, especially PUFA in a fat, the more readily are toxic substances formed (14,15). However, using growth and life span as criteria in their nutritional evaluation of oxidized fats, Kaunitz et al. (16) reported longer life spans for rats fed oxidized corn oil and soybean oil as compared to control animals. In more recent work, Kaunitz and Johnson (17) fed mildly oxidized vegetable oils and animal fats to rats to determine their long term nutritional effects. They concluded that the most severe degenerative tissue changes were brought on by the unsaturated fats.

In view of the existing controversy with respect to toxicity of an oxidized fat with a high PUFA content versus a more saturated fat, corn oil (CO) and olive oil (OO) were selected for our experiments. A relatively short feeding period of 28 days was chosen in order to determine some of the early effects of heated fat toxicity.

MATERIALS AND METHODS

Male weanling rats of the Wistar strain (Woodlyn Laboratories, Guelph, Ontario) averaging 52 g in weight were randomly distributed among six experimental groups of ten rats. Commercially available corn oil (CO) (St. Lawrence Starch Co. Ltd.) and olive oil (OO) (Pompeian Olive Oil Corp.), were used in this experiment. The animals were fed semisynthetic diets containing 15% by weight of each fresh fat, the distillable fraction of the fresh or the distillable fraction of the oxidized fats. Table I shows the composition of the diets.

Thermal oxidation of the fats and distillation in vacuo of the fresh and oxidized fats were carried out as described earlier (18). Equal volumes of corn oil (CO) or olive oil (OO) were heated in stainless steel beakers with rheostatically controlled heating mantles for 72 hr at a temperature of 180 C. The fats were stirred

TABLE I

Composition of Diet

Ingredients	Percent	Weight (g)
Casein, vitamin-free	27	1350
Sucrose	49	2450
Vitamin mix 'AM'[a]	1	50
Salt mix USP XIV[b]	3	150
Celluflour[c]	5	250
Dietary fat[d]	15	750

[a]General Biochemicals, Chagrin Falls, OH; designed to be added at 1% level.

[b]Nutritional Biochemical Corp., Cleveland, OH; supplemented with 0.75 g/kg mix of $MnSO_4$ and 0.75 g/kg of $ZnCO_3$.

[c]Chicago Dietetic Supply House Inc., Chicago, IL.

[d]Fresh fats, distillable ethyl esters of the fresh fats or distillable ethyl esters of the thermally oxidized fats.

continuously for 12 hr by means of a mechanical stirrer and by hand every hour for the next 12 hr to ensure desired aeration and mixing. The volatile fractions of the thermally oxidized and fresh fats were isolated by distillation in vacuo at a pressure of 1 Torr and a head temperature between 150 and 180 C. The distillates obtained from both the fresh and oxidized fats were converted to ethyl esters and incorporated into the diets at a level of 15%. Feed and water were provided ad libitum and feed consumption was recorded. The animals were weighed weekly and examined for any gross abnormalities.

After 28 days of feeding, the animals were killed. Preparation of tissue sections from the hearts, livers, and kidneys for histological evaluation, and grading of lesions were carried out as described previously (18). Representative portions of the organs were extracted three times with chloroform-methanol (2:1, v/v), and total lipids were determined by a modified method of Entenman (19). These were fractionated subsequently into the neutral and polar lipid fractions (20). Fatty acid methyl esters were prepared from the above fractions (21) and analyzed for component fatty acids by gas liquid chromatography (GLC) (18).

Three tissue areas were selected for histological examination, the ventricular septum of the heart, right lateral lobe of the liver, and one half of the left kidney. Grading of the lesions found in these tissues was carried out as described in a previous publication (18). The scale used ranged from 0, for normal tissue, to 3, for necrotic areas. Where applicable, statistical evaluation of results was performed using the methods of Steel and Torrie (22).

RESULTS

Distribution of the major fatty acid components of the dietary fats as determined by gas chromatography is shown in Table II. Corn oil, which is high in linoleic acid, was compared with olive oil which is high in oleic acid. The higher boiling volatiles from the fresh fats isolated by fractional distillation under reduced pressure (1 Torr) showed relatively minor changes in the concentrations of the fatty acids. The only extensive change in DCO was a 32% reduction in palmitic acid. DOO sustained a notable loss of linolenic acid and a minor loss of eicosenoic acid. The OCO showed relative increases in a number of fatty acids. While actual losses were seen only in oleic and linoleic acids, the concentration of the latter was reduced by almost 99%. A similar dramatic reduction in linoleic acid occurred in OOO where the fatty acid concentration was reduced to traces from an initial 7.7%. As expected, the oleic acid concentration of both OCO and OOO, although somewhat lowered compared to the fresh fats, did not show the extensive losses observed for linoleic acid. This indicates a slower degradation of the monounsaturated fatty acid caused by thermal oxidation. Increases in the levels of myristic, palmitic, palmitoleic, and stearic acids were found in OCO. With respect to OOO, fatty acid increases were noted only in palmitic and stearic acids.

The carbonyl values for each of the OCO and OOO were determined as an index of the degree of degradation resulting from thermal oxidation. The high values (OCO-170.0 meq/kg, OOO-190.5 meq/kg) indicated extensive degradation.

There were no significant differences in the average daily feed consumption and feed efficiencies between the test groups and their respective controls, indicating an acceptable palatability and utilization of the diets. No remarkable changes, with respect to external appearnace and behavior of the animals, were noted in the three corn oil groups. This was true also for the FOO and DOO groups; however, the rats fed OOO showed overt signs of heated fat toxicity. All of these animals exhibited rough, greasy hair, possibly due to mild seborrhea as well as a noticeable decrease in grooming time. There also was evidence of diarrhea and polyuria, concomitant with an increased water concumption.

Final body weights, relative organ weights, and percent total organ lipids are shown in Table III. Body weights in all test groups appeared to be unaffected by the diets. This was the case for the organ weights as well, with

TABLE II

Fatty Acid Composition of Dietary Fats[a]

FA[b]	FCO	DCO	OCO	FOO	DOO	OOO
14:0	tr[c]	tr	6.0	---	---	---
16:0	10.0	7.6	40.0	11.1	11.2	20.6
16:1	0.3	0.2	12.5	1.2	1.3	1.4
18:0	1.8	1.9	7.4	3.1	3.0	4.6
18:1	24.0	25.4	22.0	75.0	76.2	71.7
18:2	61.0	61.8	1.1	7.7	7.3	tr
18:3	tr	---	---	2.0	0.4	tr
20:0	1.2	1.0	1.0	0.7	0.6	0.2
20:1	1.6	1.8	1.8	1.1	tr	tr

[a]FCO = fresh corn oil, DCO = distillable ethyl esters of fresh corn oil, OCO = distillable ethyl esters of oxidized corn oil. FOO = fresh olive oil, DOO = distillable ethyl esters of fresh olive oil, OOO = distillable ethyl esters of oxidized olive oil.

[b]Expressed as % total fatty acids. Minor components have been omitted.

[c]tr = trace (<0.09).

TABLE III

Body and Relative Organ Weights and Total Organ Lipids of Rats Fed Different Dietary Fats[a]

Dietary Fat[b]	Body weights (g)	Heart		Liver		Kidney	
		Rel. Wt. %	Lipid %	Rel. Wt. %	Lipid %	Rel. Wt. %	Lipid %
FCO	$198^{1,2}$	0.38^{1}	$8.4^{2,3}$	$4.6^{1,2}$	$8.8^{1,2}$	0.93^{1}	13.1^{1}
DCO	$199^{1,2}$	0.35^{1}	$9.7^{2,3}$	3.7^{2}	10.5^{1}	0.94^{1}	$11.5^{1,2}$
OCO	205^{1}	0.36^{1}	19.4^{1}	5.7^{1}	7.1^{2}	0.97^{1}	13.7^{1}
FOO	$199^{1,2}$	0.38^{1}	11.0^{2}	4.2^{2}	$9.6^{1,2}$	0.89^{1}	12.5^{1}
DOO	190^{2}	0.38^{1}	5.8^{3}	5.1^{1}	$9.1^{1,2}$	0.95^{1}	8.3^{2}
OOO	$200^{1,2}$	0.38^{1}	15.2^{1}	$4.8^{1,2}$	$8.5^{1,2}$	0.92^{1}	13.9^{1}

[a]Each value represents an average for ten rats. Relative weights of organs expressed as % body weight.

[b]FCO = fresh corn oil, DCO = distillable ethyl esters of fresh corn oil, OCO = distillable ethyl esters of oxidized corn oil. FOO = fresh olive oil, DOO = distillable ethyl esters of fresh olive oil, OOO = distillable ethyl esters of oxidized olive oil. Values in each column with the same superscript are not significantly different from each other ($P \leq 0.05$).

minor increases found only in the relative liver weights of the OCO and DOO groups. With respect to total organ lipids, the rats in the OCO and OOO groups showed substantial increases in the percent heart lipids. None of the test groups exhibited significant changes in total liver lipids. Total kidney lipids were unaffected by feeding the thermally oxidized fats.

Fatty acid compositions of the neutral and polar organ lipids are shown in Tables IV and V. Numerous changes in fatty acid concentration were observed in the neutral organ lipids of rats that received the test diets; however, no definite pattern was evident (Table IV). The most obvious change was the substantial increase in heart arachidonic acid for the OOO group. In regard to liver neutral lipids, substantial increases were noted for both stearic and arachidonic acids when OOO was fed. These two acids also were increased in the kidney when OCO was the dietary fat. Another observation for the kidney was a drop in linoleic acid level with both OCO and OOO.

With respect to the polar lipids (Table V), the major differences between control and test groups were confined to the liver fatty acids of animals that received OCO and OOO. Palmitic, palmitoleic, and oleic acids of the liver polar lipids were elevated greatly, while the levels of stearic and arachidonic acids were reduced substantially.

Although both thermally oxidized dietary fats were very low in linoleic acid content, the livers of the OCO animals showed a remarkable increase in this fatty acid. The OOO resulted in lower levels of linoleic acid in each of the three organs examined.

Results of examinations of heart, liver, and

TABLE IV

Fatty Acid Composition of Neutral Organ Lipids of Rats Red Different Dietary Fats

Organ	Dietary fat[a]	Fatty acid[b]					
		16:0	16:1	18:0	18:1	18:2	20:4
Heart	FCO	20.5	8.7	7.0	18.9	22.2	1.9
	DCO	20.9	4.2	7.4	27.8	31.6	1.4
	OCO	28.3	6.7	6.8	22.7	21.0	1.6
	FOO	21.0	6.6	7.1	46.4	3.7	2.3
	DOO	21.1	4.6	6.2	56.7	5.6	1.2
	OOO	22.4	7.1	9.1	42.8	3.0	5.0
Liver	FCO	23.3	2.4	7.8	22.1	29.1	10.2
	DCO	17.9	1.5	7.9	17.9	30.5	17.2
	OCO	17.0	2.8	6.3	25.2	28.4	12.7
	FOO	19.6	3.7	4.6	59.1	5.6	4.1
	DOO	25.6	5.0	4.3	56.4	4.2	1.3
	OOO	16.1	2.5	9.5	47.8	5.2	13.2
Kidney	FCO	22.7	3.9	8.3	22.7	32.3	5.0
	DCO	23.9	4.8	5.6	25.5	33.5	2.3
	OCO	25.8	4.0	11.7	20.3	20.2	10.3
	FOO	20.1	4.6	5.3	56.1	6.1	4.3
	DOO	22.9	5.4	5.0	55.7	5.2	2.0
	OOO	22.1	4.2	5.0	57.3	3.2	4.3

[a]FCO = fresh corn oil, DCO = distillable ethyl esters of fresh corn oil, OCO = distillable ethyl esters of oxidized corn oil. FOO = fresh olive oil, DOO = distillable ethyl esters of fresh olive oil, OOO = distillable ethyl esters of oxidized olive oil.

[b]Expressed as % total fatty acids. Minor components have been omitted.

TABLE V

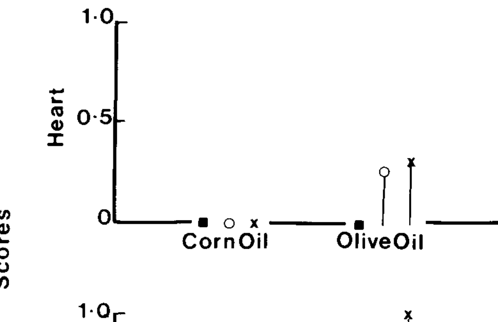
Fatty Acid Composition of Polar Organ Lipids of Rats Fed Different Dietary Fats

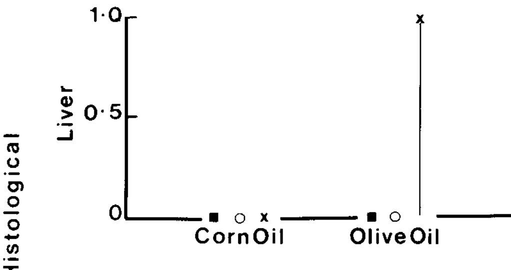
Organ	Dietary fat[a]	Fatty acid[b]					
		16:0	16:1	18:0	18:1	18:2	20:4
Heart	FCO	15.6	3.8	12.7	16.1	23.9	24.1
	DCO	10.9	1.8	21.2	20.6	23.8	20.7
	OCO	13.1	1.4	10.3	17.9	23.1	21.4
	FOO	11.4	1.3	24.8	17.9	13.5	24.1
	DOO	11.1	1.4	21.9	23.8	14.7	20.9
	OOO	10.8	1.9	23.7	19.8	10.3	24.6
Liver	FCO	17.9	2.2	21.5	8.7	15.8	20.1
	DCO	17.7	1.2	19.7	9.6	21.0	24.1
	OCO	25.0	5.4	10.2	18.2	24.5	8.9
	FOO	13.1	3.6	22.0	26.1	12.6	20.0
	DOO	13.5	2.1	24.9	21.2	7.6	21.1
	OOO	16.8	4.6	14.0	33.6	8.5	10.9
Kidney	FCO	12.2	1.1	18.6	9.5	19.5	28.5
	DCO	13.2	2.2	17.1	13.7	26.9	22.1
	OCO	16.9	1.0	23.4	9.1	14.6	30.0
	FOO	16.9	2.2	20.7	21.0	11.8	22.0
	DOO	14.4	2.8	17.8	22.9	15.9	20.7
	OOO	18.2	1.0	21.3	17.9	5.7	29.1

[a]FCO = fresh corn oil, DCO = distillable ethyl esters of fresh corn oil, OCO = distillable ethyl esters of oxidized corn oil. FOO = fresh olive oil, DOO = distillable ethyl esters of fresh olive oil, OOO = distillable ethyl esters of oxidized olive oil.

[b]Expressed as % total fatty acids. Minor components have been omitted.

kidney tissue sections from the control and test groups are represented by histological scores in Figure 1. Neither the DCO nor OCO produced any advsrse effects. There was, however, very evident liver and kidney injury due to OOO. The values shown for the heart from animals fed DOO or OOO were not statistically significant. Figure 2 shows a photomicrograph of a liver section from a test rat fed OOO. Although there was scattered but severe pyknosis of the hepatic nuclei, and cytoplasmic atrophy leading to cell death, no distinct necrotic foci were evident. The livers of these animals were undergoing extensive regeneration, as indicated by many mitotic hepatocytes. The damage to the kidneys in the OOO group consisted mainly of activated nuclei of the epithelial cells of the tubules and of the glomerular mesangial cells.





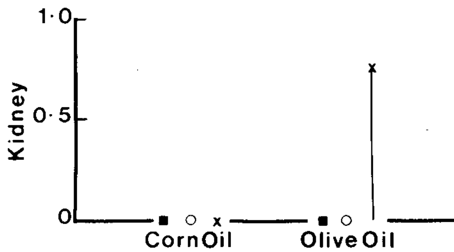


FIG. 1. Histological scores for organs of rats fed different dietary fats.

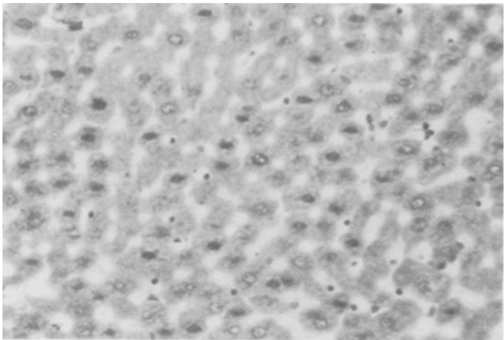


FIG. 2. There is atrophy of the hepatic cords with coarse irregular aggregations of cytoplasmic RNA and pyknosis of many nuclei. Sinusoids are dilated moderately, and endothelial and Kupffer cells are more prominent than normal. Diet: OOO. Hematoxylin and eoxin, X 344.

DISCUSSION

The heating of fats in air is known to cause the formation of volatile scission products, nonvolatile oxidized derivatives such as cyclic monomers and dimers, as well as polymers (1,23-26). However, the nature and extent of these degradation products depend on the kind of fat and the way it is heated. The thermal oxidative treatment, to which the fats used in this study were subjected, was designed to approximate the conditions used by food processors and restaurants for deep frying. The carbonyl values of the treated fats indicated that considerable oxidation had taken place during the heating and aeration.

The component fatty acids of the dietary fats are shown in Table II. It is important to realize that both the increases and decreases of fatty acid concentrations in the distillable portions, as shown in the table, are primarily relative changes resulting from loss of fatty material. The formation of volatile short chain scission products, or their cyclization during thermal oxidation of the fat, and the residue of nondistillable polymerized material, are responsible for the fatty acid changes in the thermally oxidized fat. It is known that some of the material found in heated fats is not eluted from the GLC column. Waltking (24) reported that the retained material was essentially a polymeric product of linoleic acid.

No growth depression resulted from feeding the test fats, which reflected the similar feed consumption and efficiency found in the control and test groups. The hearts of the OCO and OOO rats showed an extensive increase in cardiac lipids (Table III). This accumulation may have been due to an impared mobilization and/or metabolism of these lipids. Landes (27) and

Landes and Miller (28) found an increased serum lipoprotein lipase activity in rats that received oxidized fat. It can be concluded from these findings that the lack of these cardiac lipids as an energy source for the heart stimulated an increase in this lipoprotein lipase activity. As a result, the heart would take up additional lipids from the bloodstream leading to the fatty buildup. The fact that the hearts of the OCO and OOO animals did not show concomitant increases in percent relative weight might be explained as the result of glycogen and protein depletion as alternate energy sources. Fatty livers resulting from the feeding of oxidized fats have been reported in the literature (18,29,30); however, there was no evidence of fatty accumulation in the livers of the animals used in this study.

The effects of the dietary fats on tissue fatty acid concentrations differed greatly between the neutral and polar organ lipids. In the neutral lipids of the heart (Table IV), most of the changes observed were related to shifts in the dietary fatty acid concentrations as a result of distillation or thermal oxidation. The increased incorporation of arachidonic acid into the neutral liver lipids of animals fed OOO and neutral kidney lipids of animals fed OCO is at present unexplained. A more definite pattern of change was observed in the fatty acid concentrations of the polar liver lipids (Table V). The levels of palmitic, palmitoleic, and oleic acids were increased substantially, while the concentrations of stearic and arachidonic acids were greatly reduced compared to control values. This may indicate a reduced utilization of palmitic acid and the two monoenoic acids for energy metabolism and/or an increased incorporation of them into the polar lipid fraction. Conversely, the reduced level of arachidonic acid in the polar liver lipids indicates a preferential oxidation and/or a decreased incorporation into phospholipids. With regard to the very low level of liver arachidonic acid in animals fed OCO, it would seem evident that the heated fat has resulted in a metabolic inhibition of the conversion of linoleic acid to arachidonic acid. This is evidenced by a buildup in the level of liver linoleic acid despite a low amount in the diet of these rats. Reduced levels of linoleic acid were exhibited in all three organs from animals fed OOO and may be a direct result of the very low levels of linoleic acid in the dietary fat. The extremely low level of linoleic acid in the diet containing OOO may have resulted in an EFA deficiency of these animals and exacerbation of the toxic effects of the fat degradation products.

The results obtained in this study from the feeding of DCO and DOO indicate that they were not toxic to the rats over the experimental period. The fact that OOO proved more deleterious than OCO, as indicated by the histological scores (Fig. 1), is contradictory to reports in the literature that heated, highly unsaturated fats are more detrimental than the relatively more saturated fats (31,32). It has been shown that the polyunsaturated fatty acids, being more susceptible to oxygen attack during thermal oxidation, form scission products quite readily, which are then converted to relatively inert polymers in Diels-Alder type addition reactions.

Earlier observations in our laboratory revealed that methyl oleate, or fats containing a large concentration of oleic acid, were decidedly more toxic when thermally oxidized and fed to rats than fats with a relatively low oleic acid concentration.

It is proposed that oleate forms toxic cyclic monomers which are less inclined to polymerize than the cyclic derivatives of the polyunsaturated fatty acids. Therefore, the oleate monomeric derivatives would build up to a greater degree in fats heated in air, and this would be accentuated in fats high in oleic acid. Numerous workers have attributed the toxicity of thermally oxidized fats to the cyclic derivatives (33-36). Another factor which should be considered is that corn oil has a higher tocopherol content than olive oil (37), which would provide some protection of the unsaturated fatty acids against oxidation. Tocopherol levels were not determined, but both the OCO and OOO samples showed substantial losses of linoleic acid (Table II) due to the heating.

ACKNOWLEDGMENTS

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Sterol Composition of *Neurospora crassa*

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ABSTRACT

The sterols extracted from freeze-dried, log-phase cultures of *Neurospora crassa* were separated by thin layer and analyzed by capillary gas liquid chromatography and mass spectrometry. Ergosterol, episterol, and ergosta-7,22 dienol were the major sterols of the free sterol fraction. The major esterified sterols, which constituted 4.95% of the total sterol fraction, were ergosterol, episterol, and ergosta-7,22,24(28)-trienol, with lesser quantities of 4α-methyl-ergosta-8,24(28)-dienol. With the exception of lanosterol, all sterols were alkylated at the C-24 position.

INTRODUCTION

In recent years, the polar and fatty acid composition (1-2), carotenoids (3-4), and the major sterols of *Neurospora crassa* (5-6) have been investigated. However, nothing is known about esterified or any of the minor sterol components. In the present study, the free sterols and sterol esters have been isolated and characterized.

MATERIALS AND METHODS

The wild type 74-OR-23-1A strain was obtained from the Fungal Genetics Stock Center at Arcata, CA. Five-milliliter conidial suspensions of this strain, containing 1.5 x 10^4 cells/ml were used as inocula for 1,500 ml of standard *Neurospora* medium (7). Cultures were agitated by aeration for 3 days at 21 C. The resultant mycelium was washed in sterile distilled water, redispersed in 1,500 ml sterile H_2O, and macerated in a Waring blendor for 30 sec. A 10% inoculum (150 ml) of the macerated mycelial homogenate was used in another 1,500-ml culture for an additional 3 days' incubation. This procedure of washing, macerating, and inoculating with 10% of the following culture was repeated three times with further 1,500 ml 3-day cultures. The resultant mycelium was used as a 10% inoculum for a 4.5-liter, 3-day culture, from which the final biomass was harvested. Cultures were harvested in cheesecloth, washed with distilled water, frozen under liquid nitrogen, and lyophilized. Four-gram aliquots of dry mycelium were powdered in a mortar, then extracted by rapid agitation using a magnetic stirrer for 30 min in each of the following solvent mixtures: twice with 500 ml of acetone, twice with 500 ml 50% benzene in acetone, once with 500 ml of 50% ethyl acetate in absolute methanol, and twice with 500 ml absolute methanol. Mycelium was recovered at each step by filtration on fritted-disc filters under partial vacuum. The extracts were flash-dried and resuspended in 100 ml light petroleum ether. The fatty acids were removed by washing twice with 200 ml of aqueous NaOH (pH 8.0), then three times with distilled H_2O. The epiphase was again flash-dried and resuspended in 2 ml benzene. This was chromatographed with authentic standards of ergosterol, lanosterol, ergosterol acetate, and lanosterol acetate on a glass plate (20 x 100 cm), coated with Silica Gel G containing fluorescent indicators. The chromatogram was developed with hexane-diethylether-methanol (40:10:1). The free sterols were retrieved by washing the silica with diethyl ether on a fritted-disc filter over partial vacuum. This fraction was then flash-dried, resuspended in 2 ml benzene, and rechromatographed as above. After retrieval from the silica, the sterols were ready for acetylation.

The sterol ester band, after retrieval from the chromatogram, was resuspended in 100 ml of saponification mixture (10 ml 30% KOH, w/v, per 90 ml 95% ethanol) and left overnight. The saponification mixture was partitioned with 100 ml light petroleum, and the epiphase was washed repeatedly, dried, and resuspended in 2 ml benzene. This fraction was then rechromatographed as before.

The free sterols and sterols from esters were acetylated in pyridine-acetic anhydride (2:1) overnight. The subsequent sterol acetates were again chromatographed on silica gel with hexane diethyl ether (9:1). After retrieval from the chromatogram, the acetate derivatives were analyzed by gas chromatography using a Varian Model 2100 gas chromatograph, equipped with a flame-ionization detector. Peak areas were computed electronically by means of a Spectra-Physics Autolab Minigrator Model 23000-010.

Analyses were performed on both a 32.5 m x 0.25 mm ID glass column, coated with

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TABLE I

The Sterols of *Neurospora crassa*[a]

Sterol	Percent composition of free sterol	Percent composition of esterified sterol
Lanosterol	1.5	T[b]
24-Methylene-24,25-dihydrolanosterol	1.1	T
24-Methyl-24,25-dihydrolanosterol	0.2	t[c]
4,4-Dimethylergosta-8,24(28)-dienol	1.3	T
4α-Methyl ergosta-8,24(28)-dienol	0.8	7.1
Fecosterol	1.4	1.9
Ergosta-8,22,24(28)-trienol	0.9	1.6
Ergosta-8,22-dienol	0.4	1.4
Ergosta-5,8,22 trienol	1.0	3.1
Episterol	3.8	17.9
Ergosta-7,22,24(28)-trienol	1.7	13.3
Ergosta-7, 22-dienol	4.2	5.2
Ergost-7-enol	0.3	T
Ergosterol	81.4	47.6
Total sterol	672.5 μg/g dry wt	35.0 μg/g dry wt

[a]Values are based on 2-3 gas chromatograms per sample by comparison with 3-β-cholestanol acetate as internal standard.

[b]0.1% ⩽ T ⩽ 0.4%.

[c]t < 0.1%.

OV-101, at 245 C and a 1.83 m x 2 mm ID glass column, packed with 3% Silar 10 C on Gas Chrom Q (100-120 mesh), at 220 C. The presence of particular sterols was ascertained by comparison of relative retention times (RRTs) using 3β-cholestanol acetate as an internal standard with RRTs previously determined for yeast sterols (8,9). The amounts of individual sterols were determined by comparison of peak areas to that of the internal standard. The identity of the major components in the sterol bands was confirmed by mass spectroscopy (MS) on a Hitachi Perkin-Elmer RMU-7 mass spectrometer, using an ionization voltage of 80 eV.

RESULTS AND DISCUSSION

Repeated subculturing with macerated mycelium produced 10.4 g dry wt per liter of log-phase culture. The total lipid extracted from the freeze-dried mycelia of *N. crassa* was 10.3%, of which the free sterols comprised 0.65% and the esterified sterols .03%. These values differe from those previously published (1,5) owing to differences in the growth regime and extraction procedures. It should also be noted that the total esterified sterols comprised 13.1% of the total sterol of stationary-phase culture (1) and only 4.95% in log-phase cultures. This had been previously noted only for ergosterol in *Phycomyces blakesleeanus* (10).

With the single exception of lanosterol (Table I), all of the *N. crassa* sterols were alkylated at the C-24 position, indicating that 24 methyl-transferase activity precedes demethylation. This is unlike wild-type *Saccharomyces cerevisiae* (11), but similar to *P. blakesleeanus* (12). C-24 alkylation of lanosterol was found to constitute a minor pathway in wild type *Candida albicans* (13), and *Torulopsis glabrata* (14).

C-24 methyl derivatives of lanosterol previously have been identified in trace amounts in *C. albicans* and *C. utilis* (13). In the present study, 24-methyl-24,25-dihydrolanosterol was found in amounts which suggest that the Δ24(28) reductase may be operative on 4,4-dimethyl C-24 methylene sterols in *Neurospora* although none of the anticipated subsequent intermediates, 4,4-dimethylergost-8-enol and 4α-methylergost-8-enol, were found.

Ergosterol was the major free sterol (81.4%) with lesser amounts of ergosta-7,22-dienol (4.2%) and episterol (3.8%). With the analytical techniques used cycloartenol (15) and cholesterol (5) were not detected in any fraction. Ergosterol was also the major sterol of the sterol ester fraction, although it was not as abundant (47.6%) as in the free sterols. Episterol (17.9%), ergosta-7,22,24(28)-trienol (13.3%), 4α-methylergosta-8,24(28)-dienol (7.1%) were other abundant sterols from the sterol ester fraction. In previous work (1) the major fatty acids of the sterol esters were found to be: linoleic (58.7%), oleic (17.1%), and palmitic (10.7%), with smaller quantities of stearic (3.8%), palmitoleic (3.0%), linolenic (1.7%), and myristic (0.2%) (expressed as a per-

cent of the total fatty acids from the sterol esters). These were not further investigated in this report.

Studies with C^{14} mevalonate tracers are in progress to determine if sterol metabolism in *Neurospora* is consistent with observations (9,13) that the enzyme systems responsible for modifying the sterol intermediates will accept a variety of closely related substrates.

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Composition and Synthesis of Three Higher Ganglioside Homologs in Bovine Mammary Tissue[1]

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ABSTRACT

Three higher gangliosides were identified as constituents of bovine mammary gland. The structures of these three gangliosides were shown to be ceramide-glucose-galactose-(sialic acid)-N-acetylgalactosamine-galactose, ceramide-glucose-galactose-(sialic acid)$_2$-N-acetylgalactosamine, and ceramide-glucose-galactose-(sialic acid)$_2$-N-acetylgalactosamine-galactose. These gangliosides accounted for only a small fraction (<20%) of the lipid-bound sialic acid in mammary gland. While fatty acids with even carbon numbers from C_{14} to C_{26} were predominant in these gangliosides, they also contained C_{23} and C_{25} fatty acids. Mammary gland Golgi apparatus-rich fractions had all glycosyltransferases required for synthesis of these gangliosides starting with ceramide.

INTRODUCTION

Gangliosides are a class of glycosphingolipids that contain sialic acid. They were first identified by Klenk (2) as constituents of brain and have subsequently been found in a large number of extraneural tissues (e.g., 3-8) and in diverse cultured cell lines (e.g., 9,10).

Roseman and associates (11-15) have shown that chick brain gangliosides are synthesized in a stepwise manner by transfer of carbohydrates from sugar nucleotides to glycolipid acceptors. While many investigators assumed that gangliosides were both localized in and synthesized by surface membranes (10, 16-19), Keenan and Morré (20,21) found gangliosides to be present in Golgi apparatus, endoplasmic reticulum, and particulate-free supernatants of rat liver and bovine mammary gland. In both tissues, Golgi apparatus and rough endoplasmic reticulum fractions contained all glycosyltransferases involved in the synthesis of the common mono-sialo- and disialogangliosides (20,21).

In the present study, the composition and synthesis of three highly polar gangliosides in bovine mammary gland were examined. In earlier work, Keenan (22,23) identified three less polar gangliosides (GM_3, GM_2, and GD_3) in bovine milk and mammary tissue.

[1] Abbreviations: GM_3, N-acetylneuraminylgalactosylglucosyl ceramide; GD_3, (N-acetylneuraminyl)$_2$-galactosylglucosyl ceramide; GM_2, N-acetylgalactosaminyl-(N-acetylneuraminyl)-galactosylglucosyl ceramide; GM_1, galactosyl-N-acetylgalactosaminyl-(N-acetylneuraminyl)-galactosylglucosyl ceramide; GD_2, N-acetylgalactosaminyl-(N-acetylneuraminyl)$_2$-galactosylglucosyl ceramide; GD_{1a}, N-acetylneuraminylgalactosyl-N-acetylgalactosaminyl-(N-acetylneuraminyl)-galactosylglucosyl ceramide; GD_{1b}, galactosyl-N-acetylgalactosaminyl-(N-acetylneuraminyl)$_2$-galactosylglucosyl ceramide. These abbreviations are those of Svennerholm (1).

MATERIALS

Glucosyl and lactosyl ceramides were isolated from bovine milk fat globule membranes (24). These compounds have the structures β-glucosyl (1→1) N-acylspingosine and β-galactosyl (1→1) N-acylsphingosine (25). Ceramide, trihexosyl ceramide, and the gangliosides, N-acetylneuraminyl-galactosylglucosyl ceramide (GM_3), N-acetylgalactosaminyl-(N-acetylneuraminyl)-galactosylglucosyl ceramide (GM_2), galactosyl-N-acetylgalactosaminyl-(N-acetylneuraminyl)-galactosylglucosyl ceramide (GM_1), and (N-acetylneuraminyl)$_2$-galactosylglucosyl ceramide (GD_3), were obtained as desribed previously (22). The gangliosides, N-acetylneuraminyl-galactosyl-N-acetylgalactosaminyl (N-acetylneuraminyl)-galactosylglucosyl ceramide (GD_{1a}) and galactosyl-N-acetylgalactosaminyl-(N-acetylneuraminyl)$_2$-galactosylglucosyl ceramide (GD_{1b}), were isolated from bovine brain (26) and purified using DEAE-Sephadex and Iatrobead (Iatron Laboratories, Inc., Tokyo, Japan) column chromatography (27). The ganglioside, N-acetylgalactosaminyl-(N-acetylneuraminyl)$_2$-galactosylglucosyl ceramide (GD_2), was prepared from GD_{1b} using a rat liver β-galactosidase (28). N-acetylneuraminic acid and neuraminidase from *Clostidium perfringens* were from Sigma Chemical Company, St. Louis, MO. Gas chromatographic columns and fatty acid methyl esters were from Supelco, Bellefonte, PA. Unlabeled CMP-N-acetylneuraminic acid was prepared according to Kean (29). Unlabeled UDP-galactose (UDP-Gal), UDP-glucose (UDP-Glc), and free carbohydrates were from Sigma. UDP-[^{14}C]-galactose (285 Ci/mole) was from New England Nuclear (Boston, MA) and CMP-N-[4,5,6,7,8,9,-^{14}C]-acetylneuraminic acid (214 Ci/mole) was from Amersham/Searle (Arlington Heights, IL).

METHODS

Extraction Procedure

Lactating bovine mammary tissue, obtained at slaughter, was homogenized in water in a commercial Waring blendor. The resulting homogenate was placed in glass trays and lyophilized. The dry product was homogenized, using a Waring blendor, in 20 volumes of chloroform-methanol (2:1, by vol), and the homogenate was filtered through cheesecloth and then a sintered glass funnel. The resulting particulate was again homogenized, with a Waring blendor, in 10 volumes of chloroform-methanol (1:1, by vol) and held overnight with stirring. The combined extracts were then evaporated to dryness.

Purification of Mammary Gland Gangliosides

The residue from the homogenate was redissolved in chloroform-methanol (2:1), and gangliosides were recovered by partitioning according to Folch et al. (30). The upper phases were dialyzed, evaporated to dryness, and the ester lipids were removed by saponification in 0.5 N methanolic NaOH at 40 C for 2-3 hr. The solution was concentrated to one half its volume and was dialyzed in the cold against several changes of distilled water for 2 days. The dialysate was evaporated to dryness and the residue dissolved in chloroform-methanol (1:1, by vol). Gangliosides were then determined by sialic acid assay (31). Crude gangliosides obtained in this manner were purified to homogeneity by thin layer chromatography (TLC).

Thin Layer Chromatography

Plates coated with a 250-μm layer of Silica Gel G were used for ganglioside separation. Plates were developed either in chloroform-methanol-28% ammonia-water (60:35:7:3, by vol) or in N-propanol-28% ammonia (7:3, by vol). Gangliosides were visualized with resorcinol spray (32). For isolation, chromatoplates were placed in a tank of iodine crystals. After sublimation, bands of silica gel containing gangliosides were scraped into sintered glass funnels, and gangliosides were eluted with chloroform-methanol-water (1:1:0.2, by vol).

Analytical Methods

Sialic acid was determined by the method of Warren (31), and sphingosine was determined according to Lauter and Trams (33) using the hydrolysis procedure of Sweeley (34). Glucose, galactose, and N-acetylgalactosamine were measured separately as the alditol acetates by gas liquid chromatography (GLC) (35). For this determination, a 0.3 x 180 cm glass column packed with 3% SP 2340 on 100/120 mesh Supelcoport (Supelco, Inc., Bellefonte, PA) was used. The column was temperature programmed from 150 to 250 C at 3 C/min, at a nitrogen carrier gas flow rate of 25 ml/min in an F & M Model 402 gas chromatograph with a flame ionization detector. Fatty acid methyl esters were prepared by hydrolysis with 1 ml of methanolic BF_3 for 10 min at 100 C (36) and were separated on a 0.3 x 180 cm glass column packed with 10% SP 2340 on 100/120 mesh Chromosorb W operated by temperature programming from 150 to 250 C at 4 C/min. Protein was determined by the method of Lowry et al. (37) with bovine serum albumin as standard.

Mild Acid Hydrolysis

Gangliosides were treated with 0.001 M aqueous H_2SO_4 for 1 hr in a boiling water bath (38). Neutral glycolipids were recovered and examined by TLC. Visualization was by spraying the chromatoplates with 50% aqueous H_2SO_4. To determine if ceramide in bovine mammary gangliosides was linked to glucose, a partial acid hydrolysis was performed on samples, followed by gas liquid chromatographic analysis of carbohydrates as alditol acetates (39).

Enzymatic Hydrolysis

Reaction mixtures contained 0.1 μmole ganglioside, 500 μg sodium taurocholate, 100 μmoles of 0.05 mM acetate buffer, pH 5.2, and 0.04 units of neuraminidase in a final volume of 0.2 ml. Incubation was for 18 hr at 37 C. Reactions were stopped by addition of 3 ml chloroform-methanol (1:1), the mixture was washed and examined by TLC.

Glycosyltransferase Assays

Golgi apparatus-rich fractions were prepared from lactating bovine mammary tissue according to Keenan et al. (23). This fraction was suspended in 0.32 M sucrose 14 mM 2-mercaptoethanol and used as the enzyme source.

For incorporation of galactose into gangliosides and neutral glycolipids, complete reaction mixtures contained (in μmoles unless otherwise stated) in a *final* volume of 0.1 ml: glycolipid acceptor, 0.05; Triton CF-54-Tween 80 (2:1, w/w), 0.6 mg; cacodylate-HCl, pH 7.3, 15; $MnCl_2$, 2.5; UDP Gal, 0.05 (2.5 x 10^6 CPM/μmole); and 0.15 to 0.26 mg enzyme protein. For sialyl transferase assays, complete reaction mixtures contained (in μmoles) in a final volume of 0.1 ml: glycolipid acceptor, 0.05; Tween 80-Triton CF-54 (1:2, w/w), 0.6 mg; cacodylate-HCl, pH 6.35, 15; $MgCl_2$, 1.0;

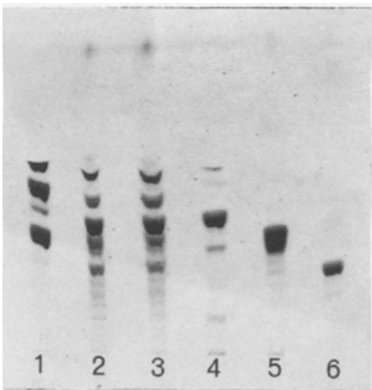

FIG. 1. Thin layer chromatogram of ganglioside fractions from bovine mammary tissue. The Silica Gel G plate was developed in *n*-propanol-28% ammonia-water (14:6:1, by vol) and sprayed with resorcinol reagent. 1: Reference GM_3, GM_2, GD_3, and GM_1 in order of decreasing mobility; 2: mammary gangliosides, 40 nmoles of sialic acid; 3: mammary gangliosides, 80 nmoles of sialic acid; 4: GD_3; 5: GD_{1a}; and 6: GD_{1b}.

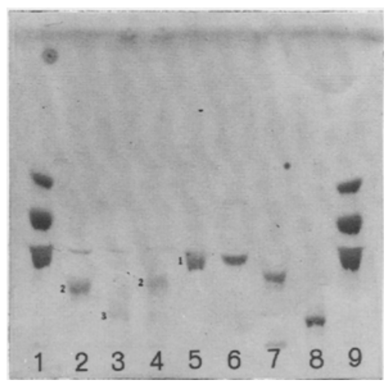

FIG. 2. Thin layer chromatogram of bovine mammary gangliosides after separation by preparative chromatography. The Silica Gel G plate was developed in chloroform-methanol-28% ammonia-water (60:35:7:3, by vol) and sprayed with resorcinol reagent. 1 and 9: Reference GM_3, GM_2, and GM_1; 2 and 4: fraction enriched in MG V; 3: fraction enriched in MG VI; 5: fraction enriched in MG IV; 6: GD_3; 7: GD_{1a}; and 8: GD_{1b}.

CMP-N-acetylneuraminic acid, 0.05 (1.8×10^6 CPM/μmole); and 0.15 to 0.24 mg enzyme protein. Incubation was for 2 hr at 37 C, after which reactions were stopped by the addition of 0.1 ml of methanol. Reaction mixtures were applied onto Whatman 3 MM paper strips which were developed (descending) overnight with 1% sodium tetraborate (40). The origins were cut from the strips, placed in vials with 15 ml of chloroform-methanol-H_2O (1:1:0.2, by vol), and incubated with shaking for 1 hr at 37 C. The extracts were evaporated, residues were solubilized in 1.0 ml of hyamine hydroxide, and radioactivity was determined by liquid scintillation techniques. Specific activity values were calculated from CPM of radioactive sugar transferred to lipids.

In order to identify the products of transferase assays, reactions were performed as described in the preceding paragraph. At that point where the glycolipid was extracted into 15 ml of chloroform-methanol-water (1:1:0.2) and evaporated to dryness, residues were dissolved in 80 μl of chloroform-methanol. Residues were then spotted on Silica Gel G plates, and the plates were developed in chloroform-methanol-28% ammonia-water (60:35:7:3, by vol). Plates were dried and overlaid with Kodak Royal Blue X-Omat X-ray film. After 7 weeks, the X-ray film was developed, and spots representing radioactive glycolipids were identified by comparison of mobilities with standards run on the same plates.

RESULTS

Distribution and Carbohydrate Composition of Gangliosides

In confirmation of previous observations (22), at least six gangliosides were observed on thin layer chromatograms obtained with mammary tissue. The three more polar gangliosides which were isolated and identified (Fig. 1) ac-

TABLE I

Molar Ratios of Ganglioside Constituents[a]

Constituent	Molar ratios		
	MG IV	MG V	MG VI
Sphingosine	1.00	1.00	1.00
Sialic acid	0.92	1.70	2.15
Galactose	2.31	0.91	1.99
Glucose	1.03	0.88	0.97
N-acetylgalactosamine	0.98	0.84	1.04

[a]Individual gangliosides were isolated from bovine mammary gland and purified to chromatographic homogeneity.

TABLE II

Fatty Acid Composition of Individual Gangliosides Isolated from Bovine Mammary Gland[a]

Acid	MG IV	MG V	MG VI
14:0	7.4	0.7	2.3
16:0	6.8	1.0	9.2
16:1	2.1	0.3	---
18:0	8.4	1.0	9.8
18:1	22.6	3.7	7.4
20:0	3.1	0.4	7.0
21:0	4.3	0.5	---
22:0	13.8	88.1	16.3
23:0	9.4	1.2	16.0
24:0	13.2	1.9	19.9
25:0	5.3	0.9	6.1
26:0	3.5	0.3	6.1

[a]Methyl esters were prepared and separated by gas chromatography as described in Methods. Data are given as weight percent of total fatty acid methyl esters. Fatty acids are abbreviated as number of carbons:number of double bonds.

count for less than 20% of the total gangliosides found in mammary tissue. The thiobarbituric acid assay (31) demonstrated the presence of sialic acid in all fractions. Glucose, galactose, and galactosamine were the only sugars observed on GLC. Glucosamine was not detected in any of the ganglioside fractions examined.

Structural Characterization

Fractions enriched in the three more polar gangliosides were obtained by Folch extraction and partition (Fig. 2). These gangliosides were designated MG IV, MG V, and MG VI (MG is for mammary gland) in order of decreasing chromatographic mobility. These gangliosides were purified to homogeneity by preparative TLC and subjected to structural chracterization.

Ganglioside MG IV had a thin layer mobility identical with that of GM_1 (Fig. 2). The molar ratios of sphingosine to sialic acid to hexose to galactosamine were 1.00:0.92:3.34:0.98 (Table I). Galactose, glucose, and galactosamine were present in a molar ratio of 2.31:1.03:0.98. Mild acid hydrolysis of MG IV yielded two neutral glycolipids, one which migrated with lactosyl ceramide and the other which migrated as a tetrahexosyl ceramide (not shown). Neuraminidase treatment of MG IV yielded unaltered MG IV (not shown). Partial acid hydrolysis of MG IV, followed by isolation of free carbohydrates and complete hydrolysis of the remaining glycolipid, showed glucose to be the last sugar removed from ceramide. Thus, the structure Gal-GalNAc-(sialic acid)-Gal-Glc-ceramide was suggested for MG IV.

Ganglioside MG V has a thin layer mobility between GD_{1a} and GD_{1b} (Fig. 2). The molar ratios of sphingosine to sialic acid to hexose to galactosamine were 1.00:1.70:1.79:0.84 (Table I). Galactose and glucose were present in a molar ratio of 0.91:0.88. Mild hydrolysis yielded two major neutral glycolipids, one which migrated with lactosyl ceramide and the other which chromatographed as a trihexosyl ceramide (not shown). Neuraminidase treatment yielded both unchanged MG V and ganglioside with the same migratory properties as GM_2 (not shown). Stepwise acid hydrolysis followed by GLC showed that glucose was linked to ceramide. These results suggest the structure of MG V to be GalNAc-(silic acid)$_2$-Gal-Glc-ceramide (GD_2).

Ganglioside MG VI demonstrated the same thin layer mobility properties as GD_{1b} (Fig. 2). The molar ratios of sphingosine to sialic acid to hexose to galactosamine were 1.00:2.15:2.96:1.04 (Table I). Galactose and glucose were present in a molar ratio of 1.99:0.97. The products of mild acid hydrolysis of MG VI were two neutral glycolipids, one with migratory properties identical to those of lactosyl ceramide and the other which chromatographed as a tetrahexosyl ceramide (not shown). Neuraminidase treatment yielded unchanged MG VI and a ganglioside with the same thin layer chromatographic mobility as GM_1. Stepwise acid hydrolysis followed by GLC demonstrated that glucose was linked to ceramide. Thus, the structure Gal-GalNAc-(sialic acid)$_2$-Gal-Glc-ceramide (GD_{1b}) was suggested for ganglioside MG VI.

Fatty Acid Composition

The individual gangliosides from bovine mammary gland were characterized by a high content of long chain (20-26 carbon atoms) fatty acids (Table II). All even carbon fatty acids from 14:0 to 26:0 were contained in ganglioside fractions, while the only odd carbon acids found in any quantity were 21:0, 23:0, and 25:0 in MG IV and MG V, and 23:0 and 25:0 in MG VI. The only unsaturated fatty acids found were 16:1 and 18:1.

In Vitro Biosynthesis

Sialyltransferases active with glycolipid acceptors were present in bovine mammary Golgi apparatus fractions (Table III). Detergent was necessary for enzymatic activity and cardiolipin and phosphatidyl glycerol enhanced activity. Ganglioside GM_1 was the most active acceptor followed in order by GM_3, GD_{1a}, GD_3, and lactosyl ceramide. Glucosyl ceramide, trihexosyl ceramide, GM_2, and GD_{1b} were inactive or showed only slight acceptor activity. The fact that GM_1 was a very active acceptor

TABLE III

Characterization of CMP-N-acetylneuraminic acid:Glycolipid Sialyltransferase Activities in Bovine Mammary Golgi Apparatus[a]

Reaction mixture	Acceptor	^{14}C incorporated (pmoles/mg protein/hr)
Complete	Lactosyl ceramide	466
Complete	None	210
Plus cardiolipin (100 μg)	Lactosyl ceramide	590
Plus phosphatidyl glycerol (100 μg)	Lactosyl ceramide	496
Minus detergent	Lactosyl ceramide	110
Heat inactivated enzyme (2 min, 100 C)	Lactosyl ceramide	72
Complete	Glucosyl ceramide	298
Complete	Trihexosyl ceramide[b]	395
Complete	GM_3	2,487
Complete	GM_2	218
Complete	GM_1	9,396
Complete	GD_3	797
Complete	GD_{1a}	1,128
Complete	GD_{1b}	234

[a]Complete reaction mixtures contained (in μmoles unless otherwise stated) in final volumes of 0.1 ml: Cacodylate-HCl, pH 6.35, 15; $MgCl_2$, 1.0; CMP-N-acetylneuraminic acid (1.8 x 10^6 CPM/μmole), 0.05; glycolipid acceptor, 0.05; Tween 80-Triton CF-54 (1:2, w/w), 0.6 mg; and 0.26 mg Golgi apparatus protein. Incubations were for 2 hr at 37 C. Other conditions are described in the text.
[b]Trihexosyl ceramide consisting of Gal-Gal-Glc-Cer.

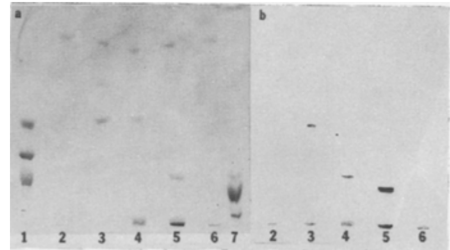

FIG. 3. (a) Thin layer chromatographic separation of the reaction products of sialyltransferases. The Silica Gel G plate was developed and sprayed as in Figure 2. 1: Reference GM_3, GM_2, and GM_1 in order of decreasing mobility; 2: products of reaction mixture with no acceptor; 3: lactosyl ceramide as acceptor; 4: GM_3 as acceptor; 5: GM_1 as acceptor; 6: GD_{1a} as acceptor; 7: reference GM_1, GD_{1a}, and GD_{1b}. (b) Autoradiogram of the thin layer plate shown in Figure 3a. Lane numbers correspond to equivalent lanes in Figure 3a.

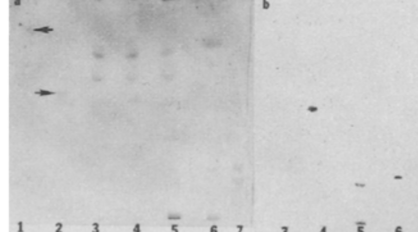

FIG. 4. (a) Thin layer chromatographic separation of the reaction products of galactosyltransferases. The Silica Gel G plate was developed as in Figure 2 and sprayed with resorcinol reagent and then anthone in sulfuric acid. 1: Glucosyl ceramide (position denoted by arrow); 2: lactosyl ceramide (arrow); 3: products of reaction mixture with no acceptor; 4: glucosyl ceramide as acceptor; 5: lactosyl ceramide as acceptor; 6: trihexosyl ceramide (GalNAc-Gal-Glc-Cer) as acceptor; 7: GM_2 as acceptor; 8: reference GM_1 and tetrahexosyl ceramide in order of decreasing mobility. (b) Autoradiogram of the thin layer plate shown in Figure 4a. Lane numbers correspong to equivalent lanes in Figure 4a.

for sialic acid may account for the failure to identify this ganglioside as a major constituent of bovine mammary gland. Autoradiograms demonstrated that lactosyl ceramide was converted to GM_3, GM_3 to GD_3, and GM_1 to GD_{1a} (Fig. 3 a and b). These were the expected reaction products in each case.

Under optimum conditions, moderate specific activities in UDP-Gal:GM_2 galactosyltransferase were observed with bovine mammary Golgi apparatus fractions (Table IV). Detergent was required for enzymatic activity, and cardiolipin and phosphatidyl glycerol enhanced activity. Heating the Golgi apparatus fractions caused a loss in activity, thus suggesting an enzymatic reaction. The best galactose acceptor was glucosyl ceramide followed by trihexosyl ceramide and GM_2; GM_3, GD_3, GD_{1a}, and GD_{1b} were inactive or showed little acceptor activity. Autoradiograms demonstrated that glucosyl ceramide was converted to lactosyl ceramide, trihexosyl ceramide to tetrahexosyl ceramide, and GM_2 to GM_1 (Fig. 4 a and b).

DISCUSSION

Of the six chromatographically distinguish-

TABLE IV

Characterization of UDP-galactose:Glycolipid Galactosyltransferase Activity of Bovine Mammary Golgi Apparatus Fractions[a]

Reaction mixture	Acceptor	^{14}C incorporated (pmoles/mg protein/hr)
Complete	GM_2	732
Complete	None	239
Plus cardiolipin (100 μg)	GM_2	933
Plus phosphatidyl glycerol (100 μg)	GM_2	1,118
Minus detergents	GM_2	296
Heat inactivated enzyme (2 min, 100 C)	GM_2	152
Minus UDP-Gal, plus UDP-Glc	GM_2	223
Complete	Ceramide	266
Complete	Glucosyl ceramide	1,730
Complete	Lactosyl ceramide	266
Complete	Trihexosyl ceramide[b]	1,520
Complete	Trihexosyl ceramide[c]	368
Complete	GM_3	281
Complete	GM_1	582
Complete	GD_3	423
Complete	GD_{1a}	180
Complete	GD_{1b}	219

[a]Complete reaction mixtures contained (in μmoles unless otherwise stated) in final volumes of 0.1 ml: Cacodylate-HCl, pH 7.3, 15; $MnCl_2$, 2.5; UDP-Gal (12.5 x 10^6 CPM/μmole), 0.05; glycolipid acceptor, 0.05; Triton CF54-Tween 80 (2:1, w/w), 0.6 mg; and 0.235 mg Golgi apparatus protein. Incubations were for 2 hr at 37 C.

[b]Trihexosyl ceramide consisting of GalNAc-Gal-Glc-Cer.

[c]Trihexosyl ceramide consisting of Gal-Gal-Glc-Cer.

able gangliosides occurring in bovine mammary gland, three have been identified previously (22). The three more polar constituents were here identified as GM_1, GD_2, and GD_{1b}. In the aggregate, these three gangliosides constitute 20% or less of the total ganglioside sialic acid in mammary gland.

Mammary gangliosides contain all even carbon fatty acids from 14:0 to 26:0 as well as substantial amounts of 23:0 and small amounts of 25:0. This fatty acid pattern is also characteristic of the other sphingolipids (sphingomyelin, glucosyl- and lactosyl ceramides, and the three previously identified gangliosides) found in bovine mammary gland and milk (22,24,41).

The sialyl- and galactosyltransferases involved in synthesis of GM_1 starting from glucosyl ceramide were present in mammary Golgi apparatus preparations. Detergent was required for enzymatic activity in both cases, and cardiolipin and phosphatidyl glycerol stimulated both transferase activities. The results are in agreement with earlier observations (22,23). UDP-GalNAc transferases have not been demonstrated in total particulate or Golgi apparatus fractions from bovine mammary gland.

This marks the first time that GM_1 has been identified as a constituent of bovine mammary gland. The low concentrations of GM_1 found in mammary tissue may be due to the very high specific activities of the sialyltransferases which convert GM_1 to GD_{1a} and to higher gangliosides. Thus, as GM_1 is formed, it may be rapidly converted to higher gangliosides. The gangliosides GD_2 and GD_{1b} could be synthesized from GD_3. GM_2 could not serve as the precursor for these gangliosides since it was a poor substrate for sialyltransferases. These data are in agreement with observations of Keenan (22) with mammary total particulate fractions and of Keenan et al. (23) with mammary Golgi apparatus.

The fact that the trihexosyl ceramide consisting of GalNAc-Gal-Glc-Cer was the best UDP-Gal acceptor would suggest that the alternate pathway of ganglioside biosynthesis demonstrated in mammals (42, 43) may function in mammary gland. Further research in this area is clearly indicated.

ACKNOWLEDGMENTS

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Studies on Lipid Biosynthesis and Cholesterol Content of Liver and Serum Lipoproteins in Rats Fed Various Phthalate Esters[1]

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ABSTRACT

The effect of various phthalate ester plasticizers on lipid metabolism in rats was studied in vivo and in vitro. Di-2-ethylhexyl phthalate (DEHP) and di-n-butyl phthalate (DBP) inhibited (30-70%) hepatic sterologenesis from ^{14}C-acetate and ^{14}C-mevalonate in liver minces from rats fed the phthalates at a level of 2.5 mmoles/100 g of chow diet for 21 days; inhibition of ^{14}C-acetate incorporation into phospholipids, triglycerides, and steryl esters was reduced (35-70%) by DEHP and DBP feeding. In addition, serum cholesterol was lowered ca. 14 mg/dl with dietary DEHP or DBP but not with dimethyl phthalate (DMP). Hepatic total cholesterol levels were reduced significantly (31%, $P<0.001$) by DMP but not by DBP or DEHP. In other studies with DEHP fed at the 0.5% level in chow diets (1.3 mmoles/100 g), the incorporation (esterification) of ^{3}H-oleate into di- and triglycerides was reduced ca. 40%. Furthermore, the addition of DEHP (2%, 5 mmoles/100 g) to a semisynthetic diet containing 10% fat (hydrogenated coconut oil) resulted in changes in serum lipoprotein composition. The percentage of serum cholesterol in LDL rose from 22% to 34% while that in HDL fell from 78% to 66%; these changes occurred without net changes in serum cholesterol levels. Possible mechanisms for the inhibitory effect of phthalates on hepatic lipid biosynthesis are discussed.

INTRODUCTION

Phthalic acid esters have a variety of industrial applications and may be found as constituents of such diverse products as paints, adhesives, cosmetics, and polyvinylchloride (PVC) plastics (1,2). Phthalates are now widely distributed throughout the environment and have been detected in human (3-6) and animal tissues (7-9). Recent studies have shown that di-2-ethylhexyl phthalate (DEHP), the most commonly used phthalate, is biologically active and capable of modifying lipid metabolism in experimental animals (10-16). The feeding of DEHP to rats results in an inhibition of hepatic lipid biosynthesis from labeled acetate and mevalonate (12-13) and a stimulation of oxidation of fatty acids by isolated hepatic mitochondria (16); in isolated heart mitochondria, DEHP feeding suppresses fatty acid oxidation (16). DEHP has also been reported to exert a hypolipemic effect in the mouse and rat (15) and to induce hepatic lipid accumulation in rats fed fat-supplemented diets (10); this chemical is also toxic to a variety of cells in culture (17-20) and may also be a vascular toxin (6,21). Observations on phthalate toxicity in animals give reason for concern in view of reports that phthalate transfer into man can occur during medical treatment involving the use of plastic devices (catheters, dialysis tubings) or the use of products stored in plastic bags (blood and blood products) (3,6,22-27). In the present study, we examined the effect of feeding three different phthalates (dimethyl phthalate, di-n-butyl phthalate, and di-2-ethylhexyl phthalate) on hepatic lipid synthesis from ^{14}C-acetate, ^{14}C-mevalonate, and ^{3}H-oleate, their effect on hepatic and serum cholesterol levels, and the distribution of cholesterol among the various plasma lipoprotein classes.

EXPERIMENTAL PROCEDURES

Animals and Diets

Male Sprague-Dawley rats with initial weights of 200-225 g were individually caged and provided with food and water ad libitum. With one exception (outlined below), the diets consisted of Purina Chow alone (control diet) or Purina Chow containing various phthalate esters which were added to the diets dissolved in diethyl ether (12). Di-2-ethylhexyl phthalate (DEHP), dimethyl phthalate (DMP), and di-n-butyl phthalate (DBP) were obtained commercially (Eastman Kodak, Rochester, NY, cat. nos. 4099, 318, and 1403, respectively). In experiments in which DEHP, DMP, and DBP were studied in parallel, the phthalates were added to the diets at a level of 2.5 mmoles/100 g which corresponded to 1.0% DEHP, 0.5% DMP, and 0.7% DBP.

In one study, the rats were fed 2% DEHP (5 mmoles/100 g) in a semisynthetic diet (28) that was modified by substituting 10% hydrogenated coconut oil for corn oil and providing 18% casein and 0.2% methionine for protein.

[1]These studies are in partial fulfillment of the requirements for a Doctorate degree in Medical Sciences, McMaster University, Hamilton, Ontario, Canada.

TABLE I

Effect of Feeding Various Phthalate Esters on Weight Gain and Liver Weight in the Rat[a]

Diet	% Weight gain[b]	Liver wt (% of body wt)	Lipid-free dry wt of liver[c] / Wet wt of liver
Control	68.0 ± 8.8 (6)[d]	4.0 ± 0.1 (9)	0.295 ± 0.006 (3)
DMP	65.7 ± 6.0 (8)	4.1 ± 0.2 (8)	---
DBP	64.6 ± 6.6 (5)	4.6 ± 0.1 (9)[e]	---
DEHP	69.9 ± 2.9 (5)	6.8 ± 0.2 (5)[e]	0.285 ± 0.004 (7)

[a]Male Sprague-Dawley rats were fed Purina Chow (control) or Purina Chow containing 2.5 millimoles/100 g of either dimethyl phthalate (DMP), dibutyl phthalate (DBP), or di-2-ethylhexyl phthalate (DEHP) for 21 days.
[b]Weight gain (g) after 21 days on the diets expressed as a percentage of initial weight (g) of the animals.
[c]Liver samples (1 g wet wt) were extracted with chloroform-methanol (2:1, v/v) and the lipid-free dry residue expressed as a ratio of the wet wt.
[d]Values are means ± SEM of the number of animals shown in parentheses.
[e]Significantly different from control values ($P<0.001$) by Student's independent *t*-test.

Tissue Preparation and Incubation

All rats were killed between 9:00 and 10:30 a.m. by decapitation while under light ether anesthesia. The livers were rapidly excised and rinsed in chilled 0.9% NaCl solution. For consistency, 500 mg of tissue were taken from the central portion of the large lobe of the liver and a tissue mince prepared (12). The liver minces (500 mg) were incubated for 3 hr at 37 C in stoppered 25 ml Erlenmeyer flasks containing either 3.5 or 4.0 ml of Krebs-Ringer-bicarbonate buffer, pH 7.4 (12,13); incubations with sodium acetate-1-^{14}C (1 μCi, 59 mCi/mM) and DL-mevalonic-2-^{14}C acid, dibenzylethylene diamine salt (0.1 μCi, 40.2 mCi/mM) were done in 3.5 ml of buffer, whereas incubations with 9,10-^{3}H(N)-oleic acid (4 μCi, 8 mCi/mM) were done in 4.0 ml of buffer. All radiochemicals were obtained from New England Nuclear Corp., Boston, MA. Penicillin and streptomycin were present at levels of 50 units and 50 μg/ml, respectively, in the incubations.

Analyses of Incubation Mixtures

Incubation mixtures containing either ^{14}C-acetate or ^{3}H-oleate as lipid precursors were homogenized in chloroform-methanol as previously described (12) and the lipid extracts washed according to Folch et al. (29). In studies with ^{14}C-mevalonate, KOH and ethyl alcohol were added to the incubation mixtures to give a final concentration of 11% and 82%, respectively, and the samples saponified 2 hr at 65 C (13). The nonsaponifiable lipid fraction (sterols and squalene) was recovered by extraction with n-hexane and the hexane extracts washed with water as previously described (13). The washed lipid extracts from all experiments described above were fractionated by thin layer chromatography on Silica Gel G-coated glass plates in a solvent system consisting of n-hexane-diethyl ether-acetic acid (146:50:4, v/v/v) (12,13). Individual lipid bands were visualized under UV light after spraying the chromatoplates with rhodamine 6G (0.05% in ethanol) (12,13). The lipid bands were scraped from the plates into vials containing scintillation fluid (Liquifluor, New England Nuclear Corp., Boston, MA, cat. no. NEF-903) and assayed for radioactivity in a liquid scintillation spectrometer (Packard Tri-Carb, Model 3375, Downers Grove, IL). Quench corrections were made by the external standardization method.

Analysis of Tissue and Serum

One gram samples of liver were extracted with chloroform-methanol (29) and an aliquot of the washed extract used for a gravimetric determination of total hepatic lipid (10); another aliquot of the same extract was taken to measure total hepatic cholesterol by the o-phthalaldehyde method (30). The lipid-free tissue residue remaining after solvent extraction was dried and used to calculate lipid-free dry wt/wet wt ratios. Serum total cholesterol levels were assayed by the cholesterol oxidase method (Calbiochem Enzymatic Cholesterol S.V.R., Calbiochem, LaJolla, CA) in serum from blood drawn from the rats by cardiac puncture under ether anesthesia.

In individual serum samples from the rats fed 2% DEHP in the semisynthetic diet, very low density lipoproteins (VLDL) and low density lipoproteins (LDL) were co-precipitated with heparin-$CaCl_2$ (31,32); total cholesterol content of the VLDL+LDL complexes and the high density lipoproteins (HDL) remaining in solution was quantitated by gas liquid chromatography (33) after saponification of the sterol esters (34). In pooled serum samples, VLDL were isolated by ultracentrifugation of serum overlayered with 1 ml of a solution of d=1.006

TABLE II

Effect of Various Phthalate Esters on Hepatic Total Lipid and Total Cholesterol Levels in the Rat[a]

Diet	Total cholesterol[b] (mg/g wet wt)	Total lipid[c] (mg/g wet wt)
Control	4.5 ± 0.2 (10)[d]	42.7 ± 1.4 (10)
DMP	3.1 ± 0.2 (8)[f]	38.5 ± 1.2 (8)[e]
DBP	3.8 ± 0.2 (5)	47.3 ± 2.2 (5)
DEHP	4.1 ± 0.1 (7)	44.2 ± 1.0 (7)

[a]Male Sprague-Dawley rats were fed Purina Chow (control) or Purina Chow containing 2.5 millimoles/100 g of either dimethylphthalate (DMP), dibutyl phthalate (DBP), or di-2-ethylhexhyl phthalate (DEHP) for 21 days.

[b,c]Liver (1 g) lipids were extracted with chloroform-methanol (2:1, v/v). Total lipid was determined gravimetrically after drying aliquots of the lipid extract; total cholesterol was measured in aliquots of the lipid extract as described under Procedures.

[d]Values are means ± SEM of the number of animals shown in parentheses.

[e,f]Significantly different from control values by Student's independent *t*-test (e, $P<0.05$; f, $P<0.001$).

TABLE III

Effect of Various Phthalate Esters on Serum Total Cholesterol Levels (mg/dl) in the Rat[a]

Diet	Duration of phthalate feeding (days)		
	7	14	21
Control	87 ± 6(5)[b]	84 ± 4(5)	87 ± 5(6)
DMP	78 ± 4(9)	77 ± 3(8)	80 ± 3(8)
DBP	83 ± 5(7)	70 ± 2(6)[c]	69 ± 3(5)[c]
DEHP	68 ± 5(10)[c]	70 ± 3(6)[c]	73 ± 5(5)[c]

[a]Male Sprague-Dawley rats were fed Purina Chow (control) or Purina Chow containing 2.5 millimoles/100 g of either dimethyl phthalate (DMP), dibutyl phthalate (DBP), or di-2-ethylhexyl phthalate for up to 21 days. Total cholesterol (free + esterified) was measured in serum from blood taken by cardiac puncture under light ether anesthesia.

[b]Values are means ± SEM of the number of animals shown in parentheses.

[c]Significantly different from control values ($P<0.05$) by Student's independent *t*-test.

(35). The VLDL-free samples were next treated with heparin-$CaCl_2$ to separate, by complexation-precipitation, the LDL from HDL remaining in solution (31,32). The three lipoprotein fractions were saponified (34) and the total cholesterol content of each quantitated by gas liquid chromatography (33).

Statistical analyses were performed using Student's *t*-test for comparing unpaired samples. Significance was given by values of $P \leq 0.05$.

RESULTS

Weight Gains and Liver Weights

The feeding of DMP, DBP, or DEHP at levels of 2.5 mmoles/100 g of chow diet for 21 days did not affect weight gain of the animals when compared with the control group; weight gains by the rats averaged 67% of their initial body weights (200-225 g) prior to initiation of phthalate feeding (Table I). Despite similarities in weight gain, increases in liver weights were observed in animals fed DBP and DEHP but not DMP (Table I). Liver weights, expressed as a percentage of body weights, were significantly increased ($P<0.001$) above control values by 15% and 70% in the DBP- and DEHP-fed animals, respectively. The large increase in liver weight relative to body weight observed in the DEHP group did not represent an edematous state since ratios of lipid-free dry wt to wet wt were similar in livers from control and DEHP-fed animals (0.295 and 0.285, respectively; Table I); this ratio was not calculated for DMP- or DBP-fed groups.

Hepatic Lipids

Hepatic total cholesterol and total lipid (mg/g wet wt) were measured in all rats fed phthalates for 21 days (Table II). Total cholesterl levels were not significantly different ($P>0.05$) from control levels in the rats fed DBP or DEHP but were reduced in the rats fed

TABLE IV

Incorporation of Acetate-1-^{14}C into Lipids by Liver Minces from Rats Fed Various Phthalate Esters[a] (dpm/g wet wt/10^5dpm in incubation medium)

Diet	n	Phospholipid	Sterol	Free fatty acid	Triglyceride	Steryl ester + squalene
Control	5	306 ± 88[b]	232 ± 82	60 ± 13	285 ± 103	271 ± 120
DMP	6	526 ± 141	330 ± 105	114 ± 25	739 ± 231	269 ± 97
Control	8	372 ± 65	237 ± 61	74 ± 8	451 ± 116	147 ± 39
DBP	10	245 ± 32	159 ± 20	65 ± 5	193 ± 29[c]	40 ± 7[e]
Control	5	253 ± 48	255 ± 54	90 ± 21	500 ± 78	204 ± 52
DEHP	5	93 ± 7[d]	73 ± 22[d]	44 ± 5	93 ± 19[f]	20 ± 4[f]

[a]Male Sprague-Dawley rats were fed Purina Chow (control) or Purina Chow containing 2.5 millimoles/100 g of either dimethyl phthalate (DMP), dibutyl phthalate (DBP), or di-2-ethylhexyl phthalate (DEHP) for 21 days. Liver minces (500 mg) were prepared as described under Procedures and incubated 3 hr at 37 C with 1.0 μCi sodium acetate-1-^{14}C in a total volume of 3.5 ml of Krebs-Ringer-bicarbonate buffer, pH 7.4. The incubation mixtures were extracted with chloroform-methanol (2:1, v/v) and the lipids fractionated by thin layer chromatography as described under Procedures.

[b]Values are means ± SEM of the number of animals, n, shown.

[c,d,e,f]Significantly different from control values by Student's independent *t*-test (c, P<0.05; d, P<0.02; e, P<0.01; f, P<0.001).

TABLE V

Incorporation of DL-Mevalonic-2-^{14}C Acid into Cholesterol and Squalene by Liver Minces from Rats Fed Various Phthalate Esters (dpm/g wet wt/10^5 dpm in incubation medium)[a]

Diet	n	C_{27} sterols	Squalene
Control	5	632 ± 297[b]	1532 ± 510
DMP	6	722 ± 133	1775 ± 231
Control	8	400 ± 47	1449 ± 230
DBP	10	279 ± 25[c]	640 ± 73[d]
Control	6	373 ± 44	1491 ± 121
DEHP	6	124 ± 30[e]	444 ± 53[e]

[a]Male Sprague-Dawley rats were fed Purina Chow (control) or Purina Chow containing 2.5 millimoles/100 g of either dimethyl phthalate (DMP), dibutyl phthalate (DBP), or di-2-ethylhexyl phthalate (DEHP) for 21 days. Liver minces (500 mg) were incubated 3 hr at 37 C with 0.1 μCi DL-mevalonic-2-^{14}C acid in a total volume of 3.5 ml of Krebs-Ringer-bicarbonate buffer, pH 7.4. The incubation mixtures were saponified with 10 ml of 15% alcoholic KOH at 60 C for 1.5 hr. The nonsaponifiable lipids were extracted with n-hexane and fractionated by thin layer chromatography to obtain C_{27} sterols and squalene as described under Procedures.

[b]Values are means ± SEM of the number of animals, n, shown.

[c,d,e]Significantly different from control values by Student's independent *t*-test (c, P<0.05; d, P<0.01; e, P<0.001).

DMP (4.5 vs. 3.1 mg/g wet wt, P<0.001). Hepatic total lipid levels were also significantly lower P<0.05) than control levels in rats fed DMP (42.7 vs. 38.5 mg/g wet wt). The lowering effect of DMP on hepatic total lipid was not observed with DBP or DEHP.

Serum Cholesterol

Rats receiving the phthalate-containing diets were bled at 7-day intervals to measure serum total cholesterol levels. A significant (P<0.05) reduction in serum cholesterol was evident after 7 days of DEHP feeding; this reduction was maintained throughout the 21 days of the study and ranged from 14 to 19 mg/dl below control values (Table III). A significant lowering (P<0.05) of serum cholesterol was also observed in animals fed DBP. The effect of DBP was observed after 2 wk of DBP feeding and was sustained until the end of the study at 21 days; cholesterol lowering by DBP ranged from 14 mg/dl at 2 wk to 18 mg/dl by 3 wk. No significant changes in serum cholesterol occurred with DMP feeding although serum cholesterol was consistently about 7 mg/dl lower in the DMP group at all intervals examined.

In Vitro Lipid Biosynthesis

Biosynthesis of lipids was studied in liver minces from rats fed DMP, DBP, and DEHP for 21 days using ^{14}C-acetate and ^{14}C-mevalonate as precursors (Tables IV and V).

^{14}C-Acetate Studies

The incorporation of ^{14}C-acetate into all lipid fractions was reduced in liver minces from rats fed DBP and DEHP, although not all the decreases in incorporation were statistically significant (Table IV). DBP feeding resulted in significant reductions of ^{14}C-acetate incorporation into the triglyceride (P<0.05) and steryl ester + squalene (P<0.01) fractions of the liver minces. With DEHP feeding, significant reductions in ^{14}C-acetate incorporation into triglycerides (P<0.001) and steryl ester + squalene (P<0.001) were also observed. In addition,

TABLE VI

Incorporation of ^{3}H-Oleate into Esterified Lipids by Liver Minces from Rats Fed 0.5% DEHP (dpm/g wet wt/10^5 dpm in incubation medium)[a]

Diet	n	Phospholipid	Diglyceride	Triglyceride	Steryl esters
Control	5	814 ± 102	1271 ± 221	3775 ± 504	684 ± 70
DEHP (3 days)	4	1037 ± 202	1622 ± 414	2622 ± 543	829 ± 165
DEHP (9 days)	5	675 ± 94	722 ± 57[c]	2388 ± 156[c]	655 ± 21

[a]Male Sprague-Dawley rats were fed Purina Chow (control) or Purina Chow containing 0.5% di-2-ethylhexyl-phthalate (DEHP, 1.3 mmoles/100 g diet) for 3 or 9 days. Liver minces (500 mg) were incubated 3 hr at 37 C with 4.0 μCi oleic acid-9,10-^{3}H in a total volume of 4.0 ml of Krebs-Ringer-bicarbonate buffer, pH 7.4. The incubation mixtures were extracted with $CHCl_3$:MeOH (2:1, v/v) and the lipids fractionated by thin layer chromatography as described under Methods.

[b]Values are means ± SEM of the number of animals, n, shown.

[c]Significantly different from control values by Student's independent *t*-test (P<0.05).

DEHP feeding significantly reduced (P<0.02) ^{14}C-acetate incorporation into phospholipids and cholesterol; incorporation into free fatty acids was also reduced by about 50% but was not a statistically significant reduction. In contrast to data obtained in livers from DBP- and DEHP-fed rats, no reduction in ^{14}C-acetate incorporation into liver lipids was observed in animals fed DMP, in fact, incorporation of ^{14}C-acetate tended to be increased in all lipid fractions except the steryl ester + squalene fraction; none of the increases, however, were statistically significant.

^{14}C-Mevalonate Studies

The effect of phthalate feeding on the incorporation of ^{14}C-mevalonate into C_{27} sterols (principally cholesterol) and squalene by rat liver minces is shown in Table V. In livers from DBP- and DEHP-fed rats, ^{14}C-mevalonate incorporation into sterols and squalene was depressed with the greatest effect observed with DEHP feeding; incorporation into C_{27} sterols and squalene in livers from DEHP-fed rats was reduced 60-70% (P<0.001) relative to control values; whereas with livers from DBP-fed rats, incorporation into C_{27} sterols and squalene was reduced 30% (P<0.05) and 55% (P<0.01), respecitvely. In contrast to the effects of DBP and DEHP feeding, incorporation of ^{14}C-mevalonate into liver sterols and squalene was essentially unaffected by DMP feeding.

^{3}H-Oleate Studies

The effect of DEHP on the esterification of fatty acids was studied by the addition of ^{3}H-oleate to liver minces from rats fed DEHP (0.5%, 1.3 mmoles/100 g diet) for 3 or 9 days (Table VI). The incorporation of ^{3}H-oleate into phospholipids, di- and triglycerides, and steryl esters was not statistically different from control values in animals fed DEHP for 3 days, although incorporation into triglycerides tended to be lower than controls while incorporation into phospholipids, diglycerides, and steryl esters tended to be elevated somewhat. However, after 9 days of DEHP feeding, there was a significant reduction (P<0.05, relative to control values) in the incorporation of ^{3}H-oleate into diglycerides and triglycerides which amounted to ca. 44% and 37%, respectively. Incorporation into phospholipids also tended to be reduced, but not significantly.

Serum Lipoproteins

The effect of DEHP feeding on the distribution of cholesterol in serum lipoproteins was studied in rats fed for 14 days with a semisynthetic diet containing 10% hydrogenated coconut oil; DEHP was present in the diet at a level of 5.0 mmoles/100 g (2% by weight). Combined VLDL+LDL fractions precipitated by heparin-Ca^{++} were analyzed and their total cholesterol content compared with that of HDL (Table VII). In rats fed DEHP, the percentage of serum cholesterol carried by VLDL+LDL was significantly elevated (P<0.01) from 22.2% to 34.4% (n=10), while the percentage carried by HDL decreased significantly (P<0.01) from 77.8% to 65.6% (n=10). A separate analysis of VLDL, LDL, and HDL isolated from pooled serum by a combination of heparin-Ca^{++} precipitation and ultracentrifugation revealed that the increase in VLDL+LDL cholesterol observed in the rats is almost entirely due to increases in LDL cholesterol (Table VII). These changes in lipoprotein cholesterol distribution were not associated with changes in total serum cholesterol levels since serum cholesterol levels were similar in the control and DEHP-fed groups on these 10% fat diets (Table VII).

DISCUSSION

Previous studies have demonstrated that the

plasticizer DEHP (di-2-ethylhexyl phthalate) is capable of modifying lipid metabolism in various animal species. DEHP feeding results in a two- to threefold stimulation of ^{14}C-palmitoyl CoA oxidation by isolated hepatic mitochondria from rats, rabbits, and pigs (16) and a 25-45% decrease in oxidation of the same substrate by isolated heart mitochondria from the rat (16). In addition, DEHP feeding to rats results in an inhibition of sterol and squalene biosynthesis from ^{3}H-mevalonate (13) and a decreased incorporation of ^{14}C-acetate into all lipid classes (12) in liver slices or minces in vitro. DEHP feeding to rats and mice is also associated with decreases in serum cholesterol and triglycerides (15) and may under some conditions result in hepatic lipid accumulation (10). The present study was undertaken to extend our investigations with DEHP and to examine the effects of two other phthalates, DMP (dimethyl phthalate) and DBP (di-n-butyl phthalate), on lipid metabolism in the rat. DMP and DBP were chosen for these studies since they also have commercial applications and have been associated with animal toxicity (20,39-41); DBP has also been found in tissues of animals (8,9) and man (5).

In rats fed either DMP, DBP, or DEHP for 21 days at a level of 2.5 mmoles/100 g of chow diet, body weight gains were similar to those observed in rats fed the control diet (Table I). However, liver weights (expressed as a percentage of body wt) were significantly increased ($P<0.001$) in rats fed DBP and DEHP (Table I). Although increases in liver weight have been associated with phthalate feeding to rats in other studies (12,15,36,37), hepatomegaly is not a general response to phthalates since no changes were observed in rats fed DMP (Table I). The hepatomegaly observed with phthalate feeding appears to represent true increases in tissue mass rather than edematous change or lipid accumulation since liver dry wt (lipid-free) to wet wt ratios were not affected by phthalate feeding (Table I) nor was hepatic total lipid concentration increased significantly (Table II). This conclusion is supported by observations that DEHP-induced hepatomegaly is associated with increases in liver mitochondrial protein (16) and proliferation of smooth endoplasmic reticulum (15). The only important change in hepatic total lipid concentration observed was seen in rats fed DMP (Table II); total lipid was reduced from 42.7 to 38.5 mg/g ($P<0.05$). DMP feeding was also associated with a significant reduction (31%, $P<0.001$) in hepatic total cholesterol levels (Table II). Although hepatic cholesterol levels were also lower (16%) in DBP-fed rats, the reduction was not statisti-

TABLE VII

Effect of 2% Dietary DEHP on Serum Lipoprotein Cholesterol (mg/dl) in the Rat[a]

Diet	Fractionation by heparin-Ca^{++} precipitation[b]			% of serum total cholesterol		Fractionation by ultracentrifugation and heparin-Ca^{++} precipitation				% of serum total cholesterol		
	VLDL+LDL	HDL	Total	VLDL+LDL	HDL	VLDL	LDL	HDL	Total	VLDL	LDL	HDL
Control	19.2 ± 1.0	68.9 ± 4.0	88.1 ± 3.8[c]	22.2 ± 1.6	77.8 ± 1.6	9.0	6.6	76.8	92.4	9.8	7.1	83.1
DEHP	31.3 ± 0.9[d]	60.2 ± 2.8	91.5 ± 3.2	34.4 ± 1.0[d]	65.6 ± 1.0[d]	9.2	19.1	62.3	90.6	10.2	21.0	68.8

[a]Male Sprague-Dawley rats were fed a semisynthetic diet containing 10% hydrogenated coconut oil with and without (control) 2% di-2-ethylhexyl phthalate (DEHP) for 14 days. Serum for lipoprotein cholesterol analysis was obtained from blood taken by jugular venapuncture (44).

[b]Very low density lipoproteins (VLDL) and low density lipoproteins (LDL) were separated from high density lipoproteins (HDL) by precipitation with heparin-$CaCl_2$ and cholesterol of VLDL+LDL and HDL measured by gas liquid chromatography as detailed in Procedures.

[c]Values are means ± SEM of data obtained from ten rats. Total cholesterol is the sum of VLDL + LDL and HDL cholesterol.

[d]Significantly different from control values ($P<0.01$) by Student's independent *t*-test.

[e]VLDL was obtained from pooled serum (n=10) by flotation at density=1.006 g/ml in the ultracentrifuge and LDL separated from HDL of the infranatant by precipitation with heparin-$CaCl_2$; cholesterol of the three lipoprotein fractions was measured by gas liquid chromatography.

cally significant at the level of DBP fed in these studies. DEHP had essentially no effect on hepatic sterol levels (Table II) thus confirming our previous observations in a study with rats fed 0.5% (1.3 mmoles/100 g diet) DEHP for 10 days (13).

Paradoxically, the effect of DMP on hepatic cholesterol levels was not paralleled by either reductions in serum cholesterol (Table III) or reductions in hepatic sterologenesis from ^{14}C-acetate (Table IV) or ^{14}C-mevalonate (Table V). In contrast, an inhibition of hepatic sterologenesis from ^{14}C-acetate and ^{14}C-mevalonate resulted from DBP or DEHP feeding (Tables IV and V), and serum cholesterol levels fell ca. 14% ($P<0.05$) over the 21 days of the study.

The serum cholesterol-lowering response in DEHP- and DBP-fed rats was maximal at 7 and 14 days, respectively (Table III). Lowering of serum cholesterol by DEHP feeding in rats and mice has been reported previously (15) but at doses above (2% and 4%) and below (0.5%) those examined here.

The reduction in serum cholesterol associated with DEHP and DBP feeding may be related to an inhibition of hepatic sterologenesis (13) since the incorporation of ^{14}C-acetate and ^{14}C-mevalonate into hepatic squalene and C_{27} sterols was reduced by DEHP and DBP feeding (Tables IV and V).

Inhibition of biosynthesis of hepatic C_{27} sterols, consisting primarily of cholesterol (38), and squalene (a C_{30} hydrocarbon precursor of sterols) from ^{14}C-acetate and ^{14}C-mevalonate was greater in rats fed DEHP (Tables IV and V) than in those fed DBP suggesting that the sterol biosynthesis inhibition effects of phthalates are related to the nature of the alkyl groups esterified to phthalic acid. Since DMP was without effect, it may be inferred that a 2-carbon alkyl chain is the minimal requirement for sterol biosynthesis inhibition. In our previous studies with DEHP feeding in rats (13), it was observed that the inhibition of incorporation of labeled acetate into hepatic sterols and squalene developed more rapidly than the inhibition of ^{3}H-mevalonate incorporation, thus suggesting that the site of action of phthalates is the enzyme 3-hydroxy-3-methyl- glutaryl CoA reductase (EC 1.1.1.34), the first rate-limiting step in hepatic sterol biosynthesis (38).

A further distinction between DMP and either DBP or DEHP was observed in the incorporation of ^{14}C-acetate into the individual lipid fractions of liver minces in vitro (Table IV). Label incorporation into phospholipid, triglyceride, free fatty acid, and the steryl ester + squalene fractions of liver were all reduced in animals fed DBP and DEHP but not in those fed DMP. In fact, incorporations of ^{14}C-acetate into all hepatic lipids of the DMP-fed animals tended to be stimulated somewhat over control values; no explanation is offered for these observations at this time. The effect of DEHP feeding on ^{14}C-acetate incorporation into hepatic lipids was greater than that observed with DBP in that, with the exception of free fatty acids, inhibition of incorporation of ^{14}C-acetate into individual lipid fractions was ca. twofold greater in DEHP-red rats than in DBP-fed rats.

The results of ^{14}C-acetate incorporation studies suggest that the inhibitory effect of DEHP and DBP on hepatic lipid biosynthesis may be related, in part, to an inhibition of fatty acid synthesis, perhaps at the level of acetate activation. This conclusion is based on the observation that reductions in ^{14}C-acetate incorporation into phospholipids, triglycerides, and steryl esters are not paralleled by accumulations of labeled free fatty acids. Since the activation of acetate is a requisite step in the de novo synthesis of sterols as well as fatty acids, inhibition of sterologenesis from acetate would also be expected. Inhibition of labeled mevalonate incorporation into sterols could be attributed either to a separate effect of phthalates or an indirect effect of impaired acetate activation. Inhibition of sterologenesis from mevalonate, which develops late with phthalate feeding (13), may reflect a decline in activity of enzymes between mevalonate and cholesterol which is induced by substrate and hence declines as mevalonate production decreases (38). Inhibition of phospholipid and triglyceride biosynthesis may also be related, in part, to an impairment in fatty acid esterification with DEHP and DBP feeding (Table VI). This suggestion is supported by studies in which liver minces from rats fed 0.5% (1.3 mmole/100 g diet) DEHP for 9 days were incubated with ^{3}H-oleate. The incorporation of ^{3}H-oleate into phospholipids tended to be reduced after 9 days of DEHP feeding, while incorporation of label into diglycerides and triglycerides was significantly reduced ($P<0.05$).

Another possibility that we have considered to explain decreases in ^{14}C-acetate and ^{3}H-oleate incorporation into saponifiable lipids involves changes in levels or activities of carnitine acetyltransferase (EC 2.3.1.7) or carnitine palmitoyltransferase (EC 2.3.1.21). Increases in these enzymes would tend to redirect acyl CoA from esterification with α-glycerol-PO_4 into carnitine esters (42,43). Our evidence that fatty acid oxidation increases two- to threefold in rabbits, rats, and pigs fed DEHP offers indirect

evidence for elevation of carnitine palmitoyltransferase with DEHP feeding (16). In addition, increases in carnitine acetyltransferase have been reported in livers of DEHP-fed rodents (15).

Although we did not study serum lipoprotein profiles or compositions in the chow-fed rats in our studies, we did examine lipoproteins in rats fed DEHP in a semisynthetic diet containing 10% hydrogenated coconut oil. Two percent DEHP feeding produced striking changes in serum lipoprotein composition (Table VII). The percentage of serum cholesterol carried by β-lipoproteins (VLDL+LDL) rose from 22.2% to 34.4%, while cholesterol carried by α-lipoprotein (HDL) decreased from 77.8% to 65.6%. Further fractionation of the lipoproteins indicated that the increased percentage of plasma cholesterol carried by the β-lipoproteins was almost exclusively a result of increases in LDL-cholesterol. The mechanism of this redistribution of serum cholesterol among lipoproteins is unclear and is unrelated to changes in total serum cholesterol levels. It is surprising that the hypocholesterolemic effect of DEHP in rats fed chow diets (Table III) (15) was not observed with the semisynthetic diet containing 10% fat. It should be pointed out, however, that dietary fat has been shown to modify DEHP responses in rats (10); Stein et al. (10) reported that the addition of lard to DEHP-containing diets in rats resulted in hepatic lipid accumulation not observed with lard diets alone. Unfortunately, no data were available on serum cholesterol levels in that study.

The data presented here provide further information on the effects of phthalates on mammalian lipid metabolism. The ability of certain phthalates to inhibit hepatic lipid synthesis and to modify serum lipoproteins raises questions of possible hazardous consequences eminating from widespread phthalate contamination of air, water, soil, foods (1), and biological fluids (blood, platelet concentrates, etc.) for administration to man (14).

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Distribution of Dietary Octadecenoate Isomers at the 1- and 2-Positions of Hepatoma and Liver Phospholipids

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ABSTRACT

Phosphatidylcholines and phosphatidylethanolamines were isolated from hepatoma 7288CTC, normal liver, and host liver of rats fed one of the following diets: fat-free diet; fat-free diet supplemented with safflower oil, safflower oil fatty acids, or partially hydrogenated safflower oil fatty acids; and commercial chow. The *cis* and *trans* octadecenoate fatty acids were isolated from the 1- and 2-positions of both phosphoglycerides and analyzed quantitatively for chain positional isomers. Octadecenoates from hepatoma and liver phosphoglycerides of animals fed fat-free or natural fat-supplemented diets contained almost exclusively two *cis* isomers: oleic and vaccenic acids. Oleic acid predominated in the 2-position octadecenoates of both phosphoglycerides from hepatoma and liver. In contrast, vaccenic acid predominated in the 1-position of normal liver phosphatidylcholine and, to a lesser extent, phosphatidylethanolamine. Host liver and hepatoma exhibited a shift to a higher percentage of oleic acid at the 1-position. Dietary *trans* fatty acids were incorporated predominately in the 1-position of both phosphoglycerides of hepatoma and liver. Except for the *cis* Δ10 octadecenoate isomer, all of the unnatural dietary *cis* isomers between Δ8 and Δ14 were incorporated into the 1-position of the phospholipids, while the unnatural *cis* octadecenoates at the 2-position consisted primarily of the Δ12 isomer. Hepatoma phosphoglycerides contained higher percentages of the *trans* Δ10 isomer that was nearly excluded from the 1-position of the two liver phosphoglycerides. All the other *trans* octadecenoate isomers were incorporated into the 1-position of both phosphoglycerides, but the small amount of *trans* fatty acids incorporated into the 2-position of liver and hepatoma phosphatidylcholine consisted of four isomers, Δ9 to Δ12, including the Δ10 isomer. Phosphatidylethanolamine exhibited a similar distribution, except for the presence of the Δ13 and Δ14 isomers at the 2-position. A combination of evidence suggests that the 1-position fatty acids in phosphatidylcholine and phosphatidylethanolamine are of similar origin. The octadecenoates at the 2-position of these two phosphoglycerides appear to be of the same origin in hepatoma but not in liver. It was also revealed that the 2-position of hepatoma phosphatidylcholine contained much higher percentages of palmitate than liver.

INTRODUCTION

This work is an extension of our studies to determine the metabolic fate of dietary fatty acids containing *cis* and *trans* double bonds (geometrical isomers) at various positions along the hydrocarbon chain (positional isomers). Lipid classes of normal liver from animals maintained on fat-free or natural fat diets have been shown to contain predominately *cis* Δ 9- and *cis* Δ 11-octadecenoates (oleic and vaccenic acids) in proportions characteristic of the individual lipid classes (1). In contrast, octadecenoates from all hepatoma lipid classes were found to contain the same approximate proportions of oleic and vaccenic acids (1,2). This loss of lipid class specificity for isomeric octadecenoates in hepatomas has also been found in a variety of other tumors (3). Recently we demonstrated (4) the preferential incorporation of dietary geometrical octadecenoate isomers into specific liver lipid classes but not in hepatoma. These studies also demonstrated the selective incorporation of positional isomers of both *cis* and *trans* octadecenoates into liver lipids and some loss of this selectivity in the hepatoma. The origin of geometrical and positional isomers of unsaturated fatty acids, our lack of knowledge about their metabolic fate, and their possible involvement in a number of pathological conditions have been reviewed recently (4). Described in this paper is the distribution of positional isomers of *cis* and *trans* octadecenoates at the 1- and 2-positions of glycerol in phosphatidylcholine and phosphatidylethanolamine from liver and hepatoma of animals fed various diets. A preliminary report of this work has appeared (5).

MATERIALS AND METHODS

Two groups of male Buffalo strain rats weighing 175-200 g were placed on each of the following diets: Purina Chow; fat-free diet (Nutritional Biochemicals Corps, Cleveland, OH); or the fat-free diet supplemented with either 0.5% safflower oil, 15% safflower oil free fatty acids, or 15% partially hydrogenated safflower oil free fatty acids (PHSOFA). One group of animals on each diet was implanted with Morris minimal deviation hepatoma 7288CTC at the time the animals were placed

on the diets. After 4 weeks, the animals were killed, and normal livers, livers of rats bearing hepatomas (host liver), and hepatomas were excised. The hepatic tissue was lyophilized, total lipid extracted by the Bligh and Dyer procedure (6), and the neutral lipids and polar lipids separated by silicic acid chromatography (7). Equal amounts of total hepatic phospholipids from three animals were pooled at this point in order to have sufficient samples for the analyses of monoene isomers at the 1-position of phosphoglyceride glycerol. Values given represent the mean of two or more analyses which usually agreed ± 10% for major components and ± 15% for minor components. Phosphatidylcholine and phosphatidylethanolamine (> 95% pure, as judged by thin layer chromatography (TLC) in two different solvent systems) were isolated by TLC (4), the fatty acids hydrolyzed from the 2-position with phospholipase A *(Ophiophagus hannah)* (8), and the methyl esters of both positions prepared and analyzed quantitatively by gas liquid chromatography (GLC) as described previously (9). Comparison of fatty acid percentages from the intact phospholipids with the percentages calculated from the 1- and 2-position compositions failed to show any selectivity in the release of isomeric fatty acids by phospholipase A. Octadecenoates were isolated by preparative GLC, separated into *cis* and *trans* fractions by argentation TLC (10), and the double bond positions determined by gas liquid chromatographic analysis of the ozonide cleavage products by the procedures detailed earlier (4). The ozonides were prepared by a modification (2) of the Beroza and Bierl procedure (11). The safflower oil consisted of 6.2% palmitate, 2% stearate, 11.3% oleate, and 80.4% linoleate. The preparation and characterization of the partially hydrogenated safflower oil free fatty acids have been described previously (4). After partial hydrogenation, the sample consisted of 12% saturates, 12% dienes, and 76% octadecenoates. The octadecenoates consisted of 67% *trans* and 33% *cis*. The *cis* fraction was composed of Δ9 (62%), Δ10 (11%), Δ11 (11%), and Δ12 (16%) isomers; and the *trans* fraction consisted approximately of Δ9 (13%), Δ10 (33%), Δ11 (34%), Δ12 (13%), Δ13 (5%), and Δ14 (2.5%). The source and purity of the safflower oil, lipid standards, solvents, and specific chemicals were the same as given previously (2,4). Other chemicals and reagents used were reagent grade or better.

RESULTS

The percentages of the major fatty acids esterified at the 1- and 2-positions of phosphatidylcholine and phosphatidylethanolamine isolated from hepatoma, normal, and host livers of animals fed various diets are given in Tables I and II. Generally, the effects of diet were marginal except for the increased percentage of 18:1 at the 1-position of both phosphoglyceride classes from animals fed the 15% PHSOFA diet. The differences in fatty acid composition between normal liver and host liver were small at the 1-position, whereas the percentage of 22:6 at the 2-position of both phosphoglycerides was increased in host liver. The increased percentage of this fatty acid demonstrates more convincingly than our earlier data (12) the effect of the hepatoma on the host liver lipids. Neifakh and Lankin (13) have reported higher levels of other polyunsaturated fatty acids (18:2 and 20:4) in host animal livers than in normal liver. Fatty acid composition of the 1-position of liver phosphatidylcholines agreed well with the corresponding position of phosphatidylethanolamines. In contrast, the 2-position fatty acids of these two phospholipids differed considerably: phosphatidylethanolamine contained higher percentages of C-20 and C-22 polyunsaturated fatty acids.

The fatty acid composition of hepatoma phosphatidylcholine and phosphatidylethanolamine differed dramatically from liver. Hepatoma phosphatidylethanolamine contained approximately half the percentage of 16:0 at the 1-position as did liver. The low percentage of palmitate confirms our earlier data (12) on this hepatoma (14) and Ehrlich ascites cells (15). Although the percentages of palmitate at the 1-position of hepatoma phosphatidylcholine were similar to liver, the percentage of this acid at the 2-position was dramatically increased in the hepatoma. The enormous decrease in the percentage of C-20 and C-22 polyunsaturated fatty acid at the 2-position of both hepatoma phosphoglycerides, which was partially offset by an increased percentage of 18:1, agrees with earlier observations (12). Our earlier results (12) had not revealed the higher percentage of palmitate at the 2-position, probably due to the kind of snake venom used in the hydrolysis. *Ophiophagus hannah* venom, used in the present study, has been shown to be preferable to other venoms for the hydrolysis of 2-position fatty acids from phosphoglycerides (16).

The *trans* fatty acids in phosphatidylcholine and phosphatidylethanolamine from hepatoma, host and normal livers of animals fed all diets (except the 15% PHSOFA diet) were undetectable or present in quantities too small to permit reliable determinations. *cis*-Octadecenoates from hepatic phosphoglycerides of animals fed

TABLE I

Fatty Acid Distribution at the 1- and 2-Positions of Phosphatidylcholine from Hepatoma, Host Liver, and Normal Liver of Rats Fed Various Diets

Hepatic tissue and diet	Position	Fatty acid percentages[a]								
		16:0	16:1	18:0	18:1	18:2	20:1	20:3	20:4	22:6
Normal liver										
Chow	1	44.5	1.4	41.2	7.0	1.9	---	---	---	---
Fat-free	1	39.9	2.0	47.0	10.8	T[b]	---	---	---	---
Fat-free + 0.5% Saff.[c]	1	40.5	2.6	43.0	11.9	1.5	---	---	---	---
Fat-free + 15% PHSOFA[d]	1	26.1	3.6	31.5	35.9	2.7	---	---	---	---
Host liver										
Chow	1	44.3	T	50.5	3.2	1.0	---	---	---	---
Fat-free	1	44.3	T	52.0	3.5	T	---	---	---	---
Fat-free + 0.5% Saff.	1	44.2	T	49.1	4.5	1.5	---	---	---	---
Fat-free + 15% Saff.[e]	1	47.5	T	46.4	3.8	1.6	---	---	---	---
Fat-free + 15% PHSOFA	1	39.5	---	43.3	16.0	1.2	---	---	---	---
Hepatoma										
Chow fed host	1	41.3	1.0	33.6	13.0	2.0	2.5	T	T	---
Fat-free fed host	1	39.3	1.7	38.5	13.9	0.8	2.8	T	0.5	---
Fat-free + 15% PHSOFA fed host	1	32.0	1.8	32.1	23.6	2.6	4.0	---	1.0	---
Normal liver										
Chow	2	2.2	0.9	---	11.4	39.1	---	0.7	36.9	6.9
Fat-free	2	2.2	7.0	T	28.6	8.5	---	15.2	31.0	3.4
Fat-free + 0.5% Saff.	2	1.8	6.5	0.5	24.5	16.6	---	11.0	28.6	6.5
Fat-free + 15% PHSOFA	2	2.4	2.7	---	22.3	27.5	---	---	30.7	7.8
Host liver										
Chow	2	3.6	T	0.6	13.9	36.7	---	1.1	31.8	10.3
Fat-free	2	3.3	2.5	0.6	26.6	18.2	---	8.5	28.2	10.8
Fat-free + 0.5% Saff.	2	2.5	T	0.7	13.4	36.1	---	1.0	26.1	18.5
Fat-free + 15% Saff.	2	2.3	T	0.7	8.2	42.8	---	0.5	25.1	17.8
Fat-free + 15% PHSOFA	2	3.5	---	---	13.0	33.3	---	---	34.8	13.8
Hepatoma										
Chow fed host	2	18.8	2.0	0.9	36.1	28.5	0.4	0.8	9.2	1.0
Fat-free fed host	2	14.1	3.8	0.9	49.0	14.2	0.7	2.9	10.8	2.0
Fat-free + 15% PHSOFA fed host	2	17.4	2.0	1.8	32.2	23.2	0.9	---	16.4	1.8

[a]The 18:2, 20:3, 20:4, and 22:6 polyunsaturated fatty esters co-chromatographed with 18:2ω6, 20:3ω9, 20:4ω6, and 22:6ω3 standards, respectively, but absolute identities were not established. The difference between the sum of any row and 100% represents minor amounts of other acids not given in the table.

[b]T represents detectable quantities of less than 0.5%.

[c]Saff. = safflower oil.

[d]PHSOFA = partially hydrogenated safflower oil fatty acids.

[e]15% Saff. = safflower oil fatty acids.

TABLE II

Fatty Acid Distribution at the 1- and 2-Positions of Phosphatidylethanolamine from Hepatoma, Host Liver, and Normal Liver of Rats Fed Various Diets

Hepatic tissue and diet	Position	Fatty acid percentages[a]								
		16:0	16:1	18:0	18:1	18:2	20:1	20:3	20:4	22:6
Normal liver										
Chow	1	41.3	T[b]	46.3	9.4	0.8	---	---	---	---
Fat-free	1	35.1	T	52.8	12.1	---	---	---	---	---
Fat-free + 0.5% Saff.[c]	1	35.8	T	51.1	12.8	T	---	---	---	---
Fat-free + 15% PHSOFA[d]	1	28.7	1.7	34.5	35.1	---	---	---	---	---
Host liver										
Chow	1	39.6	---	54.7	5.7	T	---	---	---	---
Fat-free	1	37.2	---	55.5	7.3	T	---	---	---	---
Fat-free + 0.5% Saff.	1	37.5	---	57.4	3.5	T	---	---	---	---
Fat-free + 15% Saff.[e]	1	41.0	---	53.2	4.0	T	---	---	---	---
Fat-free + 15% PHSOFA	1	36.8	---	46.3	16.0	---	---	---	---	---
Hepatoma										
Chow fed host	1	16.8	---	63.4	16.8	1.7	1.5	---	---	---
Fat-free fed host	1	17.5	---	60.3	20.2	T	2.1	---	---	---
Fat-free + 15% PHSOFA fed host	1	15.7	---	52.0	24.9	3.0	2.4	---	1.9	---
Normal liver										
Chow	2	1.3	T	0.8	3.9	24.8	---	---	51.3	14.1
Fat-free	2	1.1	1.4	0.5	7.4	2.0	---	6.8	63.3	11.1
Fat-free + 0.5% Saff.	2	2.8	2.8	2.8	11.5	8.0	---	2.9	53.4	12.2
Fat-free + 15% PHSOFA	2	2.0	---	2.4	7.6	9.9	---	---	52.1	24.6
Host liver										
Chow	2	2.5	T	1.2	3.7	18.8	---	---	45.5	25.7
Fat-free	2	1.9	0.5	1.4	6.6	7.1	---	5.9	51.4	23.0
Fat-free + 0.5% Saff.	2	0.8	---	0.9	1.8	18.1	---	---	42.5	30.4
Fat-free + 15% Saff.	2	0.9	---	0.8	0.8	17.2	---	---	36.5	38.9
Fat-free + 15% PHSOFA	2	5.5	---	8.8	3.5	12.7	---	---	38.4	29.2
Hepatoma										
Chow fed host	2	3.4	0.5	1.0	41.0	24.7	0.9	1.4	19.0	2.8
Fat-free fed host	2	2.4	1.6	0.9	57.9	13.0	1.1	2.0	16.3	2.3
Fat-free + PHSOFA fed host	2	5.1	---	3.7	40.6	20.0	1.4	---	22.2	1.3

[a]The 18:2, 20:3, 20:4, and 22:6 polyunsaturated fatty esters co-chromatographed with 18:2ω6, 20:3ω9, 20:4ω6, and 22:6ω3 standards, respectively, but absolute identities were not established. The difference between the sum of any row and 100% represents minor amounts of other acids not given in the table.

[b]T represents detectable quantities of less than 0.5%.

[c]Saff. = safflower oil.

[d]PHSOFA = partially hydrogenated safflower oil fatty acids.

[e]15% Saff. = safflower oil fatty acids.

TABLE III

The Distribution of *cis* Octadecenoate Isomers at the 1- and 2-Positions of Phospholipid Classes Derived from Hepatoma, Host Liver, and Normal Liver of Rats Fed Various Diets

Hepatic tissue and diet	Position on glycerol	Percentages[a] Phosphatidylcholine Δ9	Phosphatidylcholine Δ11	Phosphatidylethanolamine Δ9	Phosphatidylethanolamine Δ11
Normal liver					
Chow	1	24.9	73.5	45.2	53.4
Fat-free	1	19.0	79.0	38.4	59.5
Fat-free + 0.5% Saff.[b]	1	12.0	88.0	31.3	68.2
Host liver					
Chow	1	45.4	53.7	59.2	40.8
Fat-free	1	46.6	51.2	73.3	26.7
Fat-free + 0.5% Saff.	1	61.0	39.0	68.1	31.9
Fat-free + 15% Saff.[c]	1	55.9	44.1	62.3	34.7
Hepatoma					
Chow fed host	1	58.3	41.7	61.9	36.0
Fat-free fed host	1	46.1	51.4	50.7	47.0
Normal liver					
Chow	2	75.1	24.9	54.9	45.1
Fat-free	2	77.9	22.1	67.6	32.3
Fat-free + 0.5% Saff.	2	81.3	18.6	65.6	29.2
Host liver					
Chow	2	81.5	18.5	70.8	29.1
Fat-free	2	87.8	12.2	82.0	18.0
Fat-free + 0.5% Saff.	2	81.8	18.2	60.4	36.0
Fat-free + 15% Saff.[c]	2	77.3	22.7	54.1	41.0
Hepatoma					
Chow fed host	2	77.6	22.4	82.2	17.8
Fat-free fed host	2	79.8	20.2	82.6	17.4

[a]The difference between the sum of the Δ9 and Δ11 percentages and 100% represents minor amounts of other isomers.
[b]0.5% = Safflower oil.
[c]15% Safflower oil free fatty acids.

these diets contained predominately the Δ9 (oleic) and Δ11 (vaccenic) chain positional isomers at the 1- and 2-positions of the glycerol moiety (Table III). The octadecenoates at the 1-position of normal liver phosphatidylcholine consisted predominately of the Δ11 isomer. The same trend was also observed in normal liver phosphatidylethanolamine but was less pronounced. There was a shift to higher percentages of the Δ9 isomer at the 1-position of host liver and hepatoma phosphatidylcholine and phosphatidylethanolamine relative to normal liver. The 2-position of both phosphoglycerides from all hepatic tissues was predominately oleic acid. This preferential incorporation of the Δ9 isomer at the 2-position was more pronounced in phosphatidylcholine and least subject to dietary influence.

Hepatoma and liver phosphatidylcholine and phosphatidylethanolamine from animals fed the PHSOFA diet contained both *cis* and *trans* octadecenoates. The percentage distributions of the *cis* and *trans* octadecenoates at the 1- and 2-positions of both phosphoglycerides are given in Table IV. The 2-position octadecenoates of both classes in all tissues contained almost exclusively the *cis* isomers. The octadecenoates in the 1-position of liver phosphatidylcholine and phosphatidylethanolamine consisted of 70-80% *trans* isomers, whereas hepatoma phosphoglycerides contained approximately equal percentages of *cis* and *trans* octadecenoic isomers at the 1-position.

The percentage distributions of *cis* and *trans* octadecenoate positional isomers esterified at the 1- and 2-positions of phosphatidylcholine and phosphatidylethanolamine from hepatic tissue of animals fed the 15% PHSOFA diet are given in Tables V and VI. The 1-position of *cis* octadenoates from both phosphoglycerides of normal liver contained a high percentage of the Δ11 and Δ12 isomers and a low level of the Δ9 isomers similar to the 1-position of phosphoglycerides from animals on the other diets (Table III). Host liver and hepatoma showed a progressive decrease in the percentage of the Δ11 and Δ12 isomers at this position, accompanied by an increase in the percentage of oleic acid. The *cis* octadecenoates from the 2-position of both phosphoglycerides from normal liver were dominated by oleic acid as in the other diets.

TABLE IV

Distribution of *cis* and *trans* Octadecenoates of the 1- and 2-Positions of Two Phospholipid Classes from Rats Fed the 15% PHSOFA Diet[a]

Source	Phosphatidylcholine				Phosphatidylethanolamine			
	1-Position %[b]		2-Position %		1-Position %		2-Position %	
	cis	*trans*	*cis*	*trans*	*cis*	*trans*	*cis*	*trans*
Liver, normal	19.4	80.4	94.8	5.2	23.7	76.2	88.9	11.0
Liver, host	29.6	70.4	97.2	2.8	22.7	77.2	92.5	7.5
Hepatoma	57.0	43.0	97.0	3.0	52.4	47.5	96.4	3.6

[a]PHSOFA = partially hydrogenated safflower oil fatty acids.
[b]Percentages represent the mean of two or more determinations.

TABLE V

Distribution of Positional Isomers of *cis* Octadecenoates Derived from the 1- and 2-Positions of Phosphatidylcholine and Phosphatidylethanolamine from Rats Fed the 15% PHSOFA Diet[a]

Hepatic tissue	Position	Percentage of *cis* isomers[b]						
		Δ8	Δ9	Δ10	Δ11	Δ12	Δ13	Δ14
		Phosphatidylcholine						
Liver, normal	1	2.8	15.8	2.1	39.6	26.2	10.9	2.6
Liver, host	1	1.5	34.4	3.5	33.8	23.2	2.5	1.1
Hepatoma	1	1.6	54.9	2.9	24.9	12.1	2.4	1.2
Liver, normal	2	0.5	55.9	0.4	15.0	28.0	0.3	—
Liver, host	2	1.4	76.4	1.0	15.2	5.8	—	—
Hepatoma	2	1.5	68.2	2.8	19.5	6.8	1.2	—
		Phosphatidylethanolamine						
Liver, normal	1	2.6	30.9	2.5	34.2	21.8	6.7	1.3
Liver, host	1	1.2	49.0	2.7	27.0	19.9	—	—
Hepatoma	1	2.0	54.4	2.0	27.0	11.8	1.4	1.3
Liver, normal	2	2.8	48.0	1.4	27.9	19.9	—	—
Liver, host	2	0.9	60.4	1.4	27.0	10.3	—	—
Hepatoma	2	1.0	72.2	0.4	21.0	5.2	—	—

[a]PHSOFA = partially hydrogenated safflower oil fatty acids.
[b]Percentages represent the mean of two or more determinations.

The proportion of this acid was still higher in host liver and hepatoma. Vaccenic acid percentages remained the same in all three tissues in both phosphoglycerides, but the Δ12 isomer at the 2-position was decreased in host liver and hepatoma relative to normal liver.

The *trans* octadecenoate profile at the 1-position of phosphatidylcholine and phosphatidylethanolamine was characterized by an accumulation of the Δ12, Δ13, and Δ14 isomers in all tissues relative to the amounts that were in the diet. The percentage of the *trans* Δ10 isomer at the 1-position was much lower in all tissues than the relative amounts in the diet, but the hepatoma incorporated much more than liver. Although only a very small amount of *trans* fatty acids was incorporated at the 2-position of phosphatidylcholine and phosphatidylethanolamine (Table IV), the chain positional isomer distribution was surprising. Only four major isomers were incorporated into phosphatidylcholine of all tissues, and the *trans* Δ10 isomer was one of the isomers. In addition to the four major *trans* isomers (Δ9-Δ12) at the 2-position of phosphatidylcholine, liver phosphatidylethanolamine also contained the Δ13 and Δ14 isomers, but the hepatoma did not.

DISCUSSION

Positional Specificity of Natural Occurring Octadecenoates in Phosphoglycerides

Our present and previous studies (1,2,4) indicate that when rats are fed a fat-free diet or diets containing natural fats, octadecenoates from hepatoma and liver phosphatidylcholine and phosphatidylethanolamine consist almost exclusively of two *cis* isomers, namely oleic and

TABLE VI

Distribution of Positional Isomers of *trans* Octadecenoates
Derived from the 1- and 2-Positions of Phosphatidylcholine and Phosphatidylethanolamine
from Rats Fed the 15% PHSOFA Diet[a]

Hepatic tissue	Position	Percentage of *trans* isomers[b]						
		Δ8	Δ9	Δ10	Δ11	Δ12	Δ13	Δ14
		Phosphatidylcholine						
Liver, normal	1	3.1	13.9	4.9	28.5	35.3	6.8	7.4
Liver, host	1	1.8	12.9	3.0	25.3	35.8	10.4	10.5
Hepatoma	1	2.2	14.0	18.2	27.1	25.2	6.8	6.4
Liver, normal	2	2.0	29.8	31.5	17.0	16.0	3.5	---
Liver, host	2	---	34.9	21.3	28.5	15.2	---	---
Hepatoma	2	---	24.3	31.5	26.4	17.9	---	---
		Phosphatidylethanolamine						
Liver, normal	1	2.9	15.6	7.4	24.7	34.3	7.7	7.4
Liver, host	1	1.0	11.6	2.4	26.1	37.9	10.6	10.4
Hepatoma	1	2.0	13.4	15.4	29.5	27.4	6.6	5.3
Liver, normal	2	2.1	16.3	22.1	20.8	26.6	6.3	5.8
Liver, host	2	---	9.1	4.4	27.4	37.8	11.0	10.4
Hepatoma	2	---	21.6	29.4	31.9	17.2	---	---

[a]PHSOFA = partially hydrogenated safflower oil fatty acids.
[b]Percentages represent the mean of two or more determinations.

vaccenic acids. Further, the present study shows that the octadecenoates esterified at the 1-position of *normal* liver phosphatidylcholine, and to a lesser degree phosphatidylethanolamine, consist predominately of vaccenic acid, while the 2-position is dominated by oleic acid. Mass data reported by Brockerhoff and Ackman (17) for rat liver phosphatidylcholine and phosphatidylethanolamine from animals fed mackerel exhibited a preference for oleate at the 2-position. They did not observe as strong a preference for vaccenic acid at the 1-position as we observed in this study, probably due to the competing 16:1 monoene isomers present in the mackerel fed. Our results are in contrast to those reported by Spence (18), which showed that oleate occupied 70-90% of the octadecenoates at the 1- and 2-positions of phosphatidylcholine and phosphatidylethanolamine from newborn and adult rat brains. These differences probably result from differences in tissue specificity. The shift from oleate levels in normal liver to higher proportions of oleate at the 1-position of phosphatidylcholine in host liver indicates another effect of the hepatoma on the lipid metabolism of the host animals. The shift was observed in all host livers despite the extreme variations in dietary conditions. The potential diagnostic value of this observed shift will depend upon how early these changes can be detected in the host.

Positional Specificity of *trans* Octadecenoates in Phosphoglycerides

Dietary *trans* fatty acids from partially hydrogenated safflower oil fatty acids are incorporated into rat liver lipids, and the percentage of the octadecenoate fraction they represent is dependent on the lipid class (4). The present data show that the *trans* fatty acids are incorporated predominately into the 1-position of both phosphoglycerides (Table IV). This was not unexpected, since Sinclair (19) reported in 1935 that elaidic acid fed to rats replaced 25-30% of the saturated fatty acids in liver and muscle phospholipids. Bickerstaffe and Annison (20) reported the preferential incorporation of radioactive elaidic and *trans* vaccenic acids into the 1-position of phospholipids of perfused chicken livers. The preferential incorporation of *trans* fatty acids into the 1- and 3-positions of rat adipose tissue triglycerides has also been reported (21). These earlier studies, however, were carried out with only one or two isomers. We have shown (4) that not all *trans* octadecenoate isomers are incorporated into liver phospholipids, whereas all of the isomers are incorporated into triglycerides and cholesteryl esters in nearly the same proportions they occur in the diet. The very high selectivity of the acyl transferases leading to the almost exclusive incorporation of the *cis* octadecenoates into the 2-position and the *trans* octadecenoates into the 1-position of both phospholipids appears to involve more than the similarity between the conformation of the *trans* fatty acids and the saturated fatty acids. This conclusion follows from the observation that the 2-position of hepatoma phosphatidylcho-

line contains considerable quantities of palmitic acid (Table I), whereas the 2-position octadecenoates consist almost exclusively of the *cis* isomers (Table IV).

Distribution of Chain Positional Isomers of Dietary *cis* Octadecenoates

The present data appear to be the first to show the distribution of dietary chain positional isomers of *cis* and *trans* octadecenoates at the 1- and 2-positions of rat liver and hepatoma phosphatidylcholine and phosphatidylethanolamine. The interpretation of the *cis* isomeric data is complicated by the mixing of exogenous isomers with the naturally occurring endogenous isomers. However, despite this difficulty, it is still possible to see that vaccenate was more abundant than oleate in the 1-position of normal liver phosphatidylcholine and phosphatidylethanolamine and that there was a shift to higher percentages of oleate in the host and hepatoma classes. The higher percentage of oleate in the 2-position of liver and hepatoma phospholipids was also apparent. These observations are consistent with the data from animals maintained on fat-free or natural fat diets. The 2-position exhibited a higher degree of specificity for positional isomers than the 1-position in all cases. More than 95% of the octadecenoate fraction of the 2-position was made up of the Δ9, Δ11, and Δ12 isomers. These data are in partial agreement with in vitro data reported by Reitz et al. (22), who measured the rates of incorporation of the various *cis* octadecenoates into 1-acyl glycero-3-phosphorylcholine by rat liver microsomes. They showed that the Δ9 and Δ12 isomers exhibited the highest rates of incorporation, which is in agreement with our mass data. However, their data showed higher rates of incorporation of the Δ8, Δ10, Δ13, and Δ14 isomers than for the Δ11 isomer, which differs from what we have reported here. Reitz and colleagues (22) also measured the rates of incorporation of the *cis* isomers into 2-acyl glycero-3-phosphorylcholine, which showed that the Δ8, Δ10, Δ12, and Δ13 isomers were incorporated at much faster rates than the Δ9 and Δ11 isomers. These apparent discrepancies are probably attributable to differences between in vivo and in vitro data.

Distribution of Chain Positional Isomers of Dietary *trans* Octadecenoates

The positional isomers of the *trans* octadecenoates were all exogenous, which allows a clearer interpretation of the data. Normal liver and host liver show much smaller differences in the percentage distribution of the *trans* isomers at the 1- and 2-positions of both phospholipids than of the corresponding *cis* isomers. This suggests that the metabolic fate of exogenous fatty acids may be affected less by the presence of the hepatoma than is the fate of the endogenous fatty acids. Comparisons of liver and hepatoma isomeric compositions at the 1-position of both lipid classes show that the *trans* Δ10 isomer was incorporated to a greater extent in the hepatoma. If the exclusion of this isomer from the 1-position is due to enzyme specificity, then the hepatoma system has lost some of its specificty. The *trans* isomers, esterified in minor amounts at the 2-position of liver and hepatoma phosphatidylcholine (Table IV), exhibited a completely different profile from the 1-position (Table VI). The most striking observation was the occurrence of the *trans* Δ10 isomer as one of the four major isomers present. This observation indicates that the acyl transferases that esterify predominately saturated fatty acids to the 1-position of glycerol also accept and esterify *trans* monoenes at this position. In addition, the 1-position acyl transferases exhibit double bond specificity or selectivity which discriminates against the *trans* Δ10 isomer. The acyl transferases which normally esterify unsaturated fatty acids at the 2-position of glycerol virtually exclude the *trans* isomers; however, there is no discrimination of the *trans* Δ10 isomer from the small amount of *trans* acids esterified at this position. In agreement with our mass data, Okuyama et al. (23) showed that the *trans* Δ10 isomer had the slowest rate of incorporation into both 2-acyl glycero-3-phosphorylcholines and ethanolamines. There are a number of discrepancies between their in vitro data and our in vivo data. Caution must, however, be exercised in drawing conclusions from such comparisons. It must be remembered that the mass data for the various isomers represent the sum total of all absorptive, transportive, anabolic, and catabolic processes, whereas the in vitro data measured primarily the enzymatic activity of one anabolic process in the presence of excess substrate.

Origin of 1- and 2-Position Phosphoglyceride Fatty Acids

The detailed examinations of the octadecenoate isomers at the 1- and 2-positions of the two phosphoglycerides allow one to compare the origins of the fatty acids. The data strongly indicate that the fatty acids at the 1-position of liver phosphatidylcholine and phosphatidylethanolamine have the same origin, i.e., the same fatty acid pool(s) or compartment(s). This observation is supported by the similarities in: the fatty acid compositions (Tables I and II), the ratios of *cis* and *trans*

octadecenoates (Table IV), and the distribution of the chain positional isomers of the *cis* and *trans* octadecenoates (Table V and VI). Similarities in the 1-position fatty acid compositions of phosphatidylcholine and phosphatidylethanolamine have been noted previously in rat liver (11,24). The origin of heptoma phosphatidylcholine and phosphatidylethanolamine monoenes likewise appears to be similar, but the decreased percentage of palmitate at the 1-position of phosphatidylethanolamine (Table II) may indicate that one of the palmitate pools normally utilized is not available for hepatoma phosphatidylethanolamine biosynthesis. The concept of a common origin of fatty acids at the 1-position of phosphatidylcholine and phosphatidylethanolamine does not preclude the possibility that these two phosphoglycerides are derived from the same diglyceride pool. The dissimilarities of the fatty acids at the 2-position between these two phosphoglycerides can be attributed to selectivity of diglyceride species; however, most attempts to show selectivity appear to have been unsuccessful (25,26).

The similarity in the composition of the *trans* octadecenoates at the 1- and 2-positions of only host liver phosphatidylethanolamine indicates the hepatoma may modify the specificities of the host liver 2-position acyl transferases toward *trans* fatty acids to resemble the 1-position acyl transferases. On the other hand, the observed similarities could have resulted from the inhibition of the 2-position acyl transferases that esterify *trans* acids and a concomitant decrease in glycerol position specificity of the 1-position acyl transferases. The similarities in the *trans*-octadecenoate composition at the 1- and 2-positions in host liver phosphatidylethanolamine but not phosphatidylcholine suggests separate acyl transferases for these phospholipid classes. Although effects of the hepatoma on host liver metabolism are implicated and probably exist, one has to keep an open mind that some of these effects might be nutritional, brought on by decreased food consumption in the terminal stages of the host animals.

The distribution of the positional isomers of the *cis* and *trans* octadecenoates at the 2-position of liver phosphatidylcholine differs from phosphatidylethanolamine; however, they are almost identical in the hepatoma. This appears to result from another basic difference between liver and hepatoma in the biosynthesis of these two phosphoglycerides. This difference may be related to the origin of fatty acids in the two systems.

Significance

The asymmetric distribution of naturally occurring octadecenoates, unnatural dietary *cis* octadecenoates, and dietary *trans* octadecenoates at the 1- and 2-positions of normal liver phosphatidylcholine and phosphatidylethanolamine has been observed. Exclusion of some isomers and preferential incorporation of other positional isomers of the *cis* and *trans* octadecenoates at the positions of glycerol contribute to an even more select distribution. These data indicate that the acyl transferases involved in the esterification of fatty acids at these different positions recognize more than saturation and unsaturation: double bond configuration and position in the hydrocarbon chain are also determinants.

Similarities in the isomeric composition of the *cis* and *trans* octadecenoates at the 1-position of the two phosphoglycerides indicate they have a common origin, whereas the 2-position fatty acids of each phosphoglyceride appear to arise from different pools. The latter may result from class specific 2-position acyl transferases or diglyceride selectivity.

Effect of the hepatoma on host liver lipids was exhibited by a shift to higher percentages of oleate at the 1-position of the phosphoglycerides. This apparent effect of acyl transferase specificity was also noted at the 2-position of phosphatidylethanolamine: the distribution of *trans* octadecenoates was similar to the 1-position.

Hepatoma phosphoglycerides failed to exhibit the same degree of asymmetric distribution of natural and unnatural octadecenoates as liver. The exclusion of some isomers was less pronounced in the hepatoma, and the origin of the 2-position fatty acids of the phosphoglycerides appeared to be the same. The latter observation may have resulted from the loss of diglyceride selectivity or the reduction of a pool of diglycerides in the hepatoma that is normally a major source for liver phosphoglyceride biosynthesis.

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METHODS

Synthesis of a Monobrominated Analog of Dipalmitoyl Lecithin

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ABSTRACT

The synthesis of 1-palmitoyl-2-monobromopalmitoyl lecithin, where the β-chain is brominated in either the 9- or 10-position, is reported, Monobromopalmitic acid was prepared by addition of HBr to palmitoleic acid, and the anhydride of the fatty acid conjugated with 1-palmitoyl lysolecithin. The resulting lecithin was isolated by preparative thin layer chromatography and its structure confirmed by chemical and enzymatic analysis.

INTRODUCTION

Brominated oils have been used extensively as food additives (1,2) and are of current environmental interest since associated fatty acids become incorporated in the lipids of various organisms (3). Rats fed brominated oils develop lesions of the heart and liver (4), and, on the basis of toxilogical studies, it has been proposed that these oils be removed from the *Federal Register's* "safe" list (5). We describe here the synthesis of a monobrominated lecithin which could serve as a simple model for brominated lipids occurring under physiological conditions.

A monobrominated derivative can also be useful in physiocochemical studies of lipids. The most extensively studied lipid is dipalmitoyl lecithin (DPL), and the analog described here is identical in structure to DPL except for the bromine substitution in a single side chain. This analog may act as an isomorphous replacement molecule or as an anomalous scattering center in studies of thermal and structural properties of mixtures of brominated and nonbrominated lipids (Lytz, Reinert, and Wickman, unpublished). In addition, it has been shown that *Escherichia coli* membranes remain functional when a substantial fraction of the fatty acid chains, esterified in the membrane lipids, are monobrominated in the 9- or 10-carbon positions (6). Thus, derivative incorporation and X-ray study of natural membrane systems may also be feasible.

We report here the synthesis of L-α-1-palmitoyl-2-monobromopalmitoyl lecithin, where the chain in the 2-position contains a bromine atom at either the C_9 or C_{10} position. The method employed hydrobromination of palmitoleic acid, followed by conjugation of the fatty acid anhydride with lysolecithin. This procedure was arrived at following the observation that direct UV catalyzed addition of HBr to 1-palmitoyl-2-palmitoleoyl lecithin was unsuccessful due to breakdown of the lecithin molecules. Extensive deacylation of the lecithin was observed under conditions where the palmitoleoyl double bond could be quantitatively hydrobrominated. While monobrominated fatty acids are known (7), there does not appear to be any previous report of the synthesis and characterization of a monobrominated lecithin.

EXPERIMENTAL METHODS AND RESULTS

Analytical Methods

Free fatty acids were obtained from Nu-Chek-Prep (Elysian, MN), 1-palmitoyl-L-α-lysolecithin from Supelco (Bellefonte, PA), and DL-α-lecithin dipalmitoyl (DPL– from Schwarz, Mann (Orangeburg, NY). Phospholipase A (Lecithinase-A Phosphatide-2-acylhydrolase, EC 3.1.1.4, from *Naja naja* venom) was obtained from Sigma (St. Louis, MO). Dry HBr gas was obtained from Matheson (Joliet, IL), and benzene was dried by passage through an alumina column followed by redistillation. Dry benzene was saturated with HBr by bubbling dry gas through the benzene for 1 hr. N,N'-Dicyclohexylcarbodiimide (DCC) (Aldrich, Milwaukee, WI) and Na_2O (Alfa Prod-

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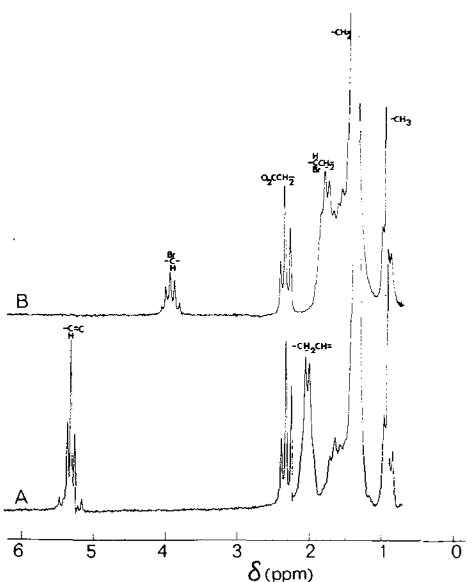

FIG. 1. NMR spectra of (A) palmitoleic acid and (B) monobromopalmitic acid produced by reaction with HBr. Concentrations are 50 mg/ml in CCl_4. Varian HA100 Spectrometer.

ucts, Beverly, MA) were obtained in 98+%. All thin layer chromatography (TLC) separations were carried out on F1500 silica gel plates from Schleicher and Schuell (Keene, NH).

Gas chromatography (GC) was performed on a HP700 chromatograph equipped with a hydrogen flame detector. Methyl esters were prepared by reacting fatty acids or lipids with excess super dry methanol containing 5% HCl gas for 90 min at 80 C. Samples were run on a 200 ft, .03 in. ID EGS liquid phase stainless-steel column at 160 C. Nuclear magnetic resonance (NMR) spectra were obtained on a Varian HA100 spectrometer using a tetramethylsilane instrument lock. Bromine content was determined by neutron activation. Samples and standards were dissolved in olive oil to equalize geometry, and bromine analyzed as ^{82}Br 48 hr after activation at 1 Mwatt power for 1 hr. Phosphorous was determined by the method of Lowry and Tinsley (8). For the phospholipase A digestions, 0.8 mg lipid was dispersed in 0.5 ml 0.1 M tris buffer pH 8.9 containing 10 mM $CaCl_2$ and 10 units of enzyme was added. After 1 hr at room temperature, the lipid material was extracted from the digestion mixture by the Folch procedure (9), the solvent removed, and the residue dissolved in $CHCl_3$. The mixture was applied to a silicic acid column (Bio-Sil A, Bio-Rad, Richmond, CA), and the liberated fatty acids eluted with chloroform.

Preparative Details

Palmitoleic acid was first hydrobrominated by UV catalysis. The acid (100 mg) was dissolved in 15 ml of a saturated solution of HBr in dry benzene. This solution was transferred to a quartz tube which was sealed and exposed to a UV lamp 30 cm distant. The extent of HBr addition was followed with NMR by monitoring the disappearance of the double bond proton resonance at 5.3 ppm. Figure 1 shows NMR spectra of the palmitoleate starting material (Fig. 1A) and the material isolated after hydrobromination (Fig. 1B). Assignments of the various resoonances are from the literature (10). The total disappearance of the resonances associated with protons on the double bond carbon and neighboring carbon (5.3 and 2.0 ppm, respectively) in palmitoleate indicates that addition to the double bond was complete within 30 min. The disappearance of these unsaturated resonances is accompanied by the appearance (Fig. 1B) of peaks associated with protons on the brominated carbon and neighboring carbon at 3.9 and 1.7 ppm, respectively. The resonances associated with the other protons in the molecule are not affected by the reaction. Integration of the various peaks in Figure 1B gives relative values for the ratio of $BrCH:CH_3:CH_2+CH_{.2}$-$CHbrH:CH_2CO_2$ of 1:2.9:25:2.0 (theoretical 1:3:24:2), and the isolated fatty acid gives one peak by GC. It is expected (7) that addition of HBr to the long chain fatty acid results in equal quantities of 9- and 10-monobromopalmitate. This mixture was isolated by preparative TLC using a diethyl ether-hexane-acetic acid, 10:90:1 eluant.

The anhydride of the brominated fatty acid was prepared by reaction with dicyclohexylcarbodiimide in CCl_4 by the method of Selinger and Lapidot (11). NMR measurements before and after the reaction indicate that preparation of the anhydride was not accompanied by dehydrobromination of the fatty acids. The anhydride was isolated by evaporation of the CCl_4 following removal of the dicyclohexylurea precipitate by filtration (11).

The anhydride was conjugated with 1-palmitoyl-L-α-lysolecithin (12). Anhydride produced from 100 mg of fatty acid was added to 25 mg of lysolecithin, and all solvent removed by evaporation. The mixture was heated to 70 C, 0.78 mg of Na_2O added, and the reaction mixture maintained at 70 C for 48 hr. The mixture was then taken up in $CHCl_3$ and examined by TLC. Very little lysolecithin remained after the 48 hr. A minor, non-phosphorous containing component migrated very close to the lecithin, so two preparative steps were used to

isolate the lecithin. In the first step, the plate was developed with chloroform-methanol-water (65:35:4). The lecithin fraction was collected and applied to a second plate which was developed with chloroform-methanol-water (50:50:6). The lecithin was then isolated free of the closely migrating contaminant.

The synthesized material exhibits one spot by TLC with the same R_f as DPL in four different solvent systems (R_f = .23 in chloroform-methanol-water, 65:35:4) visualized with iodine, Dittmer reagent for phospholipids (13), and Dragendorff reagent for choline-containing compounds (14). The isolated lipid contains 9.7% Br and 3.4% P (theoretical, 9.85% and 3.8%). GC analysis shows that the lipid contains palmitate and monobromopalmitate in a ratio of 51.5:49.5. When the lipid is digested with phospholipase A, $>$ 95% of the liberated fatty acids are monobromopalmitate, confirming its expected location in the 2-position, and indicating that the proper stereochemistry is preserved in the lecithin.

The yield of the isolated lipid was 18.5 mg (46% of theoretical). The low yield results from the necessity of the two preparative TLC cleanup steps rather than from the acylation reaction not proceeding to near completion, since little lysolecithin remained after the reaction. The minor component which migrates close to lecithin and causes the difficulty in separation is probably a side product from the anhydride synthesis. Small amounts of the dicyclohexylacylurea, for example, are produced in the reaction of fatty acids with DCC (11). While not interfering with subsequent acylation reactions of the anhydrides (11), the side products from the anhydride synthesis may complicate the cleanup of product when lecithin is produced by acylation of lysolecithin. This problem could possibly be avoided by recrystallization of the anhydride before acylation.

As noted earlier, the current preparation has yielded a lecithin mixture containing bromine at the 9- or 10-position in the β-chain. From the X-ray crystallography viewpoint, spatial resolution in lamellar phases is typically only 7 Å (15), so the 9- or 10-position should be adequate for isomorphous replacement studies. Of course, a singly substituted species at these or other chain positions is also attractive for such purposes. A possible route to such materials might employ 9-hydroxypalmitic acid as starting material. Synthesis of an ω-substituted species is also possible and would be of interest since current views of membrane structure associate pronounced thermal motions with the chain terminal groups in bilayer preparations (16). It may be shown that any of these substitutions will change (though in differing amounts) the intensities of the lamellar X-ray scattering peaks. The main criteria for utility of these species would be the fidelity with which they act as isomorphous replacement molecules in mixtures with non-brominated lipids.

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Visualization of Primate High Density Lipoproteins Isolated by Density Gradient Ultracentrifugation

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ABSTRACT

High density lipoproteins (HDL) isolated by density gradient ultracentrifugation from the plasma of monkeys ingesting semipurified diets are barely visible. This creates difficulty in separating HDL from other lower density lipoproteins following centrifugation and necessitates collecting large quantities of background density solution to insure complete recovery of the HDL fraction. A simple procedure is described involving the addition of β-carotene to nonhuman primate plasma prior to centrifugation which results in the delineation of HDL as a discrete yellow-orange band without affecting certain physical properties of HDL or interfering with standard lipid and protein assays.

INTRODUCTION

Following separation of human plasma lipoproteins by a recently described density gradient ultracentrifugation procedure (1), low density lipoproteins (LDL) and high density lipoproteins (HDL) appear as yellow-orange bands while very low density lipoproteins (VLDL) have a lactescent appearance. The yellow-orange color of LDL and HDL, which is characteristic for lipoproteins from humans ingesting an omnivorous diet, has been attributed to the plant pigment β-carotene which is transported with these lipoproteins (2-4). This particular property of human lipoproteins facilitates the identification and recovery of individual lipoprotein fractions following their isolation by density gradient centrifugation. However, when nonhuman primates ingest semipurified diets lacking β-carotene, the HDL fraction isolated by density gradient ultracentrifugation is barely visible making its identification and recovery from the centrifuge tube extremely difficult. Recent reports of altered composition and concentration of HDL in nonhuman primates due to diet (5,6) emphasize the importance of developing an improved method for isolating these HDL. This communication describes a simple procedure for adding β-carotene to plasma samples from monkeys which enables HDL to be visualized following density gradient centrifugation without interfering with standard lipid and protein assays.

MATERIALS AND METHODS

Fourteen cebus monkeys (*Cebus albifrons*) of both sexes fed a semipurified diet (7) containing 15% coconut oil and 0.1% cholesterol (30 mg/100 kcal) for two years were used in all experiments. Blood was collected from the femoral vein of fasted monkeys into syringes containing EDTA-Na_2 (1 mg/ml) and Merthiolate (1/10,000, Eli Lilly and Co., Indianapolis,

TABLE I

Effect of β-Carotene Addition on the Cholesterol and Protein Content of Nonhuman Primate Plasma[a] Lipoproteins

Lipoprotein fraction	+ Carotene	- Carotene	Percent difference
VLDL			
Cholesterol	21.6 ± 2.9[b]	20.7 ± 3.0	4.2
Protein	10.7 ± 1.2	10.8 ± 1.2	0.9
LDL			
Cholesterol	111.0 ± 8.1	110.3 ± 6.9	0.6
Protein	73.4 ± 8.0	76.3 ± 7.1	3.8
HDL			
Cholesterol	107.8 ± 0.7	112.2 ± 1.4	3.9
Protein	264.1 ± 3.0	248.5 ± 0.4	5.9
Total lipoprotein			
Cholesterol	240.3 ± 10.7	243.1 ± 8.7	1.2
Protein	348.1 ± 12.0	335.6 ± 8.6	3.6

[a]Pooled plasma from three to six monkeys per experiment.

[b]Values are the mean ± SE of three separate experiments expressed as mg/dl.

TABLE II

Effect of β-Carotene Addition on the Chemical Composition of Nonhuman Primate[a] HDL Isolated by Heparin-Manganese Chloride Precipitation

Constituent	+ Carotene	- Carotene	Percent difference
Total cholesterol	107.8 ± 5.3[b]	103.0 ± 9.4	4.5
	(35.7 ± 1.0)	(34.8 ± 1.9)	(2.5)
Phospholipid	167.2 ± 4.8	164.3 ± 5.2	1.7
	(55.4 ± 0.8)	(55.9 ± 1.5)	(0.9)
Triacylglycerol	27.0 ± 1.5	27.3 ± 1.7	1.1
	(9.0 ± 0.7)	(9.3 ± 0.9)	(3.2)
Total lipid	302.0 ± 7.7	294.6 ± 11.6	2.5
	(100.1)	(100.0)	
Protein[c]	1606.5 ± 19.1	1648.2 ± 37.8	2.5

[a]Pooled plasma from three to six monkeys per experiment.

[b]Values are the mean ± SE of three separate experiments expressed as mg/dl. Numbers in parentheses represent percent of HDL lipid mass.

[c]Represents total protein in supernatant following heparin-manganese chloride precipitation expressed as mg/dl.

IN) and the plasma harvested following low speed centrifugation at 4 C. The effect of β-carotene addition on plasma lipids, protein, and lipoproteins was examined in three separate experiments using pooled plasma from three to six monkeys per experiment.

One aliquot from each pool was mixed with finely ground β-carotene (5 mg/ml, mp 183-185 C, ICN Pharmaceuticals, Inc., Cleveland, OH) previously triturated with a mortar and pestle. Unincorporated carotene was removed by filtering the plasma through a 3μ Millipore filter equipped with a syringe adapter (Millipore Corp., Bedford, MA). Very low density lipoproteins (d < 1.006), LDL (d = 1.006-1.063), and HDL (d = 1.063-1.210) were then isolated from the filtered plasma by density gradient ultracentrifugation in a swinging-bucket rotor (SW41, Beckman Instruments, Inc., Palo Alto, CA) at 41,000 rpm for 40 hr at 20 C using a Beckman Model L5-50 ultracentrifuge. A second aliquot was treated identically but without β-carotene addition for comparative purposes. Following centrifugation, plasma samples containing β-carotene were placed beside those samples without carotene to facilitate identification of the HDL band which appeared as a yellow-orange zone above a highly pigmented d > 1.210 bottom in the carotene-supplemented samples. Each lipoprotein fraction was then collected by aspiration and dialyzed for 72 hr against 5 mM Tris/1 mM EDTA (pH 7.4) containing Merthiolate (0.01 g/1).

Analytical determinations of protein (8), total cholesterol (9), triacylglycerol (Dow Diagnostics Reagent sets, Dow Chemical Co., Indianapolis, IN), and phospholipid (10) were made for each plasma pool and lipoprotein fraction. Purity of each isolated lipoprotein was assessed by agarose gel electrophoresis (11).

The effect of β-carotene on the physical and chemical integrity of plasma lipoproteins was assessed by comparison with untreated plasma following heparin-manganese chloride precipitation, agarose gel electrophoresis, Sepharose gel chromatography, and immunodiffusion.

Plasma from monkeys with or without added carotene was subjected to heparin-manganese chloride precipitation (12); and cholesterol, phospholipid, triacylglycerol, and protein concentrations were determined on the HDL supernatant. In addition, agarose gel electrophoresis determined whether β-carotene had interfered with heparin-manganese precipitation of positively charged lipoproteins (apo B and arginine-rich apoproteins) (13) and subsequent recovery of HDL in the supernatant. Comparison of the two treatments was evaluated by percent difference calculations and by paired "t" test.

In order to determine if β-carotene affected the electrophoretic separation and Oil-Red-0 staining of plasma lipoproteins, agarose gel electrophoresis was performed on monkey plasma with or without β-carotene, and Rf values and staining intensity were compared by scanning densitometry using a Photovolt Model 52-C densitometer supplied with a Varicord Model 42-B recorder (Photovolt, New York, NY) and a Model 282 Digital Integrator (Buxco, New Haven, CT).

The influence of β-carotene on HDL particle size was assessed by applying the HDL samples

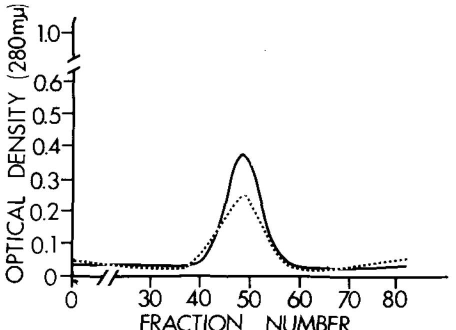

FIG. 1. Separation of carotene-treated (·····) and untreated (———) monkey HDL by gel filtration chromatography indicates that particle size was unaltered by carotene. Treated and untreated samples containing 4.7 and 5.2 mg of protein, respectively, were applied to a column containing Sepharose CL-6B. Absorbance was measured at 280 mμ.

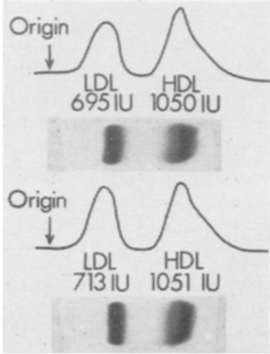

FIG. 2. Agarose gel electrophoretograms and corresponding densitometric scans of LDL and HDL from monkey plasma with (upper) or without (lower) β-carotene added reveal essentially identical patterns. Twenty μl of plasma were applied to each strip. Peak areas were quantitated as integration units (IU). Rates of migration (Rf) for treated and untreated samples were identical, i.e., LDL = 0.35 and HDL = 0.77.

harvested by density gradient ultracentrifugation to a 1.5 x 90 cm column packed with 6% (w/v) Sepharose CL-6B (Pharmacia Fine Chemicals A B, Uppsala, Sweden) at 8 C. The buffer contained 0.15 M NaCl, 0.01% EDTA-Na_2, and 0.035% NaN_3 (pH 7.0) which eluted at a flow rate of 12 ml/hr at a hydrostatic pressure of 70 cm. Two and one half ml fractions were collected with a Gilson Model FC-80K Microfractionator (Gilson Medical Electronics, Inc., Middleton, WI). HDL elution was monitored at 280 mμ with an Altex Model 150 UV monitor (Altex Scientific Inc., Berkeley, CA).

The immunologic properties of plasma lipoproteins in cebus monkeys have not been investigated, and antibodies against cebus apoproteins have not been produced. Thus, the effect of β-carotene addition on the immunologic characteristics of HDL could not be determined. However, since human apoLDL antiserum cross reacted with cebus apoLDL, the effect of added β-carotene on this immunologic parameter was determined by Ouchterlony double immunodiffusion (14) against the human antiserum.

RESULTS

Upon density gradient ultracentrifugation of plasma with or without added β-carotene, the VLDL migrated to the top while the lactescent LDL was easily discernible in the middle of the centrifuge tube. High density lipoproteins from monkey plasma with added carotene were delineated as a single yellow-orange band in the lower portion of the centrifuge tube in a pattern similar to that observed for human HDL separated from plasma containing a normal dietary component of carotene (1). As in human plasma, pigmentation from carotene was observed in the infranatant fraction (d > 1.210) of monkey plasma. This may result from the binding of other serum proteins, such as globulins, to β-carotene (2).

The VLDL, LDL, and HDL of both samples separated by ultracentrifugation migrated as single bands upon agarose gel electrophoresis. Furthermore, β-carotene had no effect on the measurement of total plasma constituents as the plasma cholesterol (264 ± 2 vs. 254 ± 2), phospholipid (486 ± 4 vs. 468 ± 4), triacylglycerol (158 ± 10 vs. 150 ± 8), and protein (6981 ± 67 vs. 7016 ± 56) values expressed as mg/dl did not differ by more than 5%. Differences in VLDL, LDL, and HDL total lipoprotein cholesterol and protein content of treated and untreated samples (Table I) were negligible, being within the limit of a 10% experimental error. In a similar manner, differences were observed neither in HDL phospholipid (164 ± 1 vs. 156 ± 2 mg/dl) and triacylglycerol (21 ± 1 vs. 22 ± 1 mg/dl) of treated and untreated samples, respectively, nor in VLDL and LDL phospholipid and triacylglycerol content. Assessment of differences between treated and untreated samples in Tables I and II by paired "t" test also revealed no significant differences.

The nearly identical elution profiles for treated and untreated HDL seen in Figure 1 suggest that β-carotene did not affect HDL particle size as measured by gel filtration chromatography. Similar Rf values and staining intensities (Fig. 2) in agarose gel electrophoresis indicated that β-carotene neither influenced lipoprotein electrophoretic mobility nor interfered

with Oil-Red-0 staining. Immunodiffusion of cebus apoLDL against human antiserum revealed a single precipitin band for both treated and untreated samples.

Table II compares the chemical composition of paired HDL samples with or without carotene isolated by heparin-manganese chloride precipitation. No significant differences were noted in either the absolute (mg/dl) or relative composition of the HDL constituents prepared by this technique. In addition, agarose electrophoresis of the supernatant revealed only alpha migrating bands, suggesting that β-carotene did not interfere with the precipitation of positively charged apoproteins, largely associated with VLDL and LDL, and subsequent recovery of HDL.

Subsequent experiments have indicated that β-carotene addition was equally effective for plasma from monkeys fed semipurified diets containing either unsaturated or saturated fat without cholesterol with the pigmented HDL band migrating to a position in the centrifuge tube comparable to that described by Redgrave et al. (1) for human HDL.

DISCUSSION

Addition of the plant pigment β-carotene to nonhuman primate plasma samples prior to ultracentrifugation appears to be a unique way of visualizing HDL. This visualization decreased the likelihood of contamination of the lower density lipoproteins with HDL during sample collection and should, therefore, be useful in isolating imperceptible HDL bands in monkeys fed semipurified diets without carotene. Delineation of HDL as a discrete band by this procedure also eliminates the necessity of aspirating large quantities of background density solution in order to effect the complete recovery of HDL. Similarities between treated and untreated HDL in elution profiles using gel filtration chromatography (Fig. 1), in electrophoretic mobilities and lipid staining intensities (Fig. 2), in ultracentrifugal migration and the unaltered character of lipoproteins separated by heparin-manganese chloride precipitation, all indicate that the physical integrity of HDL was not affected by β-carotene addition.

The addition of β-carotene at the level of 5 mg/ml plasma did not interfere with assays of cholesterol, triacylglycerol, phospholipid, or protein. On the other hand, concentrations in excess of 12 mg/ml of plasma did enhance the absorbance readings in the cholesterol assay (unpublished data) in agreement with the findings of Chen and Kane (3) who demonstrated an increased absorbance between 350 and 550 mμ for LDL in individuals ingesting diets enriched with β-carotene (60 mg/day for 3 weeks).

The exact nature of the β-carotene interaction with the lipid and protein moieties of the lipoprotein complex is not known. Carotenoid pigments have been found in association with protein of liver cell organelles (15) and with the lipid moieties of the lipoproteins as well (3,4). The selective binding of β-carotene to HDL in vitro reported in this communication may represent simple adsorption of the plant pigment by the lipoprotein complex. One could predict, therefore, that the extent of adsorption might vary with alterations in relative or absolute concentrations of HDL components, a hypothesis supported by the recent observations of Bjornson et al. (4) which demonstrated good correlations between the distribution of β-carotene and HDL total cholesterol, total lipid, and total lipid plus protein.

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Isotopic Labeling of Phosphatidylcholine in the Choline Moiety

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ABSTRACT

Two simple methods for the synthesis of phosphatidylcholine (lecithin) isotopically labeled in the methyl group of the choline moiety are described. Phosphatidylcholine is converted to phosphatidylethanolamine by enzymic transphosphatidylation, and this is methylated using either methyl iodide or diazomethane to give a product isotopically enriched in all three choline methyl groups.

The availability of radioactively labeled phospholipids is essential for many biochemical studies of biological membranes and lipid metabolism (1,2), and ^{13}C- and ^{2}H-labeled phospholipids are becoming increasingly important for biophysical studies of membranes (3,4). The phospholipid most used is phosphatidylcholine with a wide range of defined alkyl chains, and we describe two methods for labeling this lipid in the choline moiety. Stoffel et al. (5) have proposed a procedure that involves conversion of phosphatidylcholine to phosphatidyl-*N,N*-dimethylethanolamine using sodium benzene thiolate followed by methylation with labeled methyl iodide. Only one methyl group out of three is accessible to labeling by this method. Methylation of phosphatidylethanolamine was used by Stockton et al. (6); however, in order to achieve high yield, they needed to react with a large excess of labeled methyl iodide for 14 days under alkaline conditions which is likely to cause some hydrolysis of the alkyl chains. The procedures we report here also involve a methylation of phosphatidylethanolamine, but are rapid, mild, and efficient. We first describe the enzymatic preparation of phosphatidylethanolamine which we have found is most conveniently made from phosphatidylcholine utilizing the transphosphatidylation activity of phospholipase D (7).

Carbon or hydrogen isotopes are introduced from labeled methyl iodide by methylation using stoichiometric quantities of starting materials under mild conditions. Hydrogen isotopes are readily and more cheaply available as ^{2}H or ^{3}H water, and so we describe a second method allowing introduction of hydrogen isotopes with diazomethane in the presence of isotopically enriched water. These methods offer the advantage of maximum isotopic enrichment since all three methyl groups of choline head-group will be labeled. This is a particularly important point when preparing lipids for use in ^{13}C- and ^{2}H-nuclear magnetic resonance (NMR) where sensitivity is often the limiting factor.

EXPERIMENTAL PROCEDURES

Phosphatidylcholine

Pure synthetic 1,2-dimyristoyl *sn*-glycero-3-phosphocholine, 1,2-dipalmitoyl *sn*-glycero-3-phosphocholine, and 1,2-dioleoyl *sn*-glycero-3-phosphocholine were prepared by the method of Robles and Van den Berg (8).

Phospholipase D

Partially purified phospholipase D (phosphatidylcholine phosphatidohydrolase EC 3.1.4.4) from cabbage was prepared by a modification of Yang's purification (9). Chopped inner cabbage leaves are homogenized in a blender with ca. 30 ml distilled water per 100 g cabbage, and the homogenate filtered by pressing through four layers of muslin and adjusted to pH 6.5 if necessary. Aliquots of 300 ml are heated rapidly in a 1-liter conical flask to 55 C in a boiling water bath, maintained for 5 min in a 55 C water bath, and then cooled rapidly in an ice bath. The precipitate is centrifuged (10 min, 10,000 rpm, MSE 18 6 x 300 rotor), and to the supernatant are added two volumes of acetone precooled to -15 C. The precipitate is centrifuged (30 min, 2000 rpm, MSE Major 4 x 1500 rotor) and resuspended in 2 mM potassium phosphate pH 6.8 and dialyzed for 3 hr into 1 liter 2 mM potassium phosphate pH 6.8. The dialysate is clarified by centrifugation (15 min, 20,000 rpm, Sorvall RC-5 8 x 50' rotor) and freeze-dried. In a typical preparation, about 6 kg Dutch White cabbage yielded 5.2 g enzyme.

Phospholipase D of similar activity is available commercially from Boehringer, Mannheim, Germany, and Sigma, St. Louis, MO.

Transphosphatidylation

Phospholipase D (50 mg) is dissolved in 100 ml 10% (v/v) ethanolamine, 40 mM calcium chloride adjusted to pH 5.6 with acetic acid. Dioleoyl glycerophosphocholine (500 Mg) dissolved in 100 ml diethyl ether, previously washed three times with equal volumes of water, is added to the aqueous phase and left stirring overnight. Dimyristoyl glycerophospho-

choline (500 mg) or dipalmitoyl glycerophosphocholine are dissolved in water-washed chloroform and stirred with the aqueous solution as above. (Water washing of solvents is essential to saturate the solvent with water and to remove any ethanol present which competes strongly with ethanolamine in the transphosphatidylation reaction.) The reaction is followed using thin layer chromatography (TLC) to detect the disappearance of phosphatidylcholine and appearance of phosphatidylethanolamine in the organic phase. On completion, the reaction is stopped by acidification to pH 1-2, and the lipid extracted with chloroform and dried. The crude product can be used directly for the methylation reactions since the major contaminant, phosphatidic acid, does not interfere with them. However, any residual phosphatidylcholine will reduce the degree of labeling in the final product, and the phosphatidylethanolamine can be purified by chromatography on silica gel is desired. The yield of phosphatidylethanolamine is typically 50-60% after purification.

Methylation with Methyl Iodide

Phosphatidylethanolamine (300 mg) is dissolved in 10 ml tetrahydrofuran and 2 g dry silver carbonate on Celite (Fetizon's reagent) (10) added in 10 ml acetonitrile. Labeled methyl iodide (0.2 g) is added, and the flask is stoppered and stirred for 3 hr in the dark at 35-40 C. The suspension is filtered through Celite, and the filter washed with a diethyl ether-methanol mixture. The filtrate is concentrated by rotary evaporation and purified by silica gel chromatography. Dipalmitoyl glycerophosphoethanolamine (300 mg) gave [N-methyl-^{13}C]-dipalmitoyl glycerophosphocholine (240 mg, 75% yield) which was identical to authentic material by TLC. As shown by mass spectrometry (MS) and ^{13}C-NMR, the product was isotopically enriched solely in the choline moiety and to the expected degree.

Methylation with Diazomethane

Phosphatidylethanolamine (50 mg) is dissolved in 1 ml dioxane, and 50 μl ^{3}H water is added. Diazomethane in diethyl ether (11) is added dropwise to maintain a yellow color in the solution over 1 hr. Careful exclusion of light is essential for methylation of unsaturated lipids by this method. The reaction is terminated by evapoaration of excess reagent, and the labeled phosphatidyl-choline purified by silica gel chromatography. Dipalmitoyl glycerophosphoethanolamine (50 mg) gave pure dipalmitoyl glycerophospho[N-methyl-^{3}H] choline (17.9 mg, 34%) pure, identical to authentic material by TLC and MS. Using tritiated water at about 3 Ci/ml (The Radiochemical Centre, Amersham), the specific activity was 5.0 x 10^{10} dpm/mmole, and 95% of the radioactivity ran with marker phosphatidylcholine in TLC. At least 98% of the radioactivity could be removed from the lipid fraction by digestion with phospholipiase D. Similar results were obtained for dimyristoyl glycerophosphocholine and dioleoyl glycerophosphocholine. The efficiency of hydrogen isotope exchange of diazomethane under these conditions is low; however, this is amenable to improvement (13).

Purification and Analysis

Lipids were purified by chromatography on silica gel. Oven-dried silica AR (Mallinckrodt CC-4) at 100 mg/mg lipid is poured in a column in chloroform, and crude lipid loaded in chloroform. Lipids are eluted with chloroform containing increasing proportion of methanol, and the fractions are followed by TLC. Phosphatidic acid elutes at 2-5% methanol, phosphatidylethanolamine at 15-20%, and phosphatidylcholine at 70-80%.

TLC on silica gel plates was carried out using three solvent systems: (a) chloroform-methanol-water-ammonia solution, 65:30:3:1; (b) chloroform-methanol-acetic-acid-water, 75:25:8:3; (c) chloroform-methanol-water, 65:30:4. Fractions were visualized with iodine vapor or Dittmer's spray for phosphorus (12).

ACKNOWLEDGMENTS

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COMMUNICATIONS

Triglyceride Synthesis from Dihydroxyacetone Phosphate and Palmitate by Microsomes from Mammary Glands of Lactating Mice

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ABSTRACT

Both di- and triglycerides were synthesized when microsomes isolated from mammary glands of lactating mice were incubated with dihydroxyacetone phosphate (DHAP), (1-^{14}C)palmitate, ATP, CoASH, GSH, KF, $MgCl_2$, and NADPH. When NADH replaced NADPH, glyceride synthesis was very low. In the absence of either NADPH or NADH, DHAP was acylated to palmityl-DHAP. Since microsomes do not have glycerol 3-phosphate NAD:oxidoreductase activity, we inferred that glycerol 3-phosphate (GP) is not an intermediate in triglyceride biosynthesis from DHAP. This reductase, present in the cytosol, was active only with NADH. With the same concentration of either GP or DHAP, microsomes yielded essentially similar amounts of di- and triglycerides. Mitochondria, while capable of synthesizing palmityl-DHAP, did not produce di- and triglycerides.

INTRODUCTION

Recently, we reported that the presence of glucose in the incubation media caused a manyfold increase in the incorporation of not only acetate, but also decanoate and palmitate into triglycerides by slices of mammary glands from lactating mice (1). Decanoate and palmitate were mainly incorporated into the triglycerides as intact molecules, while acetate was first converted to medium and long chain fatty acids. We suggested that the oxidation of glucose via the pentose phosphate pathway (1) by the mammary cell would yield NADPH which is necessary for fatty acid synthesis from acetate. In order to explain this stimulating effect of glucose, we theorized that NADPH may also be involved in triglyceride synthesis if this gland utilizes dihydroxyacetone phosphate (DHAP) as a glyceride-glycerol precursor. NADPH is the specific cofactor required for the reduction of acyl-DHAP to yield lysophosphatidic acid (2) which is subsequently acylated to phosphatidic acid and converted to triglyceride.

Mammary glands of lactating animals synthesize large amounts of triglycerides. Although the gland can utilize glycerol 3-phosphate (GP) as a precursor, its capacity for the conversion of DHAP to glyceride has not been determined (3). The results presented in this paper show that DHAP can serve as a direct precursor of triglycerides in mammary glands of lactating mice. A preliminary report of this study has already appeared (4).

MATERIALS AND METHODS

Mammary glands were removed from C3H mice which were actively lactating for 15-17 days. The glands were sliced, washed, and homogenized in 0.25 M sucrose. From the homogenate, the mitochondria, microsomes, and cytosol were isolated by the centrifugation prodcedures described previously (5,6). Mitochondria and microsomes were washed free of cytosol by resuspension in 0.25 M sucrose and separated by centrifugation. These washed fractions prepared freshly were dispersed in 0.25 M sucrose (10 mg protein/ml), and used as enzyme sources for glyceride synthesis.

The contents of the reaction mixture to study the conversion of dihydroxyacetone phosphate to glycerides were essentially similar to those used by Hajra (7) with some modifications as given. This medium was also used to measure glyceride synthesis from glycerol 3-phosphate except that reduced pyridine nucleotides were omitted as they are not required in the reaction. Some of the changes were made to promote glyceride synthesis. These were the inclusion of (a) albumin, which results in a several-fold increase in the acylation of either glycerol 3-phosphate (8), or dihydroxyacetone phosphate (9); (b) a smaller

TABLE I

Conversion of Dihydroxyacetone Phosphate to Glycerides by Enzymes from Mammary Glands of Lactating Mice[a]

Enzyme source	Addition	nmoles (1-^{14}C)palmitate incorporated into: Acyl-CHAP[b]	PA	PC	PE	MG	DG	TG
Mitochondria	None	18	0	12	5	0	2	0
	NADPH	2	16	9	4	1	12	6
	NADH	11	8	11	4	1	4	0
Mitochondria-free Supernatant	None	7	42	27	6	2	19	96
	NADPH	5	34	22	4	1	47	382
	NADH	3	16	18	7	2	38	396
Microsomes	None	37	4	30	7	1	2	7
	NADPH	3	35	24	5	3	49	141
	NADH	14	12	28	8	2	8	36

[a]Reaction medium contained dihydroxyacetone phosphate (5 mM), potassium salt of (1-^{14}C) palmitic acid (0.33 mM), adenosine 5′ triphosphate (3.3 mM), reduced coenzyme A (0.33 mM), reduced glutathione (10 mM), $MgCl_2$ (6.6 mM), KF (6.6 mM), potassium phosphate buffer (pH 7.4, 133 mM), crystalline bovine serum albumin (3 mg), and either washed mitochondria (2 mg), mitochondria-free supernatant (15 mg), or washed microsomes (2 mg) in a total volume of 1.5 ml. Reduced nicotinamide adenine dinucleotide phosphate (NADPH) (2 mM) or reduced nicotinamide adenine dinucleotide (NADH) (2 mM) were included in the medium as indicated. Reactions were carried out in air at 37 C in a metabolic shaker for 30 min and stopped by the addition of chloroform-methanol (1:2, v/v). Lipids were extracted (12), separated by thin layer chromatography (13-15), and quantitated by their content of (1-^{14}C) palmitate.

[b]Abbreviations: acyl-dihydroxyacetone phosphate (acyl-DHAP), phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), monoglyceride (MG), diglyceride (DG), and triglyceride (TG).

amount of (1-^{14}C)palmitic acid, since higher concentrations of the acid inhibits acylation of GP or DHAP (8,10); and (c) a relatively large concentration of potassium phosphate buffer (pH 7.4) since this condition favors neutral glyceride synthesis (11).

Reactions were stopped by the addition of chloroform-methanol (1:2, v/v) and 6N HCl to the reaction mixtures, and the lipids were extracted as described by Hajra et al. (12). Various thin layer chromatographic (TLC) procedures were employed for the separation of different lipid classes. Neutral glycerides were separated from phosphoglycerides by using the TLC system described by Brown and Johnston (13); and various phospholipids such as phosphatidic acid, lysophosphatidylcholine, sphingomyelin, phosphatidylcholine, and phosphatidylethanolamine were separated, using the conditions described by Johnston et al. (14). Separation of acyl-DHAP from phospholipids was carried out on commercial silica gel plates as described by Hajra (15).

The amount of GP and DHAP added to the incubation medium was quantitated by assay with crystalline glycerol 3-phosphate:NAD oxidoreductase (16). Glycerol 3-phosphate: NAD oxidoreductase activity was estimated as described by Beisenherz et al (17). Protein concentrations of mitochondria and microsomes were determined by the method of Lowry et al. (18) with bovine serum albumin as standard. The capacity of mammary gland microsomes to acylate 2-monopalmitin was ascertained by incubation in the presence of Tween 80, [1-^{14}C]palmitate, ATP, CoASH, and GSH as described previously (19).

[1-^{14}C]palmitic acid was purchased from New England Nuclear (Boston, MA) and purified (99+%) by TLC before use. Unlabeled palmitic acid was from Applied Science Labs (State College, PA), and phosphatidic acid was from Pierce Chemical Co. (Rockford, IL). Other authentic phospholipid standards were from Supelco, Inc. (Bellefonte, PA). Palmityl-DHAP used as a standard was a generous gift from Dr. A.K. Hajra.

The dimethylketal derivative of DHAP, NADH, ATP, CoASH, GSH, and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO) and the Ca salt of L-glycerol 3-phosphate, crystalline glycerol 3-phosphate NAD:oxidoreductase and NADPH from Calbiochem (San Diego, CA). The dihydroxyacetone phosphate and L-glycerol 3-phosphate were prepared using Dowex 50-x4, H+ and converted to their potassium salts by the addition of $KHCO_3$ before use.

RESULTS AND DISCUSSION

It is now known that numerous tissues such

TABLE II

Effect of Different Glyceride Precursor and Microsomal Protein Concentrations on Di- and Triglyceride Synthesis[a]

Concentration of glyceride precursor, mM	Microsomal protein, mg	nmoles (1-^{14}C)palmitate incorporated with: Glycerol 3-phosphate		Dihydroxyacetone phosphate	
		DG[b]	TG	DG	TG
0	2	6	3	6	3
1	2	14	24	12	27
2	2	18	45	14	52
4	2	28	103	34	98
5	2	32	136	37	128
5	0.3	8	16	6	22
5	0.6	11	38	15	54
5	1.2	20	86	26	97
5	1.8	42	118	35	132

[a]Incubations were carried out as described in Table I with varying amounts of glyceride precursor and microsomal protein as indicated. NADPH (2 mM) was added to those flasks which contained DHAP and omitted from those with GP.

[b]See Table I for abbreviations.

as liver, brain, kidney, heart, adipose tissue, spleen, testis, lung (9), and intestinal mucosa (20), as well as several neoplastic tissues (21), contain enzymes which utilize dihydroxyacetone phosphate as a direct acyl acceptor for glyceride synthesis. Mammary glands of lactating mice, which actively synthesize triglycerides for milk production, also possess these enzymes for the acylation of dihydroxyacetone phosphate and produce neutral glycerides (Table I). Our results with the enzymes from mammary glands of lactating mice (Table I) are similar to those observed with adipose tissue and brain (9) since, in these tissues, the microsomal fraction is significantly more active than the mitochondrial fraction for acylation of DHAP.

Although mammary gland mitochondria are capable of synthesizing acyl-DHAP and producing phosphatidic acid in the presence of NADH or NADPH, they synthesize only trace amounts of di- and triglycerides (Table I). On the other hand, from DHAP, microsomes yield mainly di- and triglycerides in the presence of reduced pyridine nucleotides. With either organelle, NADPH is considerably more active than NADH for neutral glyceride synthesis.

With mitochondria-free supernatant obtained from mammary glands as enzyme source (Table I), both NADPH and NADH were equally active for di- and triglyceride synthesis from DHAP. In this system, certainly NADPH and perhaps to a small degree NADH could have acted as cofactors for the reduction of acyl-DHAP. In this enzyme preparation, the major role of NADH would be in the conversion of DHAP to GP, the glyceride precursor. Such a conclusion is supported by our studies of the distribution and nucleotide specificity of glycerol 3-phosphate NAD:oxidoreductase in mammary glands of lactating mice. The reductase is mostly present in the cytosol and is active only in the presence of DHAP and NADH. Washed microsomes contain no detectable reductase activity.

Rat liver microsomes prepared in presence of EDTA are able to produce neutral lipids from DHAP in amounts 3-4 fold greater than when they are prepared in the absence of EDTA (22). It has been reported that hepatic phosphatidate phosphohydrolase activity is inhibited by KF (23), and thus the presence of fluoride in the reaction mixture has been considered to inhibit neutral lipid synthesis (22). The extensive synthesis of neutral glycerides observed in our experiments with mammary gland microsomes prepared in the absence of EDTA and with KF in the reaction mixture suggest that the conclusions derived with liver enzymes do not apply in all systems.

When we used 2-monopalmitin as sole fatty acid acceptor and the microsomal protein concentration given in Table I, not more than 3 nmoles of palmitate were incorporated into diglycerides and 5 nmoles into triglycerides. On the other hand, with DHAP or GP as acceptor, extensive synthesis of these glycerides occurred (Table I and II). Thus, very little evidence for a monoglyceride pathway was obtained in our experiments with mammary gland microsomes obtained from lactating mice.

In our experiments with varying amounts of microsomal protein from mammary glands or with varying amounts of GP or DHAP (Table

II), the two precursors produced the same amounts of di- and triglycerides. Thus, the microsomal enzymes of mammary glands of lactating mice are active for glyceride synthesis from either GP or DHAP to a similar degree when provided with similar substrate concentrations. However, these glands contain about 15 times more GP than DHAP (24). It is not known whether all the intracellular GP is available to the microsomal enzymes for glyceride synthesis. In liver, in spite of a GP content which is 12-fold greater than of DHAP, glyceride synthesis prefers either DHAP or GP which is nascently produced from DHAP (25). The results presented here demonstrate clearly that the microsomes of mammary glands from lactating mice are able to synthesize triglyceride from DHAP. However, a definite conclusion regarding the importance of DHAP pathway in this gland cannot be made from the present data.

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Comparative Intestinal and Colonic Absorption of [4-^{14}C] Cholesterol in the Rat

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ABSTRACT

[4-^{14}C] Cholesterol was administered as an aqueous emulsion with triolein and dry non-fat milk either directly into the upper duodenum or into the ileocaecal junction of lymph duct cannulated rats. Lymph flow rates were similar in the two groups of animals. Whereas ca. 53% of the administered tracer dose of cholesterol was absorbed when introduced into the upper small intestine, only 0.06% appeared in lymph when administered into the caecum. Furthermore, less than 0.01% of the administered isotope was detected in urine and blood. The data demonstrate that the large intestine does not contribute significantly to the absorption of exogenous cholesterol in the rat.

INTRODUCTION

It is generally accepted that a major part of cholesterol absorption occurs in the small intestine (1,2), and that colonic absorption of dietary cholesterol is insignificant (3,4). This latter conclusion has been based on histological evidence and on analysis of lymphatic cholesterol levels.

Yamakawa et al. (3) reported that the introduction of cholesterol directly into the large intestine resulted in an increase in lymph lipid levels but not in the levels of lymph cholesterol per se. It was concluded that despite the unusual increase in lymph lipids, the colon did not have a significant role in cholesterol absorption. Byers et al. (4) reached the same conclusions using cholesterol measurements in lymph from colectomized rats. However, lymph volumes in these animals were less than half of those in control rats, and the results were highly variable.

Recent studies suggest that various dietary fibers and ionic resins may bind bile acids (5-7) and cholesterol (8), thereby reducing their availability for absorption in the small intestine. This may result in higher concentrations of sterols in the large bowel and increased levels of fecal excretion.

Because of the higher levels of colonic sterols resulting from ingestion of certain dietary fibers, the possibility of colonic sterol absorption has been reinvestigated by comparing the absorption of [4-^{14}C] cholesterol introduced directly into the duodenum or the caecum of lymph duct cannulated rats.

MATERIALS AND METHODS

Male albino rats of the Carworth Farms, (Wilmington, MA) Wistar strain, were maintained on lab chow and water ad libitum until use. Cannulation of the left thoracic lymphatic channel was performed in animals under sodium pentobarbital anesthesia as described previously (9). Subsequently, an indwelling catheter was placed in the duodenum caudad to the stomach or at the ileocaecal junction. These catheters were used for direct administration of the radioactive cholesterol.

After operation, the animals were placed in restraining cages and allowed 5% glucose-physiological saline, but no food, ad libitum. At 9:00 a.m. the following day, each animal was given, via the indwelling catheter, 0.8 ml of an aqueous emulsion containing 70 μg triolein (Sigma Chemical Co., St. Louis, MO), [4-^{14}C] cholesterol (Amersham/Searle Corp., Arlington Heights, IL), and 6.8% non-fat dry milk (10). This was homogenized prior to administration. Four animals with the duodenal catheter received a tracer dose of ca. 2 μCi of the labeled cholesterol, and four animals with the ileocaecal catheter received 5 μCi [4-^{14}C] cholesterol.

Lymph was collected at 4-hr intervals during the first 12 hr after administration of the test emulsion and as a single 12-24 hr collection thereafter. Urine was collected during the entire 24 hr test period. At the end of the study, animals were sacrificed, and blood was collected by cardiac puncture. One-ml aliquots of each sample were extracted in 20 volumes of chloroform-methanol (2:1, v/v) according to Folch et al. (11). The chloroform extract was evaporated to dryness under nitrogen, and lipids were re-extracted into hexane. Aliquots of the hexane extract were placed in scintillation vials and evaporated under nitrogen. After addition of 10-ml scintillation mixture (LSC Complete, Yorktown Research, New Hyde Park, NY radioactivity was determined in a Beckman LS-250 liquid scintillation spectrometer.

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TABLE I

Lymph Flow and Recovery of [4-^{14}C]Cholesterol after Duodenal or Ileocaecal Administration

Group	Animal weight[a] (g)	Lymph volume (ml/24 hr)	Lymph flow rate (ml/hr)	[4-^{14}C]Cholesterol recovered, 24 hr, % of dose[b]		
				Lymph[c] (%)	Urine[c] (%)	Blood[d] (dpm/ml)
Duodenal administration (4 rats)	240 ±32	128 ±28	5.3 ±1.2	53.1 ±5.6	nd[e]	nd
Ileocaecal administration	270 ±6	126 ±30	5.3 ±1.3	0.06 ±0.02	0.006 ±0.005	391 (range 3-1164)

[a]Values are means ± standard error.

[b]Administered in 0.8 ml aqueous emulsion containing 0.07 mg triolein and 6.8% non-fat dry milk.

[c]% Of administered dose recovered per 24 hr collection.

[d]dpm/ml at the end of experiment.

[e]Not detectable.

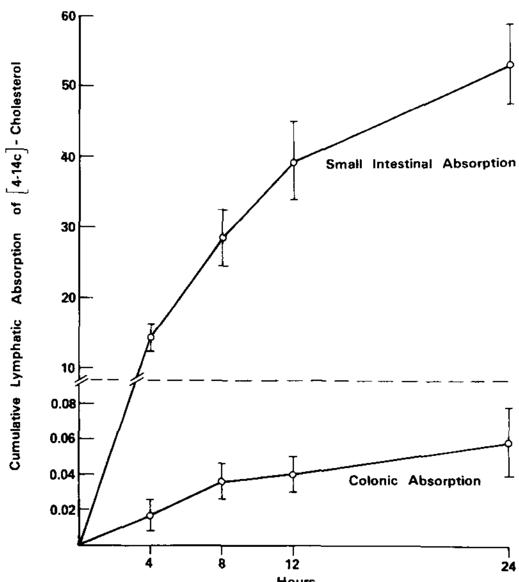

FIG. 1. Cumulative appearance of radioactivity (% of administered dose) in thoracic duct lymph of rats given an aqueous emulsion of [4-^{14}C]cholesterol directly into the duodenum or the ileocaecal junction.

RESULTS AND DISCUSSION

As shown in Table I, animal weights in the two groups were statistically the same at the time of study. Total lymph volumes over the 24-hr collection period were also identical. Lymph volumes during each of the 4-hr collection periods were not statistically different either with the same group of rats or between groups (range, 15.0 to 26.6 ml per 4-hr period). Calculation of the average flow rate per hour (Table I) showed that the hourly lymph flow in the two groups was identical at 4.3 ml/hr. Thus, the major criticism of reduced lymph flow in colectomized rats (4) was circumvented in the present study.

The time course for the cumulative lymphatic absorption of [4-^{14}C]cholesterol administered either intraduodenally or directly into the ileocaecal junction is shown in Figure 1. The smooth absorption curve for cholesterol administered via the indwelling duodenal catheter is typical of earlier data obtained using these procedures (2). Under these conditions, about 53% of the administered tracer dose of cholesterol was recovered in thoracic duct lymph during the 24-hr experimental period. The peak of absorption occurred between the 4th to 8th hr of lymph collection, which again is characteristic of this animal preparation (2). The appearance of label in thoracic duct lymph following administration of [4-^{14}C]cholesterol into the lower bowel appeared to be uniform throughout the collection period. This type of "absorption" curve is more typical of the slow isotope exchange which occurs between intestinal mucosa and lymph 96-120 hr after the administration of labeled sterol to lymph-duct cannulated rats (2). From the recovery data summarized in Table I, the lymphatic "absorption" of cholesterol from the lower bowel was only 0.06% of the administered dose of 5 μCi. This represents approximately one-nine hundredth of the absorption (53.1%) observed after adminstration of the same mixture into the upper small intestine. Furthermore, negligible levels of isotope were found in the 24-hr urine collection or in the blood sample taken at 24 hr after cholesterol administration. This latter finding was expected since it had already been shown (12) that administration of 50 μCi of

[4-^{14}C] cholesterol to lymph duct and portal vein cannulated rats did not result in significant radioactivity in either portal or systemic blood.

In the present study, the tracer dose of cholesterol was administered in an aqueous emulsion containing only 70 μgm triolein. Under these conditions, the physiological conditions in the upper small intestine were appropriate for micellization and subsequent absorption of the administered sterol. Providing a similar emulsion at the ileocaecal junction might not be appropriate since the extent of bacterial alterations, micellization, etc., cannot be totally comparable to that observed after oral or duodenal administration. Thus, it is possible that the conditions for cholesterol absorption by the colon are not entirely optimal. Nevertheless, the present comparative data suggest that the large intestine of the rat does not contribute significantly to exogenous sterol absorption either via lymph or directly into blood.

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ERRATUM

In "Analysis of Autoxidized Fats by Gas Chromatography-Mass Spectrometry: I. Methyl Oleate" [*Lipids* 12:901 (1977)], Figures 5 and 6 were printed incorrectly. The figures below have been correctly positioned.

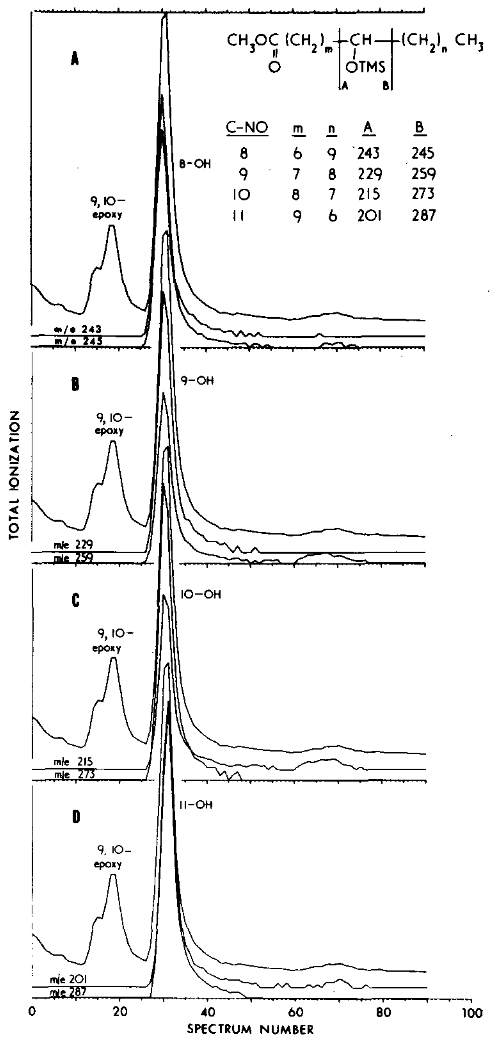

FIG. 5. Mass chromatography of hydrogenated-autoxidized methyl oleate (PV 1370) for the identification of: A. Methyl 8-OH octadecanoate (OTMS); B. 9-OH octadecanoate (OTMS); C. 10-OH octadecanoate (OTMS); D. 11-OH octadecanoate (OTMS).

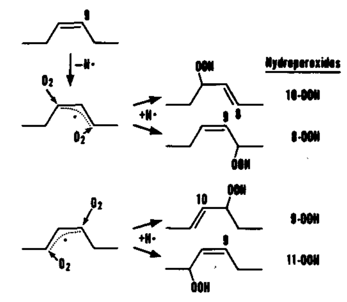

FIG. 6. Mechanism of oleate autoxidation.

Metabolism of Linoleate versus Linoelaidate in the Laying Hen[1]

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ABSTRACT

The metabolic fate in the laying hen of linoelaidic acid, the *trans,trans*-geometric isomer of linoleic acid, was compared to that of the naturally occurring *cis,cis* linoleate. In two experiments, mixtures of radioisotope-labeled linoleate and linoelaidate were orally administered to a set of three laying hens. A third mixture consisting of linoleate-^{3}H and linoleate-^{14}C was fed to three hens to measure biological isotope effects. Isotopic ratios ($^{3}H/^{14}C$) of the neutral lipid and phospholipid fractions isolated from egg yolks and of the octadecadienoic acids from these fractions were compared to those of the administered mixtures. The $^{3}H/^{14}C$ ratios indicate that linoelaidic acid and linoleic acid are equally incorporated into egg yolk neutral lipids and phospholipids. Arachidonic acid was found exclusively in the phospholipid fraction and was radiolabeled with the isotope from the *cis,cis* octadecadienoate isomer only. Further detailed analysis of individual neutral lipid components indicated: (a) discrimination against the *trans,trans* isomer in cholesteryl esters and (b) no discrimination against either isomer in triacylglycerols.

INTRODUCTION

Catalytic hydrogenation of polyunsaturated oils for formulation of commercial products such as shortenings, margarines, salad oils, and cooking oils is known to produce positional and geometrical fatty acid isomers (1-3). The *trans* octadecenoate (18:1) content of processed soybean oil ranges from 4.8 to 10.9% of the total fatty acids (2), and the *trans* octadecadienoate (18:2) isomers make up 3 to 35% of the total 18:2 content of margarines (3).

The metabolic fate of isomeric fatty acids has been the subject of scientific inquiry for some time, and these efforts are continuing on an accelerated scale. Isomeric fatty acids in hydrogenated oils are incorporated into the tissue of rats (4,5), swine (6), and humans (7). When included in the diet, specific fatty acid isomers have been found in rat liver and depot fat (8), rabbit serum and adipose tissue (9), egg yolk lipids (10,11), and human blood plasma (12). Other studies have demonstrated that isomeric fatty acids are incorporated into neutral lipids and phospholipids of rat liver mitochondria (13) and into *Escherichia coli* membranes (14).

In vitro and in vivo experiments have shown that incorporation and utilization of positional and geometric 18:1 isomers depend upon the position of the double bond in the fatty acid acyl chain. Triacylglycerols from rat liver mitochondria displayed preferential incorporation of oleic acid over other 18:1 positional isomers having the double bond from the 3- to the 13-position (13). Uptake of these isomers into rat liver mitochondria triacylglycerols decreased as the double bond was located away from the 9-position in the octadecenoic acid. In phospholipids, however, preferential utilization of the fatty acid isomer increased as the double bond was placed toward the ends of the fatty acid. Vandenhoff et al. (15) showed that only certain *trans*-18:1 isomers supported growth of bacterial and yeast mutants which were unable to synthesize unsaturated fatty acids. In addition, normal yeast growth with oleic acid was inhibited to varying extents by *trans*-18:1 isomer supplements.

Isomeric fatty acids in hydrogenated and isomerized oils have been correlated with reduced growth rate and enhancement of essential fatty acid deficiencies in rats (16), increased arterial lesions in the aortic arch of rabbits (17), increased serum lipid levels in man (18,19), and increased cholesterol levels in man (18) and swine (20).

Other researchers, however, have presented data indicating no ill effects from hydrogenated oils and isomerized fats in research animals. Anderson et al. (8) found no significant differences in body weight of rats fed diets containing linoleate along with from 1.2 to 18% of its *trans* isomers, although diets containing linoelaidate did apparently inhibit chain elongation of linoleate to arachidonate in the liver. Feeding studies with rats by Alfin-Slater et al. (21,22), with mice (23) and rats (24) by Vles and Gottenbos, and with swine by Elson et al. (25) showed no adverse effects from *trans* fatty acids or hydrogenated oils. Weigensberg and McMillan (26) reported no increase in visible atherosclerotic lesions in rabbits fed cholesterol and linoelaidic acid than in rabbits fed cholesterol and linoleic acid.

The present investigation compares the

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metabolism of linoleic acid and its *trans,trans* isomer, linoelaidic acid, in the laying hen by examining their incorporation into egg yolk total neutral lipid and total phospholipid and into individual neutral lipid components (triacylglycerols, cholesterol, and cholesteryl esters).

PROCEDURES

Experimental Design

Previous in vivo investigations at this Center by Mounts and co-workers (10,11) and Emken et al. (12) have used dual isotopic labeling to study the metabolic fate of positional and geometric octadecenoic acid (18:1) isomers found in cooking oils and margarines. The general procedure employed was to label the fatty acid isomer and the naturally occurring fatty acid with different isotopes. This allowed a mixture of the labeled fatty acids to be fed, and the metabolism of each fatty acid followed simultaneously. Thus, the natural fatty acid served as an internal standard, and its utilization could be directly compared to the isomeric fatty acid.

In these dual-labeled experiments, changes in isotopic ratios from administered ratios were used as indications of metabolic selectivity for one fatty acid isomer over the other. For example, a greater incorporation of the ^{14}C-labeled compound is indicated if the $^{3}H/^{14}C$ ratio decreases in an isolated lipid fraction compared to the isotopic ratio in the fed mixture, and conversely, an increase in the $^{3}H/^{14}C$ ratio signifies preferential incorporation of the tritiated fatty acid isomer. Previous research has demonstrated that care must be taken to assure radiohomogeniety (10,27); that is, to insure that $^{3}H/^{14}C$ ratio changes are indications of selective fatty acid isomer incorporation and not the result of discrimination caused by isotope effects or loss of radioactive label during fatty acid metabolism.

The present investigation uses this dual-labeling technique to compare the utilization of linoleic acid and linoelaidic acid in the laying hen. Mixtures of labeled fatty esters were force-fed in gelatin capsules to laying hens as described previously (10). The $^{3}H/^{14}C$ ratios of the mixtures for each feeding are given in parentheses: Feeding I, methyl linoelaidate-12(13)-^{3}H and methyl linoleate-1-^{14}C (1.35); Feeding II, methyl linoelaidate-1-^{14}C and ethyl linoleate-12(13)-^{3}H (0.84); Feeding III, ethyl linoleate-12(13)-^{3}H and methyl linoleate-1-^{14}C (0.95).

In Feedings I and II, the isotopes used to label the fatty acids were reversed. In this manner, any changes in isotopic ratios caused by metabolic selectivities should also be reversed. The reversed-labeling experiments afford duplicate results and provide an indication of radiohomogeniety. For example, if the $^{3}H/^{14}C$ ratio changes in one experiment but no opposite change occurs in a second experiment when the labels are reversed, then the change is not due to metabolic selectivities but to the loss of isotopic label or to an isotope effect. Feeding III was conducted to assess the radiohomogeniety of the ^{3}H- and ^{14}C-labeled linoleate used in these experiments.

Materials

Linoleic acid-1-^{14}C was obtained from Amersham-Searle Co. (Arlington Heights, IL). Specific activity was 61 mCi/mmol; radiopurity, 99%. After methylation using diazomethane, the ester was isolated by preparative thin layer chromatography (preparative-TLC) on silver nitrate impregnated silica gel plates (Brinkmann Instruments, Silica Gel F-254, 2 mm). The TLC plates were developed with petroleum ether-benzene (20:80).

Methyl and ethyl linoleate-12(13)-^{3}H were prepared by reduction of the corresponding ester of crepenynic acid [*cis*-9-octadecen-12-ynoic acid (28) isolated from *Crepis alpina* seed] with tritiated water (29) over Lindlar catalyst (30,31).

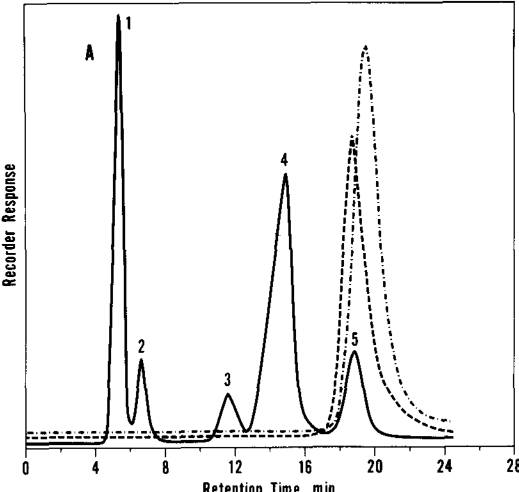
Isomerization of the radioisotope-labeled linoleate to the *trans,trans* isomer was accomplished using the nitrous acid method described by Litchfield et al. (32). The 9-*trans*,12-*trans* octadecadienoate was isolated by preparative-TLC on silver nitrate impregnated silica gel plates developed with petroleum ether-benzene (5:95).

Reductive ozonolysis, gas liquid chromatography, TLC, and infrared analysis were used to determine the position and configuration of the double bonds on unlabeled *trans,trans* 18:2 prepared in the same manner as the radioisotope-labeled esters. Radiochemical purity of all isolated esters was determined to be 99% by radiochromatogram scan (Packard Model 7200 Radiochromatogram Scanner) and gas liquid radiochromatography (33).

Methods

Each mixture of the labeled fatty esters described earlier was fed to a set of three white leghorn hens (H & N strain) which had been maintained on Purina Low Temp 6360 laying mash ration (Ralston Purina Co., St. Louis, MO). Each hen received 50-80 mg of the radioisotope-labeled fatty esters.

Six eggs subsequently laid by each hen following radioisotope administration were



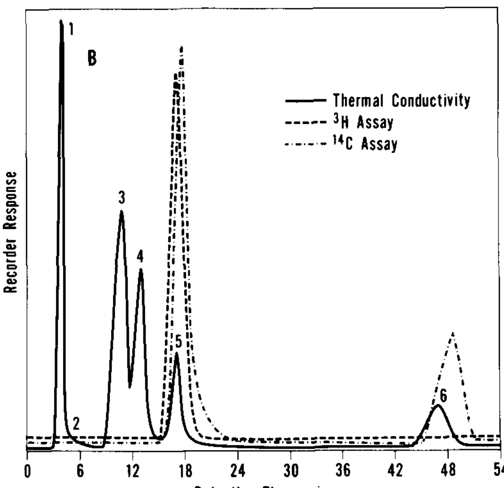


FIG. 1. Computer plot of gas liquid chromatography-liquid scintillation counter analysis of methyl esters from egg yolk (A) total neutral lipid and (B) total phospholipid fractions following administration of linoelaidate-12(13)-^3H and linoleate-1-^{14}C. Radioactive assays for ^3H and ^{14}C are superimposed over thermal conductivity trace based on retention times. The column was 10% EGSS-X (6 ft x 1/4 in. aluminum) operated at 175 C with helium flow rate of 30 ml/min. Major peaks are 1: palmitate, 2: palmitoleate, 3: stearate, 4: oleate, 5: linoleate, and 6: arachidonate.

collected for lipid analysis. Yolks from the third egg laid by each hen were combined into one sample before lipid extraction. The yolks from the fourth and fifth eggs were each individually extracted, and the yolks from the sixth egg laid by each hen were again combined before lipid extraction. Egg yolk lipid was extracted with chloroform-methanol as described by Mounts and Dutton (29).

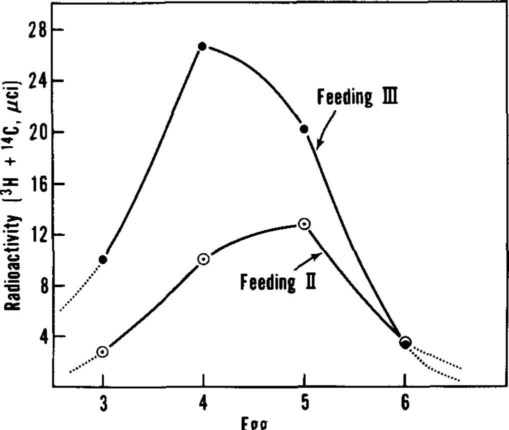


FIG. 2. Combined radioactivity (^3H + ^{14}C, μCi) recovered in egg yolk samples collected after feeding 71 μCi linoleate-^3H and 84 μCi linoelaidate-1-^{14}C (Feeding II) and 139 μCi linoleate-^3H and 145 μCi linoleate-1-^{14}C (Feeding III). These curves represent 19% (Feeding II) and 21% (Feeding III) incorporation of administered radioactivity.

Total egg yolk lipid was separated into neutral lipid and phospholipid fractions on a 25 cm x 2 cm silica gel column (60-200 mesh; J.T. Baker Chemical Co., Phillipsburg, NJ). The neutral lipids were eluted with chloroform-benzene (70:30); phospholipids were eluted with 100% methanol.

Methyl esters of the neutral lipid and phospholipid fractions were prepared by transesterification using 2N methanolic sodium methoxide and analyzed by gas liquid chromatography-liquid scintillation counting as described by Thomas and Dutton (34). This procedure couples gas chromatographic separation of the methyl esters and serial collection of the column effluent from the gas chromatograph in scintillation "cocktail" for subsequent radioisotope analysis.

Radiochemical assays were performed using a Beckman three-channel LS-250 liquid scintillation counter with previously described parameters (11). Output from the counter was interfaced to a Mod-Comp computer (Modular Computer System, Inc., Ft. Lauderdale, FL) programmed to calculate disintegrations per minute (dpm) and $^3H/^{14}C$ ratios. All dpm were corrected by external standard ratios and previously established quench correction curves. ^3H and ^{14}C dpm were tabulated along with inactive gas chromatographic data for each sample collected from the gas chromatograph. A computer plot matched peaks of the thermal conductivity recording with those of the radioactive profiles based on relative retention times,

TABLE I

Isotopic Ratios ($^3H/^{14}C$) of Egg Yolk Neutral Lipid (NL) and Phospholipid (PL)

Feeding	Administered mixture (9,12-18:2)[a]	Ratio fed	Total NL	NL 18:2 only[b]	Total PL	PL 18:2 only[b]
I	t,t-^3H + c,c-^{14}C	1.35	1.08±0.03[c]	1.36±0.02	0.64±0.01	1.32±0.03
II	c,c-^3H + t,t-^{14}C	0.84	0.71±0.01	0.83±0.03	1.45±0.08	0.81±0.01
III	c,c-^3H + c,c-^{14}C	0.95	0.84±0.01	0.96±0.01	1.01±0.02	0.99±0.05

[a]First two numbers denote position of double bonds, the number before the colon denotes chain length, and the number after the colon represents the number of double bonds.
[b]Gas liquid chromatography-liquid scintillation counting of methyl esters.
[c]Mean ± SE determined on fractions from at least six eggs (total NL and PL) and four eggs (NL and PL 18:2).

TABLE II

Isotopic Ratios ($^3H/^{14}C$) of Egg Yolk Neutral Lipid Components

Feeding	Administered mixture (9,12-18:2)	Ratio fed	Cholesteryl ester	Triacylglycerol	Cholesterol
I	t,t-^3H + c,c-^{14}C	1.35	0.99 ± 0.04[a]	1.37 ± 0.03	0.80 ± 0.03
II	c,c-^3H + t,t-^{14}C	0.84	1.71 ± 0.18	0.82 ± 0.03	0.62 ± 0.07
III	c,c-^3H + c,c-^{14}C	0.95	0.84 ± 0.02	0.95 ± 0.01	0.68 ± 0.03

[a]Mean ± SE determined on three neutral lipid fractions from eggs of different hens.

thus yielding a radiochromatogram. Typical radiochromatograms of neutral lipid and phospholipid esters are shown in Figure 1A and 1B, respectively.

Separation of neutral lipid components (cholesteryl esters, triacylglycerols, and cholesterol) from each egg yolk sample was accomplished by preparative-TLC separation of the neutral lipid fraction previously isolated by silica column chromatography. Each plate was developed with petroleum ether-benzene-acetic acid (20:80:5). Neutral lipid components were recovered from the TLC plate by repeated extraction of the silica gel with diethyl ether and chloroform-methanol (4:1) until negligible radioactivity was found in the extraction solvent.

RESULTS AND DISCUSSION

Label Incorporation

Peak incorporation of radioactive octadecadienoate into egg lipid occurred in either the fourth or fifth egg laid after feeding as shown in Figure 2. This result is in agreement with the incorporation pattern observed earlier for radioisotopically labeled octadecenoates in the laying hen (10). Total incorporation of the labeled 18:2 isomers into the egg yolks was about 20% of the radioactivity fed which is in contrast to the 6% total incorporation observed when radioisotopically labeled 18:1 isomers were fed (10).

Average $^3H/^{14}C$ ratios (mean ± SE) determined for total neutral lipid and total phospholipid fractions from each feeding experiment are presented in Table I. Similar isotopic ratios for 18:2 of the neutral lipid and phospholipid fractions (Table I) were determined from the total dpm across only the 18:2 peak of the radiochromatograms.

Neutral Lipid

Isotopic ratios for total neutral lipid decreased slightly in each of the three feeding experiments. In a preliminary dual-labeled feeding of a mixture of ^3H- and ^{14}C-labeled *cis,cis* octadecadienoates to the laying hen, Mounts and Dutton (29) also obtained $^3H/^{14}C$ ratios for total neutral lipid which were less than the $^3H/^{14}C$ ratio of the administered mixture. Analyses of the methyl esters from total neutral lipid fractions by gas liquid chromatography-liquid scintillation counting showed that radioactivity was associated only with the 18:2 peak (Fig. 1A). Ratios determined from the dpm across this peak (Table I) show no variation from the ratios of the fed mixtures. Thus, no selectivity was indicated for linoleate or linoelaidate, and no isotope effect was found.

In order to identify the cause of the decrease in $^3H/^{14}C$ ratios in all the total neutral lipid samples from the three experiments, individual neutral lipid components (triacylglycerols, cholesterol, and cholesteryl esters) from each feeding were examined. Liquid scintillation analyses of the neutral lipid components isolated from three eggs gave the average $^3H/^{14}C$ ratios presented in Table II.

Triacylglycerols

In all feedings, isotopic ratios of triacylglycerols show no deviation from the ratios of the administered mixtures which indicates no preferential uptake of linoleic acid over linoelaidic acid. These triacylglycerol data confirm the neutral lipid 18:2 data in Table I since egg yolk total neutral lipid is composed of 90% triacylglycerol. Budowski et al. (35) found that labeled triacylglycerol fatty acids deposited in the ova had the same specific activity as the plasma triacylglycerol fatty acids but that the labeled glycerol moiety found in the egg yolk had a lower specific activity than that of plasma glycerol. His data indicate that a partial hydrolysis of plasma triacylglycerols occurs in the hen's ovary followed by reacylation of the labeled fatty acids to nonlabeled glycerol. Because a large change in specific activity between the plasma and egg yolk fatty acids did not occur, it would appear that these hydrolysis and acylation reactions are not influenced by chain length or double bond configuration of the fatty acid. The lack of sensitivity for double bond configuration of these reactions is further supported by observations that the fatty acid composition of egg yolk reflects the fatty acid composition of the dietary fat (36). Our triacylglycerol data in Table II, which indicates no discrimination against either linoleic or linoelaidic acids, supports this concept that hydrolysis and acylation reactions are not affected by double bond configuration.

Cholesterol

The $^{3}H/^{14}C$ ratios determined in the cholesterol component of neutral lipid are substantially lower than the ratios of the administered esters in each of the three feedings. This decrease in the isotopic ratio indicates selective loss of the ^{3}H label. Since acetate is the primary source of carbon atoms for cholesterol biosynthesis (37), the incorporation of radioisotopic labels into cholesterol must result from catabolism of the fed labeled fatty esters and subsequent anabolism of the fragments. Budowski et al. (35) have shown that although cholesterol is synthesized mainly in the liver of the chicken, synthesis of sterols from a highly active acetate pool also occurs in the ovary and ovarian membrane. During fatty acid degradation (via β-oxidation), the ^{3}H label can readily exchange with other protons and is more easily removed from the biological system than the ^{14}C label in the acetate skeleton. The acetyl-CoA degradation product, as it enters the acetate pool, would then be expected to contain more ^{14}C than ^{3}H. Incorporation of this ^{14}C-enriched acetate into cholesterol would lower the $^{3}H/^{14}C$ ratio in this component and in the total neutral lipid. These cholesterol data explain the differences in the total neutral lipid data and the neutral lipid 18:2 data in Table I.

Cholesteryl Esters

$^{3}H/^{14}C$ ratios for the cholesteryl ester component of the egg yolk neutral lipids are shown in Table II. These ratios are lower than the ratio of the mixture fed in Feeding I and higher than the ratio fed in Feeding II. These data indicate preferential esterification of cholesterol by the *cis,cis* isomer since the ^{3}H label was on the *trans,trans* isomer in the first feeding and on the *cis,cis* isomer in the second. These results are consistent with the observations of Sgoutas (38) which demonstrated that acyl-CoA:cholesterol-*O*-transferase (EC 2.3.1) from rat liver microsomes displays a definite selectivity for linoleate over linoelaidate for esterification to cholesterol.

In Feeding III, the isotopic ratio of the cholesteryl ester component was slightly lower than the $^{3}H/^{14}C$ ratio fed. This slight difference is probably not derived from selective fatty acid incorporation or isotope effects but from esterification of the fatty acids to ^{14}C-enriched endogenous cholesterol which was observed earlier to have a lower $^{3}H/^{14}C$ ratio than the fed mixture.

Total Phospholipid

Analyses of egg yolk total phospholipid methyl esters by gas liquid chromatography-liquid scintillation counting showed radioactivity both in the octadecadienoate peak and in the elongation product of linoleate, i.e., arachidonate (Fig. 1B). In Feeding III, the $^{3}H/^{14}C$ ratios (Table I) determined for both the total phospholipid and the phospholipid 18:2 showed no significant variation from the ratio fed. The isotopic ratio found for methyl arachidonate (0.98 ± 0.05) was also the same as that of the fed mixture in Feeding III, indicating that both the ^{3}H- and ^{14}C-labeled linoleic acids were elongated to arachidonic acid at equal rates and that the ^{3}H label on the 12,13 double bond did not cause any isotope effect.

In Feedings I and II in which both labeled linoelaidate and linoleate were fed, the isotopic ratio of the 18:2 phospholipid component was identical with the administered ratio, which indicates no discrimination against either isomer for incorporation into phospholipid octadecadienoate. However, the radioactivity associated with methyl arachidonate was derived solely from the *cis,cis* isomer as seen in Figure 1B. Inclusion of the arachidonate $^{3}H/^{14}C$ ratio

with the 18:2 $^3H/^{14}C$ ratio alters the overall $^3H/^{14}C$ ratio of the total phospholipid fraction in Feedings I and II. In Feeding I, the total phospholipid ratio was lower than the $^3H/^{14}C$ ratio of the fed mixture due to the carbon-14 labeled arachidonate. The opposite effect was seen in Feeding II due to the elongation of tritiated linoleate to arachidonate. These data confirm similar observations in rats that linoelaidate is not elongated to arachidonic acid (39).

Acylation of Phospholipid

Molecular models demonstrate that the physical structure of *trans,trans*-18:2 is similar to that of a saturated fatty acid (19,40). Experiments with rats have shown that *trans,trans*-18:2 is used similarly to a saturated fat by acyltransferases in the synthesis of phosphatidylcholine in that both are acylated at the one position (39,41). In fact, in rats the preferential acylation of the one position with *trans,trans*-18:2 was found to be of the same magnitude as the selective acylation of the two position with *cis,cis*-18:2 (39). Obviously, the level of *trans,trans*-18:2 selectively incorporated into the one position in egg yolk phospholipid could be counterbalanced by an equal selectivity for *cis,cis*-18:2 in the two position, which would result in no apparent difference in the metabolism of the labeled octadecadienoates. These same acyltransferase selectivities have been reported for total rat liver phosphatidylcholine and triacylglycerols (41).

The nonselective deposition of *trans,trans*-18:2 compared to *cis,cis*-18:2 in the phospholipids may also be explained by the transport mechanisms in the laying hen for dietary phospholipid. In contrast to triacylglycerols which are reported to be hydrolyzed and reacylated in the hen's ovary, the phospholipids are transported through the ovary to the ova without modification of the phospholipid structure (35). Reiser and Dieckert (42) have suggested that triacylglycerols are the precursors of mucosa phospholipids in rats. If this premise also applies to the laying hen, one might expect the same nonselectivity shown for linoleate and linoelaidate in the triacylglycerols to carry over into the phospholipids.

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Metabolism of a n-Paraffin, Heptadecane, in Rats

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ABSTRACT

^{14}C-heptadecane incorporated in rat diet was largely absorbed, and a balance study showed extensive $^{14}CO_2$ excretion (65%). There was no elimination of the hydrocarbon in the urine, and only minute quantities of labeled metabolites. Radioactivity in the feces was entirely in heptadecane. About 7% of the heptadecane absorbed was stored in the carcass, whereas the rest was ω-oxidized to heptadecanoic acid. This fatty acid was incorporated into neutral lipids and phospholipids, underwent the normal fatty acid degradation pathway, and contributed to the synthesis of lipids, including fatty acids, squalene and cholesterol, and nonlipids (7-10%). Heptadecanoic acid was desaturated to heptadecenoic acid. The even distribution of radioactivity in the fatty acids of the various phospholipid classes indicated that heptadecane did not interfere with the biochemical mechanisms of these functional lipids.

INTRODUCTION

Hydrocarbons normally occur in foods from plant origin mostly. Increasing amounts of these substances are ingested by animals and man due to the use of mineral oil in food technology and to some sources of food contamination such as n-alkanes from yeasts grown on paraffins. Aliphatic hydrocarbons are absorbed well (1-6) when incorporated at low doses into the diet of mammals; absorption varies with the length of the carbon chain (7) and with the animal species (8).

In 1942, Stetten (9) suggested that rat liver oxidized hexadecane. Kolattukudy and Hankin (10) observed that nonacosane was partially absorbed and oxidized to fatty acids, in particular to heptadecanoic acid. More recent studies with ^{14}C-hexadecane and octadecane have established the process of terminal oxidation of n-alkanes to corresponding fatty acids (11) and have localized in liver (12-15) and kidney (15) the microsomal enzymatic systems involved. Mitchell and Hubscher (16) showed that mucosa cells of the small intestine of guinea pig oxidize hexadecane also. n-Paraffins accumulated preferentially in the fat of rats fed diets containing low doses of eicosane (17) or green algae rich in heptadecane (18).

Until now, no metabolic balance nor systematic research on metabolites has been undertaken; and it cannot be stated a priori that oxidation to the corresponding saturated fatty acid constitutes the sole metabolic pathway of n-paraffin degradation. Neither is there any data on the extent of this process. This investigation attempts to fill these gaps by studying the metabolism of this class of hydrocarbons in more detail. Heptadecane was chosen because it is widespread in the marine food chain (19-23), it is one of the main components of the n-paraffin series used as a substrate for producing alkane yeasts, and because until now the metabolism of odd-chain normal paraffins had not been studied.

METHODS

(8-^{14}C) n-heptadecane was synthesized by the Service of Labeled Molecules at the CEA (Saclay, France) (specific activity, 27 mCi/mM). Chemical purity was checked by gas liquid chromatography (GLC) on two columns of different polarities and radiochemical purity was tested by thin layer chromatography (TLC) (24).

Wistar male rats weighing about 200 g were fed as libitum on a semisynthetic diet (casein

TABLE I

Gross Distribution of ^{14}C 7 Days after Ingestion of ^{14}C-heptadecane

Dose of heptadecane	Percentage of ^{14}C fed (mean ± SE, n = 6)			
	Feces[a]	Urine[a]	Carcass[b]	Total
1 mg - 10 μCi	0.96 ± 0.21	1.12 ± 0.04	36.9 ± 5.8	39.0 ± 5.5
200 mg - 30 μCi	28.0 ± 5.3	0.47 ± 0.07	25.2 ± 4.2	53.7 ± 2.3

[a]Cumulative for 6 days.
[b]Residual at day 7.

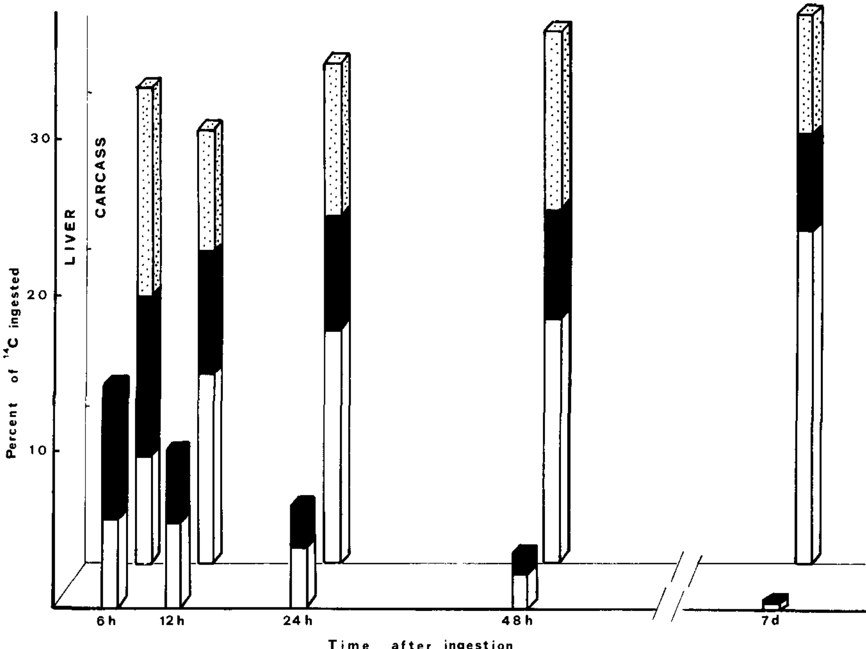

FIG. 1. Evolution and distribution of ^{14}C in liver and carcass of rats fed ^{14}C heptadecane; ■ hydrocarbon, ▩ lipids, □ nonlipids.

18%, wheat starch 39%, sucrose 24%, peanut oil 8%, cellulose 3%, minerals and vitamins 8%). (8-^{14}C) n-Heptadecane was diluted with analytical grade (99% min) n-heptadecane (Baker) and incorporated in the peanut oil of the diet. In all experiments, the animals were fed lightly the night before the experimental diet was given so that the ^{14}C-heptadecane, administered in a small amount of the feed, would be ingested within 15 min. The animals were given the control diet ad libitum immediately afterwards. Rats were kept in metabolism cages, urine and feces were collected daily for 6 days, and the rats were killed on the 7th day. Lipid incorporation kinetics were studied in animals killed 6 hr, 12 hr, 24 hr, 48 hr, and on the 7th day, respectively, after the ingestion of labeled n-heptadecane.

Urine was measured directly by liquid scintillation counting with an Intertechnique SL 32 instrument, in the form of a gel stabilized by Triton X 100. Lipid fractions isolated by chromatography were measured in a PPO-dimethyl-POPOP liquid scintillation cocktail in toluene. Total radioactivity in tissues, feces, and residues of lipid extraction was measured after combustion of 500 mg samples in an automatic apparatus (Intertechnique Oxymat). Radioactivity was localized on thin layer chromatograms using a Berthold thin layer scanner.

Total lipids of tissues and feces were extracted with chloroform-methanol (2:1) according to Folch's method (25) and saturated hydrocarbons, neutral and complex lipids were separated on a silica gel column (Kieselgel 60, Merck) by successive hexane, chloroform, and methanol elutions. Hydrocarbons were analyzed by GLC using the technique already described (6). Neutral lipids were separated into classes on a Florisil column using Carroll's method (26). Purity of fractions was checked by TLC on Silica Gel G using hexane-ethyl ether-formic acid, 120:30:1.5.

Fatty acid methyl esters, prepared by transmethylation, were purified on silica gel plates (elution with hexane-ethyl ether, 90:10), then analyzed by GLC on 1/8 in. x 3 m stainless-steel column packed with 10% DEGS-PS on 80/100 Supelcoport. Separation according to the number of double bonds was performed by TLC on silica impregnated with 20% silver nitrate (27). Saturated and unsaturated fatty acid methyl esters were separated according to the number of carbon atoms by reversed phase chromatography on a Lipidex 5000 column (Packard) (elution with methanol-water-methylene chloride, 80:20:10). Squalene and cholesterol, in the nonsaponifiable fraction, were identified by GLC on a ¼ in. x 1.5 m glass column packed with 3% Dexsil 300 on 80/100 W AW DMCS Chromosorb. Phospholipids were

TABLE II

Distribution of ^{14}C in Liver Neutral Lipids
24 hr after Ingestion of 20 μCi ^{14}C-heptadecane

Fraction	Percent of [a] total ^{14}C	Specific activity [b]
Monoglycerides	1.2	880
Diglycerides + Cholesterol	25.8	5510
Free fatty acids	20.4	12440
Triglycerides	34.6	1250
Cholesterol esters + Squalene	18.0	3980

[a]Neutral lipids separated from saturated hydrocarbons.
[b]dpm/mg.

TABLE III

Radioactivity of Fatty Acids and Total Cholesterol in Liver Neutral Lipids

	Specific activity[a]		
Time after ingestion	6 hr	24 hr	7 days
Fatty acids	2810	1280	81
Cholesterol	500	1050	190

[a]dpm/mg.

separated into fractions on a silicic acid column (Mallinkrodt) (28). Purity of fractions was tested by TLC on Silica Gel G (elution with chloroform-methanol-acetic acid-water, 100:55:16:4). Fatty acids were analyzed after transmethylation using the technique described above.

RESULTS

The metabolic balance was measured after a single administration to a rat of two doses of ^{14}C-heptadecane, and was calculated from the radioactivity excreted in urine and feces for 6 days, and from the residual radioactivity measured in the whole carcass at the end of the experiment. Table I indicates that heptadecane was highly absorbed. The radioactivity measured in the feces was only in the form of heptadecane. In this and a previous study (6) involving an intermediate dose (15 mg), the absorption level was closely related to the administered dose.

In both studies (6), no heptadecane was found in urine. The high amount of unaccounted radioactivity (61% and 46.3%, respectively) must be due to $^{14}CO_2$ excretion, which implies that heptadecane was completely metabolized. Fifty-five percent of the ingested ^{14}C was eliminated in the first 6 hr as $^{14}CO_2$. A small amount of the nonrecovered ^{14}C could be explained by hydrocarbon excretion in sweat (29-30) and sebum lipids (31).

Figure 1 shows the distribution of radioactivity in the liver and in the remaining carcass. Total radioactivity decreased rapidly in liver; it leveled off within 6 hr, then changed little over a 7 day period in the carcass. ^{14}C was mainly incorporated in the lipid fraction of the liver, whereas labeling of the nonlipid fraction of the remaining carcass was already significant at 6 hr and thereafter (33.7% to 44.4% of total radioactivity).

Radioactivity in the form of heptadecane decreased rapidly in the liver but remained almost unchanged in the carcass between the 12th hour and 7th day, showing retention of heptadecane and other n-paraffins (C_{20}, C_{21}, C_{24}) in rat (6). The concomitant increase of the radioactivity of the lipids in the carcass, and decrease of labeled heptadecane and lipids in the liver over a 7 day period, confirm the transformation of heptadecane into lipid components.

Neutral Lipids

In liver and adipose tissue, 65% of the radioactivity was bound to saturated acids and the rest to monounsaturated acids. The distribution of radioactivity according to length of fatty acids was established only in the most radio-

TABLE IV

Distribution of ^{14}C in Saturated and Monounsaturated Fatty Acids from Neutral Lipids of Liver and Adipose Tissue

Fatty acids	Liver[a]		Adipose tissue[b]	
	Radioactivity	Composition	Radioactivity	Composition
<15	1.5[c]	1.8	ND	2.1
15:0	6.0	ND[d]	11.3	ND
16:0	3.7	27.3	16.1	23.0
16:1	4.8	2.7	6.3	4.7
17:0	48.5	ND	37.1	ND
17:1	22.8	ND	19.3	ND
18:0	5.1	5.0	2.1	3.7
18:1	5.6	46.0	7.8	44.0
>18	2.0	17.2	ND	22.5

[a]6 Hr after ingestion.
[b]7 Days after ingestion.
[c]Mainly $C_{13:0}$
[d]ND = non detectable.

active samples, liver after 6 hr and adipose tissue after 7 days.

Table II indicates that heptadecanoic acid and heptadecenoic are strongly labeled. This indicates the oxidation of heptadecane to heptadecanoic acid and an active desaturation process. The presence of labeled 15:0 and 13:0 in the liver shows that heptadecanoic acid undergoes the usual β-oxidation. Radioactive saturated and unsaturated even fatty acids must result from resynthesis involving ^{14}C-acetate. Although neutral lipids of liver and adipose tissue exhibit similar fatty acid compositions, a difference in the distribution of the radioactivity was noticed (Table II). Six hr after ^{14}C-heptadecane administration, about 80% of the ^{14}C was in liver odd fatty acids, whereas 7 days later, the even fatty acid resynthesis in adipose tissue, especially of palmitic acid, was substantial.

Neutral lipids of liver and adipose tissue freed from hydrocarbons were separated into their different classes. Radioactivity in adipose tissue was mainly (90%) in the triglycerides, whereas it was widespread in the lipid classes of the liver (Table III). Specific activity of free fatty acids was about 7 times higher than that of triglyceride fatty acids. This preferential labeling must correspond to the initial metabolic stage of heptadecane ω-oxidation, to heptadecanoic acid. Separation of cholesterol, diglycerides on one hand, squalene and cholesterol esters on the other, established that cholesterol was strongly labeled and ^{14}C-squalene was present.

Cholesterol specific activity increased after

TABLE V

Distribution of ^{14}C in Saturated and Monounsaturated Fatty Acids from Liver Phospholipids 24 hr after Ingestion of ^{14}C-heptadecane

Fatty acids	Radioactivity[a]	Composition[b]
14:0	0.5	0.3
15:0	9.3	0.2
16:0	37.1	35.0
16:1	ε	0.6
17:0	31.9	0.6
17:1	9.6	ND
18:0	11.2	52.9
18:1	0.4	10.4

[a]p.100 of ^{14}C in fatty acids.
[b]p.100 of saturated + monounsaturated fatty acids.

TABLE VI

Distribution of ^{14}C in Liver Phospholipids 6 hr after Ingestion of ^{14}C-heptadecane

	Percent of total ^{14}C	Specific activity [a]	Palmitic + stearic acids / total fatty acids
Phosphatidic acid	1.9	292	0.08
Phosphatidyl ethanolamine	27.1	815	0.49
Phosphatidyl serine	3.7	807	0.49
Phosphatidyl choline	64.2	1050	0.52
Sphingomyelin	3.1	620	0.30

[a]dpm/mg.

the 6th hour, whereas that of fatty acids decreased rapidly (Table IV). This time lag is due to the successive stages of ω-oxidation to heptadecanoic acid, β-oxidation, ^{14}C-acetate production, and synthesis of squalene and cholesterol.

Phospholipids

The incorporation of ^{14}C in hepatic phospholipids was studied 24 hr after ingestion of heptadecane. Radioactivity was entirely in fatty acids. The separation of fatty acid methyl esters according to the degree of unsaturation showed that 90% of the radioactivity was associated with saturated acids and the rest with monounsaturated acids. The distribution by chain length (Table V) indicates major ^{14}C incorporation into heptadecanoic and pentadecanoic acids (about 3200 dpm/μg for both acids), and a strong labeling of nonmeasurable quantities of heptadecenoic acid. Twenty-four hr after ingestion of heptadecane, resynthesized ^{14}C even fatty acids, especially palmitic acid, were evident.

Analysis of phospholipid classes (Table VI) shows that most of radioactivity was in phosphatidylcholine, but specific activities indicate a more homogeneous distribution closely related to saturated fatty acids contents of each fraction.

DISCUSSION

Metabolism of heptadecane was studied at low levels of the diet encountered in food contamination. This is in contrast to most experiments on n-paraffins which have been carried out with high doses given either by gastric intubation or intravenous injections, with or without previous emulsification. Considerable variations in the absorption rates result from the nonnutritional conditions (7,32). Our results show decreased absorption with increased dose, over a 200-fold range, indicating that low doses of n-paraffins incorporated in the diet are largely absorbed.

Heptadecane accumulates during the first 6 hr and then levels off, whereas labeled lipids accumulate progressively. Heptadecane probably follows the lymphatic route and reaches the tissues, especially adipose tissue, where it accumulates due to its lipophilic nature (5,7). Because of the limited oxidation capacity of the liver, the proportions of heptadecane and labeled lipids are related to extent and rate of ^{14}C-heptadecane absorption. To ensure a fast absorption Savary and Constantin (5) used a gastic probe of 200 mg emulsified octadecane and found that rat adipose tissue retained ^{14}C-heptadecane but very little labeled lipids even after 48 hr. Pokrovskii (33), in similar experimental conditions, found that 94% of fat radioactivity was still bound to ^{14}C-heptadecane 15 days after ingestion. Under the food contamination conditions of our experiments, heptadecane metabolism is more rapid.

Our experiments clearly show that the first stage of heptadecane metabolism is ω-oxidation to heptadecanoic acid, which undergoes the same fate as normal fatty acids (34). The presence of the homologous 15:0 and 13:0 odd acids indicate the first stages of β-oxidation. Squalene and cholesterol labeling imply the availability of ^{14}C-acetyl CoA. Due to label on carbon 8 of heptadecane, ^{14}C-acetate is only produced after four successive steps, which means that heptadecanoic acid is thoroughly metabolized. The extent and intensity of $^{14}CO_2$ production show that heptadecane is well used by the rat as an energetic source.

Preferential incorporation of ^{14}C-octadecane radioactivity into lecithin fatty acids has been reported (35) based on total counts per fraction. Measurement of specific activities shows that the odd fatty acids resulting from the oxidation of heptadecane are distributed in phospholipid classes the same as the other fatty acids. Thus, no modification of structural lipids role should be expected.

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Effect of Dietary Vitamin C on Adrenal Cholesteryl Ester Content in the Guinea Pig

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ABSTRACT

Male guinea pigs fed a vitamin C-deficient diet for 3 weeks had lower concentraitions of cholesteryl esters in their adrenals than did control animals fed the recommended intake of the vitamin. Not all esters were affected to the same degree, and the fatty acid profiles of the esters from control and deficient guinea pigs differed; there was proportionately more palmitic and linoleic acids and less docosatetraenoic acid [22:4 (n-6)] in the deficient guinea pig adrenal esters. [Fatty acids are designated as X:Y (n-Z), where X and Y are the numbers of carbon atoms and olefinic bonds in the acid and Z is the number of carbon atoms after the terminal olefinic bond.] A 100-fold excess of vitamin C in the diet also resulted in lower concentrations of adrenal cholesteryl esters than did the control diet, but they were not as low as in the deficient animals. Fatty acid profiles were similar for esters from control and excessively supplemented guinea pigs. Vitamin C deficiency apparently imposes a long term stress which results in a depletion of adrenal cholesteryl esters, possibly specific esters, to meet the requirements for glucocorticoid synthesis.

INTRODUCTION

The adrenals of many species are rich in cholesteryl esters, and it has been postulated that these act as a reservoir of cholesterol for glucocorticoid synthesis (1). Stress results in a decrease in adrenal cholesteryl esters concomitant with an increase in plasma corticosterone in the rat (2-4). In rats fed an adequate diet, there is also an inverse relationship between adrenal cholesteryl ester concentration and plasma corticosterone throughout the normal circadian cycle of the glucocorticoid in the blood (5). Adrenal cholesteryl esters do not decrease in a uniform pattern in these situations of enhanced glucocorticoid synthesis, but specific esters appear to be preferentially utilized (3-5).

Ascorbic acid is involved in the synthesis and release of glucocorticoids by the adrenal (6-10). Since the guinea pig requires a dietary source of this vitamin, plasma glucocorticoid concentration should reflect the level of ascorbic acid in the diet and, by analogy with the relationship of plasma corticosterone and adrenal cholesteryl esters in the rat, should also modify the cholesteryl ester content of the adrenal. In a recent study (11), we demonstrated a diurnal rhythm for plasma cortisol in the guinea pig. This rhythm was independent of the ascorbic acid content of the diet although the actual levels of cortisol in the plasma throughout the day did reflect the dietary vitamin C supply. However, adrenal cholesteryl esters did not exhibit an inverse relationship to plasma cortisol in the guinea pig during the daily variations in this plasma component. Although no circadian cycle was evident for these esters, their concentration in the adrenal did reflect the vitamin C status of the guinea pig. Moreover, the fatty acid composition of the adrenal cholesteryl esters also depended on the level of vitamin C in the diet as is illustrated in this report.

MATERIALS AND METHODS

Male guinea pigs (High Oak Ranches, Goodwood, Ont.), weighing 200 to 250 g, were randomly assigned to three groups and fed ad libitum the semipurified diet of Reid and Briggs (12). One group received this diet devoid of vitimin C, the second group was fed 0.2 g of ascorbic acid per kg diet [NRC recommended intake (13)], and the final group was fed 20.0 g of ascorbic acid per kg diet, a 100-fold excess of the vitamin. The animals were accustomed to the diet by mixing it in increasing proportions with ground guinea pig chow (Ralston Purina Co., St. Louis, MO) until, 1 week after their arrival, they were consuming the assigned semipurified diet ad libitum. The guinea pigs were housed individually in stainless-steel cages and maintained on a constant photoperiod of 12 hr light (0800 to 2000) and 12 hr darkness. They were weighed weekly, fresh water was available at all times, and clean cages were supplied every 10 days.

After 21 days on the experimental diets, when the vitamin C-deficient animals were scorbutic, groups of five animals from each of the three dietary treatments were killed at 4 hourly intervals over a continuous 24 hr period. Sodium pentabarbital (30-60 mg) was injected intraperitoneally; the adrenal glands were removed and immediately frozen in liquid nitro-

TABLE I

Effect of Dietary Vitamin C on the Concentration of the Major Adrenal Cholesteryl Esters in the Male Guinea Pig[a]

Ester	Dietary treatment				
	C-deficient	Control	Excess-C	$S\bar{x}$	ω^b
	(μ moles/g tissue)				
14:0	0.52	1.65	1.16	0.07	0.24
16:0	2.56	4.62	3.12	0.17	0.56
16:1	0.78	2.53	1.87	0.10	0.34
18:0	0.56	1.25	0.84	<0.01	0.01
18:1	2.72	7.03	4.71	0.24	0.80
18:2	1.51^A	2.46	1.38^A	0.09	0.29
18:3(n-6)	0.84^A	1.37	0.70^A	0.06	0.19
20:3(n-6)	0.38	1.27^A	1.13^A	0.07	0.22
20:4(n-6)	2.58	6.85	4.87	0.26	0.87
22:4(n-6)	0.71	3.53	2.66	0.16	0.55
Total esters	14.68	37.65	25.60	1.21	4.06

[a]each value is the mean for 35 animals. $S\bar{x}$ is the overall standard error of the mean. Minor components have been omitted from the table.
[b]ω is Tukey's honestly significant difference. Differences between means must exceed ω to be significant ($P<0.05$). Treatment means followed by the same superscript are not significantly different ($P>0.05$).

gen. They were stored in 0.9% saline at -10 C until analyzed.

After removal of perirenal fat, the adrenal lipids were extracted with chloroform-methanol (2:1) in an all-glass extraction apparatus (14) by the procedure of Folch et al. (15). After addition of a known amount of cholesteryl pentadecanoate as an internal standard, the total lipid extract was fractionated by thin layer chromatography (TLC) (4) and the cholesteryl esters isolated; they were transesterified with boron fluoride-methanol (16). The resulting methyl esters were fractionated by gas liquid chromatography (GLC) in a 3 m x 2 mm ID glass column packed with 3% EGSP-Z on 100-120 mesh Gaschrom Q (Applied Science Labs, Inc., State College, PA) operated isothermally at 180 C. Relative peak areas were measured with a computing digital integrator, and the concentration of each ester was calculated from the ratio of its peak area to that of the pentadecanoate internal standard. Identification of the esters was effected by comparison of retention data with those of known standards chromatographed under the same conditions as the samples.

In order to further characterize the trienoic esters in the adrenal, the total methyl ester sample from the control group was chromatographed on thin layers of Silica Gel G impregnated with 10% by weight of silver nitrate, using petroleum ether-diethyl ester (4:1) for development. After detection with 2′,7′-dichlorofluorescein, the esters with the same mobility as a methyl γ-linolenate reference sample were extracted from the gel with diethyl ether, purified by rechromatography on silica gel-silver nitrate and re-extracted with diethyl ether. This fraction was then characterized by reductive ozonolysis and gas chromatography of the products (17).

The data were subjected to an analysis of variance, and comparisons of the individual means were made at the 5% level of significance using Tukey's ω test (18).

RESULTS

Since there was no significant effect of sampling time on the concentration of cholesteryl esters in the adrenals of animals on the three diets, the data have been combined to yield a single analysis for each individual diet. Data for the major esters are presented in Table I.

The major cholesteryl esters present in the guinea pig adrenal were palmitate, oleate, and arachidonate, with smaller concentrations of myristate, palmitoleate, stearate, linoleate, γ-linolenate, eicosatrienoate (n-6), and docosatetraenoate (n-6). Several minor esters were also present but have been omitted from Table I. The presence of γ-linolenate [18:3 (n-6)] was initially inferred from the appearance of a peak with the same retention characteristics on two different gas liquid chromatographic columns as an authentic sample of this ester. This component was present in the trienoic ester fraction isolated by argentation TLC. GLC of the products of reductive ozonolysis of the trienoic ester fraction yielded three major peaks, the first two of which corresponded to those ob-

TABLE II

Effect of Dietary Vitamin C on the Distribution of Fatty Acids in Adrenal Cholesteryl Esters in the Male Ginea Pig[a]

Acid	Dietary treatment			$S\bar{x}$	ω[b]
	C-deficient	Control	Excess-C		
		(Mole %)			
14:0	3.5	4.4^A	4.5^A	0.15	0.49
16:0	17.6	12.7^A	11.9^A	0.34	1.13
16:1	5.3	6.8^A	7.3^A	0.14	0.47
18:0	3.8	3.4^A	3.3^A	0.13	0.45
18:1	18.5^A	18.9^A	18.5^A	0.34	1.13
18:2	10.4	6.6	5.4	0.19	0.65
18:3(n-6)	5.7	3.7	2.7	0.15	0.50
20:3(n-6)	2.6	3.4	4.4	0.20	0.68
20:4(n-6)	17.7^A	18.2^A	19.1^A	0.47	1.57
22:4(n-6)	4.8	9.2	10.4	0.34	1.14

[a]Each value is the mean for 35 animals. $S\bar{x}$ is the overall standard error of the mean. Minor components have been omitted from the table.

[b]ω is Tukey's honestly significant difference. Differences between means must exceed ω to be significant ($P<0.05$). Treatment means followed by the same superscript are not significantly different ($P>0.05$).

tained on ozonolysis of the methyl γ-linolenate standard and presumably represented the C6-aldehyde and C6-aldehyde-ester; the third peak was the C8-aldehyde-ester from 20:3 (n-6), the other major component in the triene ester fraction.

There was significatly more cholesteryl ester in the adrenals from animals fed the control diet than in those from animals fed either of the other two diets; there was less adrenal cholesteryl ester in the vitamin C-deficient than in the excessively supplemented group. The general pattern in total adrenal cholesteryl ester concentration was also evident in the individual major esters, i.e., on an absolute basis, the greatest concentration of a given ester was found in the control animals, with the lowest concentration in the deficient guinea pigs; the excessively supplemented animals yielded ester concentrations between these two extremes. The concentrations of cholesteryl linoleate in the deficient and excessively supplemented animals, and of cholesteryl eicosatrienoate (n-6) in the control and excessively supplemented animals, were not significantly different. For all the other major esters presented in Table I, differences among guinea pigs on the three diets were significant ($P<0.05$).

The differences in the concentrations of the individual adrenal cholesteryl esters among the three dietary groups were not proportional to the differences in the total cholesteryl esters, and dietary vitamin C modified the distribution of fatty acids in the adrenal esters (Table II). In general, the fatty acid profiles of the adrenal cholesteryl esters from guinea pigs fed the control and excessively supplemented diets were similar, whereas that of the esters from vitamin C-deficient animals was different from the other two. The major differences were in the greater proportions of palmitic, linoleic, and γ-linolenic acids and smaller proportions of 22:4 (n-6) and, to a lesser extent, of 20:3 (n-6), in adrenal cholesteryl esters from vitamin C-deficient guinea pigs.

DISCUSSION

The overall fatty acid profile of guinea pig adrenal cholesteryl esters recorded in this study was generally consistent with those reported by other investigators for this species (19,20). However, in the present study, γ-linolenate [18:3 (n-6)] constituted as much as 5% of the total esters. This ester elutes from polar gas chromatographic columns before α-linolenate. In a previous study on guinea pig adrenal lipids (19), an unspecified octadecatrienoic ester (18:3) accounted for 4-5% of the cholesteryl esters and arachidate (20:0), which would have similar retention characteristics to 18:3 on a polar gas chomatographic column, has also been reported to account for 6% of adrenal cholesteryl esters in this species (20). It is possible that in both of these previous investigations, γ-linolenate was found.

The occurrence of substantial amounts of γ-linolenic acid, although unusual in tissue lipids, is not unique to guinea pig adrenals; it has also been reported in the cholesteryl ester fraction of human adrenals (21). Indeed, several higher metabolites of linoleic acid, including 20:3 (n-6), 20:4 (n-6), 22:4 (n-6), and 22:5 (n-6), are relatively abundant in the adrenal choles-

teryl ester fraction from several different species (4,19-24). The physiological and biochemical bases for the incorporation of the less common polyunsaturated fatty acid metabolites by the adrenal are not known.

The apparent utilization of adrenal cholesteryl esters during glucocorticoid synthesis has been readily demonstrated in the rat by the net decrease in this tissue component associated with stress-induced steroidogenesis (2-4) and with the normal circadian variation in plasma corticosterone (5). No fluctuation in adrenal cholesteryl ester concentration accompanied the daily cycle of plasma cortisol in the guinea pig (11), nor did short-term stimulation of cortisol production by adrenocorticotrophic hormone injection effect a change in adrenal cholesteryl ester concentration (Wilbur and Walker, unpublished data). Apparently the short-term requirements for cholesterol for glucocorticoid synthesis in the guinea pig are readily met by extra-adrenal sources (25) or adrenal cholesterol is rapidly replenished during steroid hormone synthesis.

The long-term stress of vitamin C deficiency (26) did, however, induce a sustained elevation in plasma cortisol (11). The significantly lower concentration of cholesteryl esters in the adrenals of these animals is consistent with a demand for cholesterol for glucocorticoid biosynthesis. Moreover, the modification in the fatty acid profile of the ester fraction implies a selective metabolism of adrenal cholesteryl esters during corticosteroidogenesis. On the basis of the smaller proportion of the 22:4 (n-6) ester in the adrenals of the ascorbic acid-deficient guinea pigs relative to those fed the control diet, this appears to be one of the esters which decreased preferentially as a result of the stress imposed by the deficiency. This contrasts with work on the rat which showed that the cholesteryl esters of 18:1, 18:2, and 20:4, but not of 22:4 (n-6), were preferentially depleted during stress-induced glucocorticoid synthesis (4,5). In vitro, rat adrenal cholesteryl ester hydrolytic activity exhibited a higher specificity for unsaturated than for saturated esters (5). A similar preference for unsaturated esters was observed with the rat liver enzyme (27) and positional isomers of the octadecenoic esters were hydrolyzed at different rates in that tissue (28). The fatty acid specificity of guinea pig adrenal cholesteryl ester hydrolase activity has not been reported previously, but the current experiment does indicate a possible preference for longer chain polyunsaturated esters. It must be pointed out, however, that this conclusion is based on net changes in concentration of the esters, and these could have resulted from differences in the rates of resynthesis of the esters rather than differences in their rates of hydrolysis.

The effects on adrenal cholesteryl ester concentration of the diet excessively supplemented with vitamin C are not readily explained. There was no indication of any stimulation of glucocorticoid synthesis since the plasma cortisol concentration in these animals was similar to that of the controls (11). Nevertheless, excessive dietary vitamin C did depress adrenal cholesteryl ester concentration, though not to the extent observed in the dificient guinea pigs. Moreover, the decrease in adrenal cholesteryl ester was reasonably uniform, with relatively few small changes occurring in fatty acid distribution in this fraction. This result was quite unexpected since it has been postulated that ascorbic acid inhibits the synthesis and release of glucocorticoids by the adrenal (8-10). Lack of utilization of adrenal cholesteryl esters resulting in an increase in their concentration was anticipated in the animals fed the diet excessively supplemented with vitamin C but was not observed.

It is apparent that dietary vitamin C exerts an effect on adrenal cholesterol metabolism, with both deficiency and excess inducing a decrease in total cholesteryl esters and the deficiency also modifying the fatty acid profile of the esters. This latter observation is consistent with stress-induced utilization of the esters for cortisol synthesis, with some degree of specificity for the esters utilized. The effects of the high dietary level of vitamin C are not readily accounted for.

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Eicosenoic and Docosenoic Acid Incorporation in Serum Lipoproteins in Rats Fed Rapeseed Oil

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ABSTRACT

Rats were fed rapeseed oil rich in eicosenoic (20:1) and docosenoic (22:1) acids for 7 days, and the fatty acid composition of the lipid classes of serum and serum lipoproteins was determined. Concentrations of 20:1 and 22:1 acids in the lipid classes were variable, especially among lipoproteins, and were a direct function of the alimentary state of the animal. The results suggest differences in the incorporation of the above acids among the major lipoprotein types and various lipid classes within a given lipoprotein type. The quick partial disappearance of very low density lipoproteins (VLDL) and of low density lipoproteins (LDL) containing 20:1 and 22:1 acids upon starvation and the preferential incorporation of these acids in the triacylglycerols of high density lipoproteins (HDL) are discussed.

INTRODUCTION

Several workers have demonstrated that young rats fed rapeseed oil showed a transient lipidosis and residual fibrosis of cardiac muscle (1-5). The effect was related to the long chain fatty acids of that oil and included an accumulation of eicosenoic (20:1) and docosenoic (22:1) acid in cardiac and other tissues (1-5). As there appears to be a correlation between cardiac fatty infiltration and circulating lipids in rats (6), it appeared to us of interest to investigate in detail the serum lipids in rats fed rapeseed oil diets high in 20:1 and 22:1 acid. In this paper, the incorporation of 20:1 and 22:1 acids in serum lipoproteins and their lipid subfractions is described.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing ca. 200 g were placed on an experimental diet consisting of rat pellets containing 20% rapeseed oil by weight. The rapeseed oil had a % fatty acid composition as follows: palmitic 3.6, stearic 1.9, oleic 12.0, linoleic 14.6, linolenic 1.7, 20:1 17.2, and 22:1 46.5. After 1 week of feeding the experimental diet, the animals were separated into two groups. One group was bled without prior starvation; the other group was bled after 16 hr food deprivation. Controls were maintained on Standard Purina Laboratory Chow, and they were bled without prior starvation. Three such experiments were done at different time periods.

Blood was obtained from the abdominal aorta of the rats anesthetized with sodium pentobarbital. The blood was pooled (5-6 rats) according to group and allowed to clot. After centrifugation, the upper milky layer was removed and washed three times in 0.15 M NaCl by recentrifugation. Lipoproteins were then separated by sequential ultracentrifugation at progressively increased densities in a Spinco Model L ultracentrifuge as described by Havel et al. (7). The lipoproteins were separated into three density classes: VLDL (d < 1.006), LDL (d 1.019-1.063), and HDL (d 1.063-1.21). The VLDL, LDL, and HDL fractions were washed by recentrifugation at their respective upper densities of 1.006, 1.063, and 1.21 to remove any possible contaminating serum proteins. This washing procedure was repeated one to

TABLE I

Distribution of Serum Lipids[a]

Group	Triacylglycerols	Phospholipids	Cholesterol
Control (3)[b]	34 ± 2.1	90 ± 4.2	67 ± 3.1
Rapeseed oil Unfasted (4)	75 ± 2.8[c]	93 ± 3.9	69 ± 2.8
Rapeseed oil Fasted (4)	28 ± 1.8	87 ± 3.1	70 ± 1.6

[a]Each value represents the mean ± SEM, expressed as mg per dl serum.

[b]Numbers in parentheses indicate number of observations.

[c]Significantly different from controls (P<0.05).

TABLE II

Fatty Acid Composition of Serum Lipids[a]

Fatty acid[c]	Rapeseed oil unfasted (4)[b]			Rapeseed oil fasted (4)			Control (3)		
	CE	TG	PL	CE	TG	PL	CE	TG	PL
16:0	8.5 ± 0.6	13.9 ± 1.2	20.2 ± 2.0	10.8 ± 1.0	14.0 ± 1.3	20.7 ± 2;1	11.0 ± 1.0	14.5 ± 1.2	20.5 ± 2.0
18:0	4.2 ± 0.8	5.9 ± 0.6	21.1 ± 1.9	3.3 ± 0.3	7.6 ± 0.8	22.6 ± 1.9	3.8 ± 0.2	5.6 ± 0.9	22.8 ± 2.1
18:1	21.1 ± 1.9	28.6 ± 2.3[d]	7.8 ± 1.0	19.9 ± 2.1	25.3 ± 2.2	8.8 ± 1.0	19.5 ± 1.8	20.4 ± 2.5	7.8 ± 1.0
18:2	21.9 ± 2.1	16.6 ± 1.8[d]	21.9 ± 1.9	20.9 ± 2.2	20.1 ± 2.1[d]	16.6 ± 1.3	22.3 ± 2.1	26.0 ± 2.3	18.5 ± 1.6
18:3	3.0 ± 0.9	3.1 ± 0.3		2.1 ± 0.2	2.2 ± 0.3		1.8 ± 0.2	2.1 ± 0.2	
20:1	2.2 ± 0.3[d]	5.8 ± 0.8[d]	4.3 ± 0.3[d]		3.0 ± 0.2[d]				
20:4	29.9 ± 1.9[d]	1.4 ± 0.2[d]	9.9 ± 0.8[d]	38.4 ± 3.0	1.1 ± 0.1[d]	19.1 ± 1.9	43.0 ± 3.8	5.0 ± 0.6	16.8 ± 1.5
22:1	3.5 ± 0.3[d]	19.6 ± 1.9[d]	4.1 ± 0.2[d]		3.9 ± 0.6[d]				
24:1	2.0 ± 0.3[d]	3.1 ± 0.4[d]	4.2 ± 0.3[d]	1.5 ± 0.2[d]	2.3 ± 0.3[d]	2.3 ± 0.5[d]			1.1 ± 0.6
22:6	4.8 ± 0.4		6.2 ± 0.4	4.2 ± 0.3		5.9 ± 0.8	4.2 ± 0.2		5.8 ± 0.4

[a]Percent of total fatty acid in the mixture ± SEM. Minor components have been omitted.

[b]Numbers in parentheses give the number of observations.

[c]The number before and after the colon indicates the number of carbon atoms and double bonds, respectively.

[d]Significantly different from controls ($P<0.05$).

two times until lipoprotein fractions were free of albumin, as demonstrated by immunodiffusion. The LDL and HDL fractions were dialyzed against 0.15 M NaCl containing 0.05% EDTA, to remove excess salt. Samples of lipoprotein fractions were subjected to agarose electrophoresis (8) in order to confirm proper gradient separation in the ultracentrifugal fractions in serum.

Hearts, livers, and epididymal fat pads were removed immediately, washed in ice-cold 0.15 M NaCl, and homogenized in distilled water using a Waring blender.

Lipids from serum lipoproteins, tissues, and other preparations were extracted and purified following the method of Bligh and Dyer (9) after the addition of ^{14}C-labeled tripalmitin as an internal standard. They were dried under vacuo at 40 C and then redissolved in chloroform. Suitable aliquots of the chloroform extract were applied to microcolumns of silicic acid (Unisil, Clarkson Chemical Co., Williamsport, PA). Neutral lipids in the chloroform eluate were separated further by Silica Gel G thin layer chromatography (TLC) using a solvent system of heptane-isopropyl ether-glacial acetic acid (60:40:1, v/v). After migration, the plates were dried under nitrogen, then sprayed with Rhodamine 6G, and the lipids were identified under UV light. Each fraction was then scraped and quantitatively transferred into methylation tubes. Methylation was carried out as previously described (10,11). Fatty acid methyl esters were quantitated by gas liquid chromatography (GLC) in the presence of methyl pentadecanoate as the internal standard. Two stainless-steel columns (6 ft x 2 mm) were employed. One column was packed with 15% EGSS-X on 80-100 Chromosorb W and column, detector, and flash heater temperatures were at 180 C, 250 C, and 240 C, respectively. The second column was packed with 3% SE-30 on 60-80 Chromosorb W (1:6), and its temperature was programmed from 180 C to 220 C at 2.5/min. The silicone column was used to separate arachidonic from 22:1 acid, a mixture which did not, in our hands, separate well on polar columns. The analysis by chain length on polyester and silicone columns generally agreed within one unit %. Peaks were quantitated by use of a minicomputer (Hewlett-Packard 2100 Data System) and identified on the basis of retention time of known methyl esters. The relative error of the technique was 1.5%.

Cholesteryl esters, free cholesterol, triacylglycerols, and phospholipids were determined as previously described (12-14). Total protein was determined according to Lowry (15). Total lipoprotein was obtained by adding total lipid

TABLE III

Serum Lipoproteins and Chemical Composition[a,b]

Group	CE	TG	C	PL	Protein
VLDL					
Control (3)[c]	55.2 ± 0.9	36.8 ± 2.1	2.8 ± 0.3	1.5 ± 0.2	6.0 ± 0.8
Rapeseed oil Unstarved (4)	3.8 ± 0.8	56.5 ± 2.4[d]	2.9 ± 0.4	1.8 ± 0.3	11.2 ± 0.7
Rapeseed oil Starved (4)	3.8 ± 0.6	39.2 ± 2.5	3.2 ± 0.2	1.3 ± 0.2	10.4 ± 0.4
LDL					
Control (3)	28.3 ± 1.2	8.8 ± 1.4	5.6 ± 0.8	12.0 ± 1.2	14.6 ± 1.8
Rapeseed oil Unstarved (4)	26.2 ± 1.4	16.4 ± 2.1[d]	6.8 ± 0.9	13.8 ± 1.4	13.8 ± 1.0
Rapeseed oil Starved (4)	32.3 ± 1.5	6.8 ± 1.6	6.0 ± 1.1	14.2 ± 0.8	14.8 ± 1.1
HDL					
Control (3)	58.9 ± 2.3	8.9 ± 0.8	8.9 ± 0.4	59.1 ± 2.3	89.3 ± 2.6
Rapeseed oil Unstarved (4)	62.3 ± 2.4	12.1 ± 1.2[d]	7.6 ± 0.8	62.5 ± 3.1	91.5 ± 2.1
Rapeseed oil Starved (4)	59.4 ± 2.1	7.4 ± 0.9	9.5 ± 0.5	63.4 ± 2.5	84.6 ± 3.2

[a]Values are the mean ± SEM, expressed as mg per dl serum.

[b]Abbreviations are as follows: VLDL = very low density lipoproteins, LDL = low density lipoproteins, HDL = high density lipoproteins, CE = cholesteryl esters, TG = triacylglycerols, C = cholesterol, and PL = phospholipids.

[c]Number in parentheses indicates number of observations.

[d]Significantly different from controls ($P<0.05$).

and total protein in each fraction.

Sections of hearts were stained with hematoxylin and erosin for routine histological examination (16). Whenever fatty infiltration was presumed, frozen sections stained with Sudan red were used to confirm the diagnosis.

Statistical analyses were performed by Student's t-test (17).

RESULTS

After 1 week on the rapeseed oil diet, histological examinations revealed the characteristic cardiac lipidosis which has been extensively described (1,16). For the same time interval, fatty acid analysis of cardiac lipid subclasses in rats fed the rapeseed oil diet showed that the 20:1 and 22:1 acid were incorporated in triacylglycerols to the amounts of 2.8 and 42.0% of total fatty acids, respectively; in cholesteryl esters, 1.7 and 27.8%; and in phospholipids, 1.6 and 3.6%. These results are in agreement with previously published data (1,5,18,19). In liver triacylglycerols from rats fed rapeseed oil, the 20:1 and 22:1 acid concentration was 7.3 and 4.7%, respectively; in cholesteryl esters, 5.5 and 3.8%; and in phospholipids, 1.3 and 1.0%. In adipose tissue triacylglycerols, 20:1 and 22:1 acids comprised 7.1 and 5.9% of total fatty acids, respectively. Starvation for 16 hr did not change any of the above values.

The concentration of various serum lipids is shown in Table I. Rats fed rapeseed oil showed an increase in triacylglycerols which was significant ($R < 0.05$) only in the unfasted animals. No change in cholesterol or phospholipid level was observed.

Table II shows the fatty acid composition of serum lipid fractions in unfasted and fasted rats fed the rapeseed oil diet as they compare to controls. Dietary 20:1 and 22:1 acids clearly accumulated into all serum lipid classes in unfasted rats. These levels were considerably less than the levels of 20:1 and 22:1 acid content of the diet. They were also lower than those of the cardiac lipids but significantly greater than the hepatic and adipose tissue lipids. In blood and cardiac tissue, the 22:1 acid concentration was higher than the 20:1 acid, but the reverse was true for hepatic and adipose tissue lipids. In rats fasted for 16 hr, dietary 20:1 and 22:1 acids were found only in serum triacylglycerols.

It is of interest to note that the incorporation of 20:1 and 22:1 acids in serum lipids caused a decrease in 18:2 and 20:4 acids, suggesting that differences in concentrations of fatty acids in serum lipids after rapeseed oil feeding resulted apparently from substitution

TABLE IV

Fatty Acid Composition of Serum Lipoproteins in Unfasted Rats Fed Rapeseed Oil for 1 Week[a,b]

Fatty acid	CM			VLDL			LDL			HDL		
	CE	TG	PL	CE	TG	PL	CE	TG	PL	CE	TG	PL
16:0	9.9 ± 1.5	14.7 ± 1.0	18.4 ± 1.6	9.0 ± 0.6	16.6 ± 1.3	17.6 ± 1.6	11.7 ± 1.2	11.8 ± 1.8	19.5 ± 1.9	7.2 ± 0.6	13.2 ± 0.9	18.8 ± 1.8
18:0	4.4 ± 0.3	4.6 ± 0.6	16.6 ± 1.3	5.3 ± 0.4	4.6 ± 0.3	20.6 ± 2.2	8.2 ± 0.9	6.8 ± 0.6	20.5 ± 1.5	2.5 ± 0.2	9.5 ± 0.8	22.5 ± 2.2
18:1	28.7 ± 1.9	17.5 ± 1.8	16.0 ± 1.2	39.7 ± 3.0	19.2 ± 2.0[c]	17.1 ± 1.8[c]	21.9 ± 2.2	38.9 ± 3.0[c]	14.5 ± 0.7	9.5 ± 0.6	38.2 ± 3.1[c]	8.2 ± 0.9
18:2	16.5 ± 1.2	18.3 ± 1.9	18.0 ± 1.8	16.0 ± 1.9	17.8 ± 2.1[c]	15.7 ± 1.8	24.3 ± 2.2	18.6 ± 2.1[c]	16.4 ± 1.2	23.6 ± 2.0	12.8 ± 1.3[c]	18.1 ± 2.1
20:1	4.6 ± 0.4	6.8 ± 0.9	4.2 ± 0.6	4.2 ± 0.3[c]	6.5 ± 0.4[c]	7.6 ± 1.3[c]	2.2 ± 0.3[c]	5.9 ± 1.0[c]	5.2 ± 1.0[c]	3.3 ± 0.6[c]	5.7 ± 1.1[c]	4.6 ± 0.6[c]
20:4	21.2 ± 1.9	1.7 ± 0.3	12.9 ± 1.7	9.5 ± 1.0[c]	3.2 ± 0.3[c]	11.1 ± 1.8[c]	21.6 ± 2.0[c]	4.8 ± 0.6[c]	8.2 ± 1.0[c]	32.2 ± 3.6[c]	3.2 ± 0.5[c]	13.2 ± 1.3[c]
22:1	7.3 ± 1.2	26.8 ± 2.8	9.4 ± 0.8	7.6 ± 0.8[c]	22.8 ± 2.2[c]	7.2 ± 0.6[c]	1.4 ± 0.2[c]	12.7 ± 1.0[c]	6.4 ± 0.4[c]		15.8 ± 1.3[c]	
22:6	1.2 ± 0.4		4.7 ± 0.5	1.9 ± 0.2	2.6 ± 0.1	4.0 ± 0.2	6.8 ± 0.3		6.4 ± 0.2	4.6 ± 0.4		6.8 ± 0.2

[a]Values are the mean of four observations ± SEM, expressed as percent of total fatty acid in the mixture. Minor components have been omitted.

[b]See Tables II and III for identification of abbreviations.

[c]Significantly different from control values ($P < 0.05$).

of 20:1 and 22:1 acid for 20:4 in cholesteryl esters and phospholipids and for 18:2 and 20:4 in triacylglycerols . An increase in 18:1 and 24:1 was observed which may be attributed to transformation (20) of 22:1 by chain shortening or lengthening, respectively.

Table III shows the lipid composition of isolated VLDL, LDL, and HDL in rats fed the rapeseed oil and control diets. Each of the lipoproteins was identified by electrophoresis on agarose gel (8) and by chemical composition studies. From control rats, compositional studies gave the following results for various protein to lipoprotein ratios: 11.5% for VLDL, 21.5% for LDL, and 40.9% for HDL, respectively. These values were in agreement with those reported by Lombardi and Ugazio (21). The results indicate that in unfasted rats, there is an increase in triacylglycerols of all types of lipoproteins after rapeseed oil feeding. In fasted animals, no significant difference was observed when lipid classes of any lipoprotein type were compared to the corresponding controls.

Table IV presents the fatty acid composition of the individual lipoproteins and their lipid subfractions in unfasted rats receiving the rapeseed oil diet. Dietary 20:1 and 22:1 acids were incorporated into all fractions with the exception of 22:1 acid which did not incorporate into the cholesteryl esters and phospholipids of HDL. In the other lipoproteins, both acids incorporated in the order: triacylglycerols > phospholipids > cholesteryl esters. The triacylglycerols of chylomicrons and VLDL had almost identical % of 20:1 and 22:1 acids, as well as a similar overall fatty acid composition. The triacylglycerols of LDL and HDL also had similar fatty acid composition. By comparing Tables IV and V (control), it becomes apparent that the increase in 20:1 and 22:1 acids resulted in a decrease in 18:2 and 20:4 with an occasional increase in 18:1 and 24:1 acids.

In fasted rats (Table VI), 20:1 and 22:1 acids were present in only the triacylglycerol fraction of HDL. Other fatty acids displayed consistent differences between lipoproteins, particularly in cholesteryl esters. Phospholipids did not show these differences.

The absolute concentration levels of 20:1 and 22:1 acids in triacylglycerols of serum and serum lipoproteins are given in Table VII. These levels were determined with the aid of two internal standards (^{14}C tripalmitin and decapentanoic acid). Table VII also gives the % deposition of dietary 20:1 and 22:1 into serum and serum lipoprotein triacylglycerols. No determinations were made for phospholipids and cholesteryl esters. Since 22:1 is an exogenous fatty acid, the presence of this acid in

TABLE V

Fatty Acid Composition of Serum Lipoproteins in Control Rats[a,b]

Fatty acid	VLDL			LDL			HDL		
	CE	TG	PL	CE	TG	PL	CE	TG	PL
16:0	10.5 ± 0.8	18.8 ± 1.2	18.8 ± 1.2	9.3 ± 0.4	13.5 ± 1.2	20.3 ± 1.8	3.8 ± 0.3	18.2 ± 1.2	22.5 ± 1.8
18:0	4.2 ± 0.4	4.2 ± 0.2	19.2 ± 1.3	7.2 ± 1.0	4.2 ± 0.6	19.9 ± 2.1	1.8 ± 0.2	8.8 ± 1.8	22.3 ± 2.0
18:1	37.0 ± 2.0	21.8 ± 1.9	15.0 ± 1.4	22.8 ± 1.8	30.9 ± 2.1	11.2 ± 1.8	10.5 ± 0.8	31.2 ± 2.9	8.3 ± 1.1
18:2	20.5 ± 1.6	28.5 ± 2.0	15.2 ± 1.2	23.5 ± 2.1	26.6 ± 2.4	18.2 ± 1.9	22.2 ± 1.8	19.8 ± 1.8	23.1 ± 1.9
20:4	23.2 ± 1.1	7.3 ± 0.6	20.4 ± 2.3	19.9 ± 1.5	8.2 ± 1.0	18.2 ± 1.9	41.5 ± 2.6	7.2 ± 1.2	18.8 ± 1.9
22:6	1.3 ± 0.5	2.9 ± 0.3	7.2 ± 1.1		1.3 ± 0.2	6.5 ± 0.8	3.6 ± 0.8		4.8 ± 0.2

[a]Values are the mean ± SEM, expressed as percent of total fatty acid in the mixture. Minor components have been omitted. The results are from three observations.
[b]See Tables II and III for identification of abbreviations.

TABLE VI

Fatty Acid Composition of Serum Lipoproteins in Fasted Rats Fed Rapeseed Oil for 1 Week[a,b]

Fatty acid	VLDL			LDL			HDL		
	CE	TG	PL	CE	TG	PL	CE	TG	PL
16:0	9.1 ± 0.9	19.5 ± 1.9	21.4 ± 1.9	13.4 ± 0.9	16.4 ± 1.8	21.2 ± 1.8	8.4 ± 0.6	16.8 ± 1.8	16.8 ± 1.6
18:0	1.5 ± 0.2	2.5 ± 0.2	20.2 ± 1.8	6.1 ± 0.3	2.9 ± 0.2	22.4 ± 1.8	1.9 ± 0.3	10.3 ± 1.9	22.1 ± 2.0
18:1	33.6 ± 1.8	21.3 ± 1.8	14.8 ± 1.1	22.6 ± 2.0	32.2 ± 2.2	9.2 ± 0.9	7.5 ± 0.8	23.6 ± 1.8[c]	9.4 ± 1.2
18:2	21.4 ± 1.82	24.6 ± 1.7	14.2 ± 1.1	21.9 ± 1.9	27.5 ± 2.5	16.3 ± 1.2	21.5 ± 1.8	13.7 ± 1.9[c]	18.5 ± 1.7
20:1		1.9 ± 0.2[c]			2.7 ± 0.3[c]			6.4 ± 0.6[c]	
20:4	20.9 ± 1.2	3.4 ± 0.8[c]	19.6 ± 1.8	22.0 ± 1.2	5.1 ± 1.0[c]	21.4 ± 1.8	47.5 ± 3.1	3.3 ± 0.5[c]	18.6 ± 1.8
22:1		1.5 ± 0.2			2.1 ± 0.4			16.4 ± 1.6[c]	
22:6	1.3 ± 0.5	5.4 ± 0.4	6.9 ± 1.2		5.9 ± 0.6	4.8 ± 0.5		2.2 ± 0.4	4.2 ± 0.6

[a]Values are the mean ± SEM, expressed as percent of total fatty acids in the mixture. Minor components have been omitted. Results are from four observations.
[b]See Tables II and III for identification of abbreviations.
[c]Significantly different from controls ($P<0.05$).

TABLE VII

Incorporation of 20:1 and 22:1 Acids into Triacylglycerols[a,b]

Fatty acid	Serum	VLDL	LDL	HDL
Unfasted				
20:1	0.26(0.13)[c]	0.20(0.1)	0.031(0.02)	0.009(0.004)
22:1	0.72(0.13)	0.52(0.1)	0.084(0.02)	0.022(0.004)
Fasted				
20:1	nd[d]	nd	nd	nd
22:1	0.026(0.005)	nd	nd	0.010(0.002)

[a]Values express mg of fatty acid incorporated in each rat. Each value represents the mean of two observations. Rats of 200-250 g weight give ca. 4.2 ml serum.

[b]See Tables II and III for identification of abbreviations.

[c]Numbers in parentheses give the percent incorporation of the daily consumed dietary fatty acid (see text).

[d]Not determined because of insufficient quantity.

lipid fractions could be related to dietary consumption of 22:1 acid.

The latter was determined as follows: In one experiment (24 rats), the consumption of the rapeseed oil diet was measured and it was found to be 6.4 ± 0.8 (mean ± SEM) g/rat/day. The diet contained 20% rapeseed oil, of which 90% was fatty acids. Of the total weight of fatty acids, 46.5% was 22:1 and 17.2% was 20:1. Hence, the daily consumption of 22:1 acid was 0.54 g and of 20:1 acid was 0.20 g per rat.

DISCUSSION

A 0.13% of each daily consumed 20:1 and 22:1 acid was deposited in serum triacylglycerols, and this result suggested that rats fed rapeseed oil incorporate a very small proportion of 20:1 and 22:1 acid in their blood lipids. This finding is in agreement with previous work which showed that rats, except for their cardiac tissue, incorporate only a small proportion of dietary 22:1 into their tissues (1-5). Furthermore, a recent acute feeding experiment (20) has demonstrated that at 30 min after injection of ^{14}C-labeled 22:1 acid intravenously into rats, only 1.3% of the injected acid was incorporated as 22:1 acid into blood triacylglycerols.

The current results demonstrate that the accumulation of dietary 20:1 and 22:1 acids in serum, lipoproteins, and their lipid subfractions is highly dependent on the alimentary condition of the animal. Unfasted rats incorporated 20:1 and 22:1 acids in all lipoproteins and their lipid subclasses, whereas starved rats had 20:1 and 22:1 acids only in HDL triacylglycerols.

It is well known that blood lipids are subjected to diurnal variation when animals digest fat-containing meals (22,23). It is also reported that the effect depends on dietary fat, and it is mostly noticeable in VLDL and LDL (11,24,25). In previous results (11), onset of starvation left appreciable amounts of dietary fatty acids in plasma VLDL and LDL. Contrary to this, practically no 20:1 and 22:1 acids were seen in the VLDL and LDL isolated from starved animals in this study. This suggests that the rapeseed oil diet had caused an increased rate of clearance of chylomicrons and VLDL containing 20:1 and 22:1 acids. Indeed, Struijk et al. (26) have demonstrated that diets rich in 22:1 acid, when fed to rats for 1 week, caused an increased postheparin lipoprotein lipase activity in vitro, resulting in a rapid degradation of VLDL. Whether the in vitro situation is applicable in vivo is presently unknown, and further studies will be required to solve this problem.

The striking finding of the present study is the deposition of 20:1 and 22:1 acids in the triacylglycerol fraction of HDL both in unfasted and fasted animals. It is well known that HDL comprise a highly heterogeneous class of substances (27) which reflect structurally and metabolically distinct subclasses. Work performed in other laboratories (27-30) indicated that at least part of HDL in plasma originates from chylomicrons and VLDL. To date, it is unknown whether properties of the precursor chylomicron and VLDL bear a predictable relationship to the physicochemical properties (including flotation rates) of the products of their processing. An interesting possibility, that the fatty acid composition of the precursor chylomicron or VLDL is a dominant factor on the density range of the post-lipolysis particles, is presently under active investigation in our laboratory. Alternate possibilities for the synthesis of HDL particles rich in triacylglycerols containing 20:1 and 22:1 acids exist, and we are currently investigating the liver endo-

plasmic reticulum pathway (31).

The present data also suggest a slow catabolic rate for HDL triacylglycerols rich in 20:1 and 22:1 acids during starvation. Similar observations were made in previous studies (Szlam and Sgoutas, unpublished data), where HDL triacylglycerols rich in docosadienoic acids were found in the serum of rats fed 20% partially hydrogenated soybean fat with 35% of trans monoenoic acids. Half-lives exceeding 12 hr were indicated in contrast to 6 hr half-lives reported for the average HDL (32). Other investigators have also reported a preferential enrichment of HDL triacylglycerols in saturated fatty acids in rats fed various diets (33). Experimentation dealing with this question is currently being pursued.

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Evidence for Singlet Oxygen in the Air Oxidation of Surface Adsorbed 2,5-Diphenylfuran

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ABSTRACT

Heating 2,5-diphenylfuran on a silicon or titanium dioxide surface results in its conversion to *cis*-dibenzoylethylene. This is considered an indication of singlet oxygen intermediacy. No significant reaction occurs with the solid or in solvents other than acetone. A singlet oxygen quencher inhibits the reaction, and a free radical scavenger has little effect. The differences between the surface reaction and lack of reactivity in solution may arise from several causes. These include local forces involved in adsorption, topographical and geometric effects, and metal catalysis. Knowledge of factors influencing singlet oxygen formation under nonphotochemical conditions has potential practical application.

INTRODUCTION

When unsaturated lipid is spread on silica gel and allowed to oxidize, the reaction rate (1) and product distribution (2-8) differ from bulk phase autoxidation. Moreover, there is little or no induction period (9). It is known that singlet and triplet oxygen react with unsaturated materials at different rates (10) and form different products (10,11). Singlet oxygen reactions have no induction period (10,12) compared to traditional autocatalytic autoxidation (13). Our observation (Slawson, unpublished) of light emission (wavelength undetermined) from silica gel that had been heated to about 150 C and cooled to room temperature, suggested the possibility of the conversion of triplet to singlet oxygen by a silica gel surface. To test this possibility, we examined the oxidation of 2,5-diphenylfuran (2,5-DPF) adsorbed on silica gel. This is known to give a unique product on reaction with singlet oxygen.

2,5-DPF reacts rapidly with singlet oxygen (14,15). The photooxidation product is *cis*-dibenzoylethylene (*cis*-DBE) (16). The formation of this compound in an aqueous system has been used as evidence for the production of singlet oxygen during an enzymatic reaction (17), and should occur in the same manner in a nonenzymatic reaction.

MATERIALS

2,5-DPF (Eastman, Kingsport, IN), *trans*-dibenzoylethylene (*trans*-DBE) (Aldrich, Milwaukee, WI), nickel dibutyldithiocarbamate (NiDBC) (Pfaltz and Bauer, Flushing, NY), Sensitox (Hydron, New Brunswick, NJ), methyl linoleate (me $C_{18:2}$) (Nu Chek Prep, Elysian, MN), methyl eicosanoate (Me C_{20}) (Nu Chek Prep, Elysian, MN), and reagent grade solvents were used as received. Titanium dioxide was Baker and Adamson 1283. Silica gel was Baker 3405 (100 ppm Fe, 120 ppm Mg, 45 ppm Ca, and a trace of copper), or Baker 1-3405 (Phillipsburg, NJ) (100 ppm Fe, 100 ppm Mg, 250 ppm Ca, and a trace of copper). We consider 0.1 ppm or less of Cu to be a trace. 2,6-Di-*tert*-butyl-p-cresol (BHT) (Koppers, Pittsburg, PA) was recrystallized from pentane. *Cis*-DBE was prepared by nitric acid oxidation of 2,5-DPF (18).

METHODS

Although *cis*-DBE is converted to the *trans*-isomer in some gas liquid chromatographic (GLC) systems, it was stable when chromatographed in a Varian 2100 equipped with a 6 ft glass column packed with 3% OV 101 on 100/120 Gas Chrom Q. The retention time of 2,5-DPF was about 3 min at 220 C. *Cis*- and *trans*-DBE emerged shortly after, in that order. They were identified by comparison of their retention time with those of authentic *cis*- and *trans*-DBE. The GLC response, on a weight basis, was within 10% of that of Me C_{20}. No corrections were made.

Breakdown of the endoperoxide formed by reaction of 2,5-DPF with singlet oxygen can under some conditions regenerate the parent hydrocarbon (19) rather than carbonyl compounds (20). Since it seemed desirable to verify the product of singlet oxygen reaction, GLC was used for identification.

Singlet oxygen is frequently generated by photochemical reaction in the presence of dyes. Immobilization of the dye on an insoluble support simplifies analysis or purification of a reaction mixture because simple filtration removes the dye (21). Rose Bengal is com-

TABLE I

Conversion of DPF to *cis* and *trans* DBE on Solid Supports—Dark Incubation at 55-60 C

	Control on glass[a]		TiO_2[b]		SiO_2[c]	
	cis[d]	*trans*	*cis*	*trans*	*cis*	*trans*
2 days	---	---	---	---	0.71	1.46
4 days	---	0.23	---	0.14	1.11	2.83
8 days	---	0.15	---	0.13	2.38	4.15
42 days	---	0.17	1.02	0.78	38.6	21.1
71 days	---	0.36	4.25	3.83	35.3	50.6

[a]3.9 mg 2,5-DPF in centrifuge tube.
[b]3.9 mg 2,5-DPF on 0.6 g TiO_2.
[c]3.9 mg 2,5-DPF on 0.6 g SiO_2.
[d]GLC ratio: $\frac{\textit{cis}\text{-(or }\textit{trans}\text{-)DBE}}{\text{2,5-DPF} + \textit{cis}\text{-DBE} + \textit{trans}\text{-DBE}}$ (100)

mercially available on a polymeric support (Sensitox). In a Pyrex Erlenmeyer flask, a solution of 3 mg 2,5-DPF, 3 mg Me C_{20}, and 28 mg Sensitox in about 5 ml CCl_4 was stirred in sunlight for 45 min, then filtered. Only *cis*-DBE and Me C_{20} were found on GLC. The yield was below theory. The loss is attributed to reaction of singlet oxygen with *cis*-DBE.

Ozonized diisopropyl ether decomposes to release singlet oxygen identified by bleaching of rubrene (22) and by light emission (23). A carbon tetrachloride solution of 0.3 mg 2,5-DBF and 0.3 mg Me C_{20} was chilled to -20 C. About 2 ml of ozonized diisopropyl ether (-70 C) was added, and the mixture was warmed to room temperature. 2,5-DPF disappeared, and *cis*-DBE was formed. The yields were low in the presence of excess singlet oxygen.

Silica gel or titanium dioxide was coated with 2,5-DPF by slurrying with acetone, adding an acetone solution of the furan, and evaporating the solvent at room temperature with rotation under partial vacuum. The freely flowing powder was transferred to a clean Erlenmeyer flask. Controls were prepared by pipetting an acetone solution of 2,5-DPF into the bottom of a centrifuge tube and evaporating the solvent with nitrogen. Coated samples and controls were heated at 55-60 C in an unlighted oven. At intervals, portions of the coated adsorbents were removed and extracted with acetone. The controls were sampled by adding acetone and removing an aliquot. The solvent was removed with a stream of nitrogen, and samples and controls were returned to the oven. All solutions were handled under diffuse daylight.

RESULTS

The pronounced influence of type of support on the conversion of 2,5-diphenylfuran to *cis*- and *trans*-DBE is shown in Table I.

The significant findings are that no *cis*-DBE was found in the controls, and that it appeared on both silica gel and titanium dioxide, although more rapidly on silica gel. We believe this is evidence for production of singlet oxygen on these absorbents. Several factors may influence the relative ratio of the components; among them are number and activity of adsorption sites and surface area of the adsorbents. Volatility from the surface may be important. Although the melting points of 2,5-DPF, *cis*-DBE, and *trans*-DBE are higher than the incubation temperature of 55-60 C (87-89 C, 129-131 C, and 108-111 C, respectively), the vapor pressures may be significant.

The effect of a singlet oxygen quencher was investigated. NiDBC quenches singlet oxygen $^1\Delta_g$ rapidly (12). It did not elute from our gas chromatographic column, probably due to decomposition, but it did not interfere with quantitation of a standard mixture containing 2,5-DPF, NiDBC, and Me C_{20}.

Silica gel was coated with 2,5-DPF, NiDBC, and Me C_{20} (0.98, 1.1, and 0.81 g/g silica). It was heated in the dark at 55-60 C, along with solid and liquid (acetone) controls (i.e., substrate but no silica). After 33 days, a small amount of *cis*- and *trans*-DBE (about ½% of each) was found in the material eluted from the silica gel. Traces of *trans*-DBE appeared in the controls. By 47 days, the amount of *cis*-DBE was 1.0%, with 1.3% *trans*-DBE. Again, only traces of *trans* were found in the controls. This is significantly less *cis*-DBE than producted in a similar interval at higher coverage (Table I), where rate of conversion should be slower. (Table II shows the inverse correlation between loading and rate of conversion.) NiDBC has almost completely eliminated production of *cis*-

TABLE II

Effect of Coverage on Products of 2,5-DPF Oxidation on Silica

	GLC areas (cm^2)			
	2,5-DPF	*cis*-DBE	*trans*-DBE	$\frac{cis\text{-(or } trans\text{-)DBE}}{2,5\text{-DPF} + cis\text{-DBE} + trans\text{-DBE}}$ (100)
Washed SiO_2, low load (0.1 mg/g SiO_2)	16.3	0.99	0.92	10.5
Washed SiO_2, high load (1.0 mg/g SiO_2)	33.4	0.20	0.37	1.7
Stock SiO_2, low load (0.1 mg/g SiO_2)	15.6	1.20	1.53	14.9
Stock SiO_2, high load (1.0 mg/g SiO_2)	32.3	0.38	0.38	2.3

DBE on silica gel. However, NiDBC also prevented the dark autoxidation of methyl linoleate in benzene. A benzene solution containing NiDBC, Me $C_{18:2}$, and Me C_{20} (3.46, 4.44, 2.21 mg/ml, respectively) and a control solution containing Me$C_{18:2}$ and Me C_{20} (4.68 and 2.35 mg/ml) were heated in the dark at 55-60 C. By 13 days, oxidation in the control was evidenced by a decrease in the ratio $\frac{\text{Me } C_{18:2}}{\text{Me } C_{20}}$. After 26 days, the value was 72% of the original ratio. There was no significant change in the solution containing NiDBC.

Failure of the NiDBC to function solely as a singlet oxygen quencher prompted repetition of the experiment incorporating a free radical scavenger in the 2,5-diphenylfuran coating of the silica gel. BHT is an efficient free radical scavenger having little or no effect on singlet oxygen reactions (11). Silica gel coated with 2,5-DPF and BHT (0.1 mg each/g silica) was incubated in the dark at 55-60 C. After 15 days, both *cis*- and *trans*-DBE were found. Although some of the BHT was lost by sublimation or by reaction, the amount remaining was about 25% of the 2,5-DPF + *cis*-DBE + *trans*-DBE. More BHT than *cis*-DBE was found. *Cis*- and *trans*-DBE were also found on the control silica coated with 2,5-DPF but no BHT. Curiously, the *cis/trans* ratio was higher in the presence of BHT than in the control, suggesting that adsorption at specific sites may influence production of *cis*-DBE.

The possibility that the unreactivity of the solid 2,5-DPF in the control experiments was caused by the low surface area exposed to oxygen was considered. Solutions of 2,5-DPF were incubated and examined for oxidation products.

Solutions of 2,5-DPF (0.01M) containing methyl eicosanoate (Me C_{20}) as an internal standard were sealed in glass vials with sufficient headspace air to furnish at least a tenfold excess of oxygen. Me C_{20}, presumably not oxidized under these mild conditions, was added so that reaction of the furan to form products not seen by GLC would be detected by a decreasing ratio of $\frac{\text{2,5-DPF}}{\text{Me } C_{20}}$. The solvents were chosen to provide variation of polarity and singlet oxygen lifetime.

The tubes were heated at 55-60 C in the dark for 6 or more weeks. Samples were removed at intervals. GLC of the solution furnished the ratio $\frac{\text{2,5-DPF}}{\text{Me } C_{20}}$ and the appearance of *cis*- or *trans*-DBE. No significant changes were found for carbon tetrachloride, carbon disulfide, benzene, 1.7 M ethanol in benzene, and 1.6 M ethanol + 0.3M H_2O in benzene. The lifetime of singlet ($^1\Delta_g$) oxygen varies from 700 μsec in carbon tetrachloride to 2 μsec in H_2O (24).

However, a change occured in acetone (lifetime $^1\Delta_g$ is 26 μsec). 2,5-DPF was stable for about 3 weeks. *Cis*- and *trans*-DBE then appeared as the ratio $\frac{\text{2,5-DPF}}{\text{Me } C_{20}}$ decreased. Conversion continued until complete disappearance of the furan. We believe this is due to oxidation by accumulated acetone peroxides.

Although thermal autoxidation of 2,5-DPF can yield *cis*-DBE (25), failure to react in all but one of the solvents listed suggests that experimental conditions were insufficient for thermal oxidation.

A possible cause for the reactivity of 2,5-DPF in acetone is the formation of peroxides or hydroperoxides in the acetone itself by free radical processes during the extended incubation. Combination of secondary peroxy radicals is known to form a cyclic transition state from which singlet oxygen is eliminated (26,27). This would provide a continuing supply of reactive oxygen.

This hypothesis was tested by addition of a

free radical scavenger to an acetone solution of 2,5-DPF and Me C_{20}. It should function by preventing free radical production of peroxide, and subsequent singlet oxygen production. One hundred μl portions of distilled acetone containing 2,5-DPF, BHT, and Me C_{20} (3.8, 4.8, 3.1 mg/ml) were sealed in glass tubes and placed in a dark oven at 55-60 C. The volume of headspace air was sufficient to provide a tenfold excess of oxygen for reaction. BHT was present after 6 weeks, no *cis*-DBE was seen, and the ratio $\frac{\text{2,5-DPF}}{\text{Me } C_{20}}$ was identical with the starting ratio.

Silica gel occasionally contains traces of mineral acid sufficient to affect acid sensitive compounds during chromatography. A portion of the stock silica was washed with distilled water in a Soxhlet extractor for 30 hr, air dried, then dried at 78 C overnight. Washed and unwashed silica gel was coated with two loads of 2,5-DPF (deposited from benzene). The "low" and "high" coverages were 0.1 and 1.0 mg 2,5-DPF/g SiO_2, respectively. The coated samples were placed in Erlenmeyer flasks and heated at 55-60 C in the dark for three days, 0.07 mg and 0.7 mg portions of 2,5-DPF in the bottom of two test tubes, corresponding to the total amounts coated on the supports, were incubated as controls. The reaction products were extracted with acetone and evaporated at room temperature with nitrogen. GLC of the solid controls showed traces of *cis*- and *trans*-DBE. Unequivocal production of *cis*-DBE is shown in Table II. It is clear that conversion of 2,5-DPF to *cis*-DBE is not eliminated by a prolonged water washing of the silica gel before coating. (However, the water washing did remove an unidentified extraneous peak extractable by acetone from the stock silica gel). The table shows roughly similar *cis*-DBE and total conversion for the same coverage. Assumption is made that the surface area has not been grossly altered by the treatment. Proportionally more *cis*-DBE is formed at "low" than at "high" coverage. This is consistent with a limited number of sites available for adsorption or capable of catalysis.

Effect of Other Oxidizing Agents

Acetone solutions of three common oxidants were mixed with an acetone solution of 2,5-DPF and Me C_{20}. They were allowed to stand for 3 days in the dark at room temperature. The solutions were extracted with diethyl ether, the extract was washed with water, then dried over anhydrous $MgSO_4$. Traces (<1% conversion) of *cis*- and *trans*-DBE were found in the materials treated with potassium permanganate and hydrogen peroxide. In the reaction with dibenzoyl peroxide, however, 6-10% of the 2,5-DPF was converted to the *cis*-DBE. This production of *cis*-DBE in the presence of dibenzoyl peroxide is consistent with singlet oxygen production by secondary peroxy radicals previously referred to (26,27). Failure to find *cis*-DBE in the solution containing hydrogen peroxide may have been due to failure of the peroxide to produce hydroperoxy-free radicals (superoxide) spontaneously. However, superoxide leads to production of triplet state oxygen and hydrogen peroxide (ibid.), and *cis*-DBE is not produced from 2,5-DPF in the Fenton system (17). *Cis*-DBE was also produced in CCl_4 solutions of benzoyl peroxide and 2,5-DPF.

DISCUSSION

The formation of *cis*-DBE from 2,5-DPF on silica gel or titanium dioxide suggests that singlet oxygen is formed on the surface. This conversion does not occur when solid 2,5-DPF is heated under the same conditions or in solvents other than acetone. The surface reaction is not decreased by a free-radical scavenger. It is inhibited by a singlet oxygen quencher (NiDBC).

Our results show that (a) DPF is essentially unreactive toward ordinary oxygen both in the neat state and in solution (provided that oxidation of the solvent itself does not generate peroxides); (b) DPF on silica gel reacts with oxygen relatively rapidly to give some of the product (*cis*-DBE) characteristic of the homogeneous reaction with singlet oxygen; (c) the surface reaction does not proceed primarily through a free radical mechanism; (d) it is faster with low than with high surface coverage; and (e) acid and trace metal impurities on the surface, if involved, are not removed by water washing. Finding (b) suggests that the mechanism of the surface-catalyzed reaction involves oxygen in a singlet or singlet-like state. It is possible, for example, that metal or other sites catalyze the triplet-singlet oxygen equilibrium, the singlet oxygen than reacting with adjacent DPF. Alternatively, but in low efficiency, singlet oxygen escaping into the gas phase could recollide with the surface to react in a Rideal (28) type mechanism. This mechanism implies that the surface oxidation is activated by at least the triplet-singlet oxygen energy difference (22 kcal/mole for $^3O_2$-$^1\Delta O_2$). Also, however, the singlet oxygen equilibrium population would be low (about 10^{-8} mole fraction of oxygen in this form), and it is questionable whether this is high enough to sustain the observed reaction rate. A variant of the equilibrium mecha-

nism is that the active sites supply energy for the triplet-singlet oxygen conversion, rather than acting purely as a catalyst. In this event, the concentration of singlet oxygen could be much higher; also, the oxidation need not be activated. Such participative reaction, however, implies consumption of active sites; such consumption would be consistent with observation (d).

Ordinary oxygen is triplet because the highest occupied molecular orbital (2 pπg) is doubly degenerate with only two electrons present. These, therefore, do not spin pair (which in this case takes energy). It seems quite possible that in surface adsorbed oxygen this orbital degeneracy is removed so that these two electrons now may spin pair. In effect, in the surface-absorbed state, the singlet configuration may become the one of lower energy. This possible influence of adsorption on the properties of oxygen is analogous to the unique reaction of strained acetylenes producing singlet oxygen by a thermal route (29). It then would be understandable that surface oxygen could show the same reaction chemistry that singlet oxygen does in homogeneous media.

Singlet oxygen ($^1\Delta_g$) initiation of autoxidation has been suggested previously (10,11,17,30,31), with photochemical and enzymatic product of the singlet oxygen. Our demonstration of the formation of singlet oxygen in the apparent absence of such factors has important implications. If singlet oxygen is similarly formed on the surface of packaged foods like corn chips, mashed potato flakes, and freeze dried meats, addition of singlet oxygen quenchers to the standard free radical inhibitors could result in a significant increase of shelf-life. There are also applications to the health field. Silicon dioxide has been incriminated in the primary membrane damage leading to clinical silicosis (32). It was shown to increase lipid peroxidation of red blood cell model system significantly. Although the results were attributed to free radical formation, they are also consistent with singlet oxygen initiation by the silica and subsequent free radical autocatalysis. The protective effect of polyvinylpyridine-N-oxide is explicable if it quenches or reacts with singlet oxygen. A positive correlation was shown between a high intake of unsaturated (peroxidation susceptible) dietary fat and cancer when the diet contained a known carcinogen (33). Such a correlation has also been suggested in a long term human study (34). This was conducted in the Los Angeles area where there is both smog and petroleum industry. Thus the polycyclic aromatic hydrocarbon concentration in the air might have been significantly higher than the United States average. It is known that cancer mortality is higher in areas with petroleum industry than in control areas (35), and a mechanism proposed for the carcinogenicity of polycyclic aromatic hydrocarbons involved production of singlet oxygen (36). It appears that there may be practical reasons for defining necessary determinants of singlet oxygen production by nonphotochemical processes.

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Effect of Chronic Ethanol Administration on Cholesterol and Bile Acid Synthesis In Vivo

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ABSTRACT

Chronic administration of ethanol failed to stimulate the hepatic rate of cholesterol synthesis in meal-fed rats. In contrast, chronic ethanol feeding caused a 50% inhibition in the rate of incorporation of [4-^{14}C] cholesterol to bile acids in the bile-duct cannulated rats. It is, therefore, suggested that the decreased rate of cholesterol degradation to bile acids may play an important role in ethanol-induced accumulation of cholesterol in liver.

It has previously been shown that chronic ethanol feeding to rats causes the accumulation of esterified cholesterol in the liver and blood (1). This effect of ethanol was thought to be due to an increased rate of hepatic cholesterol synthesis or to a decreased rate of cholesterol degradation or both. The major site of cholesterol synthesis and degradation to bile acids is the liver. 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (EC1.1.1.34) and cholesterol 7α-hydroxylase are considered to be the rate-limiting enzymes of cholesterol synthesis and its degradation to bile acids, respectively (2-4). We have recently demonstrated that chronic ethanol feeding to rats neither affected the diurnal rhythm nor stimulated the hepatic activity of HMG-CoA reductase (5). In contrast, hepatic cholesterol 7α-hydroxylase activity was decreased by 56% suggesting that the hepatic rate of cholesterol degradation to bile acids is impaired after chronic ethanol feeding. In the present report, we have investigated the effects of chronic ethanol feeding upon (a) the in vivo rate of hepatic cholesterol synthesis and (b) the in vivo rate of conversion of cholesterol to bile acids in bile-duct cannulated rats.

EXPERIMENTAL PROCEDURES

Materials

[4-^{14}C] cholesterol (56 mCi per mmole), tritiated water, Econofluor and Aquasol scintillation fuilds were from New England Nuclear, Boston, MA. [7β-^{3}H] 5-cholestene-3β, 7α-diol ([^{3}H] 7α-hydroxycholesterol) was a gift from Dr. H. Danielsson, Karolinska Institutet, Stockholm, Sweden. Cholesterol (>99% pure) and Tween 80 were from Sigma Chemical Co., St. Louis, MO. All other chemicals and reagents were of analytical grade.

Animals and Treatment

All animals used in this study were male Wistar rats from Carworth, Wilmington, MA. The experimental procedures for producing chronic ethanol-fed rats and the corresponding pair-fed controls have been described (5).

TABLE I

In Vivo Rate of Hepatic Cholesterol Biosynthesis as a Function of Time after the Start of Meal-Feeding Period[a]

Time of tracer injection (hr)	Time of killing (hr)	Cholesterol synthetic rate (μmol acetyl units/g^{-1}h^{-1})	
0900	1000	2.07 ± 0.16	(13)
1200	1300	2.37 ± 0.20	(5)
1400	1500	4.26 ± 0.34[b]	(16)
1700	1800	2.63 ± 0.37	(5)

[a]Rats were meal-fed between 0900 and 1200 hr for a week, and on the day of the experiment each rat was intraperitoneally injected with tritiated water (20 mCi per kg body weight) at indicated times. Sixty minutes later, the rats were killed, and the tritium incorporation into hepatic sterol was determined as described in the Experimental Procedures section. Each value is the mean ± SE. Figures in parentheses represent the number of animals used in each group.

[b]Significantly different from the value observed at 1000 hr; $p<0.001$.

TABLE II

Effect of Chronic Ethanol upon Bile Acid Synthesis

Treatment	Labeled bile acids synthesized[a] from			
	[4-^{14}C]cholesterol		[^{3}H]hydroxycholesterol	
Control	100	(10)	100	(7)
Ethanol	50.6 ± 6.3	(10)	112.5 ± 6.7	(7)

[a]Tracer amounts of [4-^{14}C]cholesterol (7.5x10^{5}-3x10^{6} dpm) and/or [^{3}H]-hydroxycholesterol (3x10^{5} dpm) were dissolved in 0.5 ml of 0.2% Tween 80 in 0.9% NaCl and injected into the portal vein at 0 time in bile-duct cannulated rats, and labeled bile acids were analyzed as described in the Experimental Procedures section. Conversion rate in ethanol-fed animals from each precursor is expressed as percent of the rate found in the control animals. Each value is the mean ± SE. Figures in parentheses indicate the number of animals used in each group.

Measurement of the In Vivo Rate of Hepatic Cholesterol Synthesis

Initially a group of rats (body weight 170-190 g) were meal-fed between 0900 and 1200 hr for 7 days. On the last day they were subdivided into 4 groups. The rats in each group were injected intraperitoneally with tritiated water (20 mCi per kg body weight) at indicated times (see Table I) after the start of the meal-feeding period. Sixty minutes later, the rats in each group were killed, and the incorporation of tritium into hepatic sterol was determined (6).

In the second experiment, at the end of the last meal-feeding period, the rats in the 3-week chronic ethanol-fed group and pair-fed control group were injected intraperitoneally with tritiated water (40 mCi per kg body weight) at 1400 hr. One hour later, each rat was killed, and the incorporation of tritium into hepatic cholesterol was determined (6).

Measurement of the Rate of Conversion of Labeled Cholesterol to Labeled Bile Acids In Vivo

At the end of 3 weeks of chronic ethanol feeding, cannulation of the bile duct, administration of labeled compounds, and the collection of the bile was performed on one rat from ethanol-fed group and the corresponding pair-fed control animal simultaneously. Thus, after the cannulation of the bile duct, indicated amounts (see Table II) of [4-^{14}C]cholesterol and/or [^{3}H]hydroxycholesterol were injected into the portal vein. The rat was sutured and kept in a restraining cage. Bile was collected in an ice-chilled tube for 4 hr. At the end of 4 hr the rat was killed by decapitation. The liver and blood were saved for the analysis of ^{14}C or ^{3}H specific activity. Bile was processed for bile acids as described by Mosbach et al. (7). The bile acids were separated from cholesterol by thin layer chromatography (8) and analyzed for ^{14}C and/or ^{3}H content in a Beckman Model LS-350 liquid scintillation spectrometer. Calculation of dual labeled experiments was carried out by the external standard-channels ratio method using automatic quench compensation. Chloroform-methanol (2:1) extracts of liver and serum were analyzed for ^{14}C or ^{3}H specific activity (dpm per g liver or per ml serum).

RESULTS AND DISCUSSION

The overall rates of hepatic cholesterol synthesis and degradation to bile acids in vivo were investigated in chronic ethanol-fed and pair-fed control rats in an attempt to understand the possible cause(s) for the accumulation of cholesterol in the liver after chronic ethanol feeding.

The effects of chronic ethanol feeding upon the body weight, liver weight, liver and serum cholesterol concentrations have previously been described (5). As can be seen in Table I, hepatic cholesterol synthetic rate in vivo exhibited a circadian rhythm being low before the start of the meal-feeding and reaching the peak around 1400 hr. This correlates well with concomitant changes in hepatic HMG-CoA reductase activity (5) and confirms that HMG-CoA reductase activity reflects the overall rate of cholesterogenesis under most physiological conditions (9).

The in vivo rate of hepatic cholesterol synthesis in six chronic ethanol treated meal-fed rats and corresponding six pair-fed control animals were 2.23 ± 0.54 and 2.76 ± 0.24 μmoles acetyl units/g^{-1}h^{-1}, respectively. There was no statistical difference in the rates of cholesterol synthesis between chronic ethanol-fed and pair-fed control groups. These results clearly demonstrate that the increased accumulation of hepatic cholesterol following chronic ethanol feeding is not due to an increased rate of hepatic cholesterol synthesis. This is sub-

stantiated by our earlier finding that chronic ethanol feeding did not appreciably alter hepatic HMG-CoA reductase activity (5).

Since chronic ethanol is known to increase only the esterified cholesterol but not the free cholesterol in the liver (1), it is obvious that the pool size of free cholesterol in the liver is not changed by chronic ethanol feeding. Cholesterol 7α-hydroxylase is a microsomal enzyme, and it has been shown that the pool size of free cholesterol in the liver microsomes is also not changed significantly under different nutritional conditions (10,11). Therefore, it is reasonable to assume that our studies on the conversion of cholesterol to bile acids after the administration of tracer dose of labeled cholesterol reflect the overall rates of cholesterol degradation to bile acids although they do not represent the absolute rates.

The effects of chronic ethanol feeding on the conversion of [4-^{14}C] cholesterol and/or [^{3}H] hydroxycholesterol into bile acids in vivo are shown in Table II. Since there were individual variations in the conversion rates from one pair of rats to another, the conversion rate for each ethanol-fed rat was recorded as percent of the corresponding pair-fed control. However, the hepatic or serum specific activity of either ^{14}C or ^{3}H in any ethanol-fed rat did not differ significantly from that in the corresponding pair-fed control animal.

Table II shows that the chronic ethanol feeding markedly impaired the conversion of [4-^{14}C] cholesterol to bile acids in each of the ethanol-fed rats compared to the corresponding pair-fed control. In contrast, the conversion of [^{3}H] hydroxycholesterol to bile acids was virtually unaffected by chronic ethanol feeding compared to the corresponding pair-fed control (Table II). These results suggest that chronic ethanol affects mainly the conversion of cholesterol to 7α-hydroxycholesterol but not the subsequent steps involved in bile acid formation. These conclusions are strongly supported by our earlier finding that the hepatic activity of cholesterol 7α-hydroxylase, which is considered to be the rate-limiting enzyme for bile acid synthesis, is in fact markedly decreased after chronic ethanol feeding (5).

It has previously been demonstrated in vivo with the use of ^{14}C-labeled cholesterol that at least 90% is excreted in the feces as a saponifiable component of the bile (12). This study showed that the conversion of cholesterol to bile acids constitutes the major pathway for cholesterol degradation. Excretion of cholesterol per se or as coprosterol represents only a minor pathway. Moreover, since bile acids are not oxidized to CO_2 by animal tissues, they represent the true end products of cholesterol metabolism.

Thus, it may be concluded from the present investigation that an impaired degradation rather than enhanced synthesis seems to be the major cause for ethanol-induced accumulation of cholesterol in the liver. Such a block in degradation would affect not only the disposal of endogenously synthesized cholesterol but also the exogenous cholesterol derived from the diet.

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Autoxidation and Effects of Pro- and Antioxidants in Lyophilized Red Blood Cell Membranes

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ABSTRACT

Oxygen uptake and effects of pro- and antioxidants have been compared at 80 C in lyophilized red blood cell (RBC) bilayer membranes (ghosts). In this dry and relatively immobile system, catalytic metals have pronounced effects. When ghosts are prepared in the usual manner, in phosphate buffer, hypotonic saline, or deionized water (DI), oxygen uptake is extremely slow and limited unless: (a) catalytic metal, e.g., cobaltous ion, is supplied in the absence of metal-complexing buffers and (b) residual phosphate buffer is removed by repeated deionized water or hypotonic saline washes. Ghosts adsorb d-α-tocopherol strongly from 1% alcohol emulsion in buffer in amounts far above normal RBC concentrations, whereas synthetic antioxidant uptake seldom exceeds the normal tocopherol level. Uptake and effectiveness of antioxidants introduced by either perfusion or in the vapor phase, and resistance of ghosts to autoxidation are discussed as they relate to protection of lyophilized membranes and freeze-dried foods.

INTRODUCTION

The development of rancid off-flavor due to autoxidation is the principal reason for consumer rejection of stored freeze-dried foods. Microbiological spoilage, browning, and hydrolytic rancidity do not generally occur because of lack of free water. Water is present at 2 or 3% of dry weight and is considered to be "bound" in a coating of about a monolayer thickness.

Because of the removal of free tissue water, oxygen gas in freeze-dried foods has nearly equal access to all membrane lipids of the cell. In contrast, triglycerides are present in finely divided bulk phases in adipose depots or oil droplets, which present a diffusion barrier to oxygen (1-4). In addition, membrane phospholipids usually have more polyunsaturation. It is not surprising, therefore, that the autoxidation of membrane lipids causes most of the early rancidity in whole cooked and freeze-dried meats (5-8).

Membranes of refrigerated or freeze-dried foods usually have been physically disrupted to some degree, whether in the water-washing, shredding, cutting, or precooking prior to refrigeration, freezing, or freeze-drying, or in the freezing and drying steps. In these processes, the usual integrity of the membrane may be breached, and catalysts (metal ions, and prooxidant metal complexes like hemoglobin and myoglobin, and possibly even ascorbic acid) may come into contact with sensitive double bonds, and remain during refrigeration or subsequent freezing or freeze-drying (7).

Lipids in freeze-dried whole tissue foods (uncomminuted foods where tissue integrity is largely preserved) are often not well protected by added conventional antioxidants, whether naturally occurring or laboratory synthesized (9-11). However, the antioxidants usually are effective when they can be readily introduced into the lipid, as in bulk fats like lard (12). Lack of ready access to the sites of autoxidation in the membrane may be one of the reasons for the poor protection in whole tissue foods, particularly those with low moisture.

As a model system for membrane lipid autoxidation, we and others have previously studied autoxidation and antioxidant effectiveness in a linoleic acid monolayer or sub-monolayer adsorbed on silica (13-18). We desired to compare these results with those in a freeze-dried natural membrane system.

For this reason, we sought an easily prepared, dry, pure, undamaged membrane system, representative of food membranes, which would give reproducible and substantial oxygen uptake arising largely from membrane lipid autoxidation. The system reported here is lyophilized red blood cell (RBC) membranes (ghosts), activated with cobaltous ion.

Shimasaki and Privett (19) have stimulated autoxidation of RBC suspended in saline-buffer by adding linoleate hydroperoxide. Destruction of the cells was closely related to oxidation of endogenous or added vitamin E.

Barker and Brin (20) have used the buffer-suspended RBC membrane activated enzymatically or by dialuric acid (hydroxybarbituric acid) as a model for the peroxidative breakdown of other biological membranes.

McCay et al. (21) have shown hemolysis of saline-suspended RBC coupled to microsomal NADPH oxidation, and inhibited by vitamin E supplementation of the RBC.

TABLE I

Standard Method of Lyophilized RBC Ghost Preparation

1. Centrifuge whole blood and aspirate supernatant – 1000 x g, 10 min.
2. Wash 3x 310 imOsm phosphate buffer, 5 vol., pH 7.4, 1000 x g, 10 min.
3. Hemolyze in 20 imOsm phosphate, 5 vol., pH 7.4, centrifuge 16,000 x g, 40 min.
4. Wash 5x in 20 imOsm phosphate, 5 vol, 16,000 x g, 40 min.
5. Freeze-dry in minimum buffer.

Karel and Labuza (22) and Martinez and Labuza (23) have studied oxygen uptake, browning, and astacene pigment loss in whole tissue freeze-dried salmon. This system does not, of course, discriminate between membrane and depot lipid oxidation.

The lyophilized RBC system was also chosen for its ability to discriminate among antioxidants in uptake and effectiveness in membranes. Lucy and Dingle (24) found that several long chain terpenoid substances including dl-α-tocopherol acetate, squalene, vitamin K_1, ubiquinone-30, and phytol were apparently well adsorbed by RBC membranes suspended in isotonic saline and were effective in preventing the rapid hemolysis caused by vitamin A uptake. Substances like menadione without a side chain were ineffective in their RBC system, although they possess vitamin K_1 activity in blood-clotting tests. Vitamin A is apparently very strongly adsorbed in amounts sufficient to give extensive vacuolation.

In contrast, Tinberg and Barber (25) have shown that 25 times as much tocopherol as either butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT) was bound by liver microsomes suspended in buffer to which alcohol emulsions of the antioxidants were added.

The research reported here is part of a four part program to find the factors which limit uptake and effectiveness of antioxidants in whole foods of low moisture. The parts are: (a) testing of a wide selection of antioxidants in a lipid monolayer on silica gel, a dry model system (15,18); (b) testing in a simple relatively undamaged dry natural membrane, freeze-dried red blood cell ghosts; and (c) selection of presently available natural or synthetic antioxidants compatible with membrane uptake and effectiveness; and (d) testing in a whole tissue food, cobalt-activated freeze-dried carrots. The latter system is now fully developed and in use for antioxidant testing.

PROCEDURES

For preparation of ghosts, human venous blood was procured as out-dated samples from the Blood Bank Processing Division of Framingham Union Hospital. This was used at random, whether preserved in citrate-phosphate-dextrose (CPD) or acid-citrate-dextrose (ACD) anticoagulant solution and regardless of blood type. It was usually used within 1 week of receipt, hence, about 1 month from date of withdrawal. Ghosts were prepared by the Dodge, Mitchell, and Hanahan procedure (26). In this method, red blood cells are washed in 310 ideal milliosmolar (imOsm) phosphate buffer, pH 7.4 (for meaning and preparation, see Ref. 26). They are lysed, and the resulting ghosts are washed in 20 imOsm phosphate buffer, at the same pH.

This procedure was varied by the occasional use of isotonic saline (Travenol Laboratories, Inc., Morton Grove, IL) for washing of RBC and 20 imOsm saline for lysis and washing. Deionized, distilled water (DI) (Ion Exchanger, Illinois Water Treatment Co., Rockford, IL, conductivity less than 1.2 μmho/cm at 25 C, equivalent to 0.6 ppm NaCl) was used often in tests of stress, and was the choice for the final two washes before addition of cobalt and final freeze-drying in the standard method. This water has a pH of 5.1, whereas the pH of the saline used, although variable, was measured at about 5.7.

Cobaltous chloride hexahydrate (Mallinckrodt, Analytical Reagent Grade specified as 0.002% Cu, 0.001% Fe) was used as received and dissolved in DI or isotonic saline, as appropriate. Antioxidants were procured and purified as in the silica monolayer studies (15,18) by recrystallization or redistillation when thin layer chromatography, melting point, or UV spectrophotometry indicated impurity.

Trolox C (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and Topanol-354 (3,5-di-t-butyl-4-hydroxyanisole) were graciously supplied for testing by Hoffmann La Roche, Inc. and Imperial Chemical Industries, Ltd., respectively.

The standard ghost preparation procedure adopted after extensive investigation for study of uptake and effectiveness of pro-oxidants and antioxidants is shown in Table I.

Just before freeze-drying, the suspension was very faint pink and the supernatant nearly colorless and clear. A red plug of coagulum was removed from each centrifuge tube before the ghosts were transferred to a 500 ml, wide-neck, freeze-drying flask (Thermovac Industries Corp., Copiaque, NY), shell-frozen over dry ice-alcohol and lyophilized at 100μ (13.3 Pa) for

12 hr over a dry ice-alcohol trap. The ghosts were often a faint pink at this point, but the hemoglobin content is reported to be usually less than 4000 ppm (26).

Effectiveness of antioxidants was tested either after vapor phase uptake or after perfusion uptake from a slurry of ghosts in 20 imOsm phosphate buffer.

The standard perfusion uptake method of pro- and antioxidant testing is shown in Table II.

Ghosts were rehydrated in a solution, emulsion, or suspension containing either the antioxidants, or no antioxidant for control. Four hundred mg dry ghost weight (DGW) of ghosts were added to 400 ml 20 imOsm phosphate buffer. DGW refers to dry weight of ghosts minus calculated weight of phosphate. At times for experimental purposes, 20 imOsm saline or deionized water was used for rehydration, without antioxidant.

When antioxidant was added by perfusion uptake, it was at a final concentration of 25 μg/ml, added as a total of 10 mg in 4 ml ethanol for 400 mg of ghosts. The usual antioxidant was very soluble under these conditions, but for tocopherol a fine emulsion was formed by adding the buffer slowly to the alcoholic tocopherol solution. Alcohol was 1% of final buffer volume and was shown to have no effect on oxidation rate with or without antioxidant.

For BHT and Topanol-354 (3,5-di-t-butyl-4-hydroxyanisole), about 100 mg food grade lecithin (Clearate, WDF, W.A. Cleary corporation, New Brunswick, NJ) was dispersed in 400 ml 20 imOsm buffer by bubbling with a nitrogen stream, and the ethanolic antioxidant solution was added dropwise to this with stirring to give a clear suspension with little turbidity, and no tendency to break during the adsorption period.

Ghosts were shaken for 1 hr at 20 C in the adsorption medium (Warner Chilcott Reciprocal Shaker, 100 cycles/min), centrifuged at 16,000 x g for 40 min, washed once with 50 vol of DI and once with 7 vol of DI; the wash amount in each case being 150 ml. The difference in wash ratio arises from the small size of the first pellet after rehydration (3 ml), whereas after the first water wash, the pellet is about 20 ml and after the second wash, 35 ml. This is a consequence of the packing effect of the phosphate buffer or saline, apparently a function of ionic strength since after three DI washes the pellet is progressively more diffuse and less discretely bounded. After about four DI washes, the membranes are obviously damaged, since there are wispy trails and foaminess in the supernatant, whereas the usual pellet, even after the standard two DI washes, has a sharp boundary and a clear, nonopalescent and nonfoamy supernatant.

TABLE II

Standard Method of Perfusion Uptake of Antioxidants by RBC Ghosts

1. Rehydrate 400 mg DGW[a] in 400 ml 20 imOsm phosphate containing 10 mg antioxidant (alcohol, 1% buffer vol.), shake 1 hr, 20 C.
2. Centrifuge 16,000 x g, 40 min. Decant buffer (98%).
3. Wash 2x DI water (150 ml) 16,000 x g, 40 min.
4. Add 25 mg cobalt chloride hexahydrate in 25 ml DI water to ghosts in 75 ml DI water. Swirl 5 min.
5. Freeze-dry and oxidize.

[a]Dry weight of ghosts.

Typically, after the second DI wash, the ghosts from each tube were transferred separately in about 37 ml DI to a 250 ml wide mouth freeze-drying flask, and 12 ml of cobaltous chloride solution (1 mg/ml in DI) was added with 5 min of swirling. Subsequent rapid weighing of the dried ghosts shows that about 125 mg DGW are present at this point, resulting in the ratio of ca. 25 mg cobaltous ion/g DGW. The added solution and the resulting suspension are faintly pink. The flask contents are shell-frozen and lyophilized as before (12 hr, 100 μ, 13.3 Pa). After this, the excess anhydrous cobaltous chloride is the bright blue color of the dried salt, an excellent monitor of low water activity that persists during the subsequent oxidation-flask filling operation.

Experience showed that to insure reproducible oxygen uptake, the following five procedures were mandatory:

1. Glassware should be thoroughly cleaned with a nonphosphate, nonchelating detergent, since very small amounts of residual phosphate can strongly affect reproducibility, presumably by metal chelation or precipitation.
2. After rehydration in 20 imOsm phosphate, and subsequent centrifugation, the phosphate supernatant should be totally decanted, leaving a very small (3 ml) pellet. Less than total decantation results in phosphate contamination and lack of reproducibility in oxidation of the ghosts after the second freeze-drying.
3. After rehydration, water-washing, and centrifugation, the contents of each centrifuge tube (typically 125 mg of ghosts in 37 ml DI in a 250 ml tube) should be placed in a separate 250 ml freeze-dry flask and ultimately a separate 10 ml oxidation flask.

TABLE III

Perfusion Uptake of Antioxidants and Phytol by Ghosts

Compound	Initial concentration μg/ml[a]	Mean uptake mg/g DGW	MD[b]
d-α-tocopherol	25	10.3	±0.5
BHA	25	0.54	0.08
BHT	25	Trace	---
TBHQ	25	Undetectable	---
Phytol	24	21.1	1.3
Phytol	111	39	6.0

[a]Standard conditions of uptake except for last phytol entry.
[b]Mean deviation of two experiments.

4. Cobaltous chloride solution should be added directly to the DI suspension of the ghosts in the freeze-drying flask after complete transfer of ghosts from the centrifuge tube.
5. Ghosts should be transferred quite rapidly to a previously tared oxidation flask (see infra) to avoid water and oxygen uptake after breaking freeze-drying vacuum. It was not, however, found necessary to break vacuum with dry nitrogen or to work in a glove box.

An exactly and rapidly weighed portion (ca. 100 mg) of lyophilized ghosts is introduced into a 10 ml pear-shaped oxidation flask, which is closed with a combination of two rubber serum bottle stoppers, one reversed. This has been found to prevent any air leakage through sampling syringe needle holes during the usual study period. It was found important to load the ghosts rapidly into the flasks and to obtain their weight by difference from a tared flask weight, in order to minimize water and oxygen uptake during the loading process. For the delicate process of loading the freeze-dried ghosts, which are extremely light and vulnerable to scattering by static charge, preassembled and fitted cones of coated weighing paper (S and S Type, VWR Scientific, Inc., Boston, MA) were inserted into the necks of the flasks, to permit quick transfer with minimum loss and crushing.

Sampling of oxygen and plotting of uptake were done as described for the silica system (13). No evidence was found for restriction on oxygen diffusion in ghosts, regardless of the fragment size, provided that the standard loading was not exceeded. Uptake readings were normalized for a 10 ml volume and 100 mg DGW, and an empty stoppered flask was sampled as thermal and stopper control (rubber oxidation). The volume chosen corresponds to 85 μmoles of oxygen, and the 100 mg DGW would contain on the average 18 mg of total fatty acid and ca. 10 mg of cholesterol (26).

For testing of vapor phase uptake of antioxidants, 1 ml of a solution or emulsion containing 0.1 mg/ml of antioxidant in the appropriate solvents or emulsifying agents was introduced into the 10 ml conical-tipped oxidation flask prior to introduction of ghosts. The solvent was removed at reduced pressure (RP) on the Büchi Rotovapor, leaving an evenly distributed antioxidant coating on the walls of the flask. Melting points of these antioxidants are shown in Table VI. One hundred mg of freeze-dried ghosts were then introduced, and oxygen uptake readings were carried out as above.

To find how much cobaltous ion was directly adsorbed, the ghosts were rehydrated and DI washed as described in the standard method, but were centrifuged at 16,000 x g for 40 min immediately after cobalt addition and swirling, instead of the usual lyophilization. Twenty-six mg of cobaltous chloride hexahydrate was added in 205 ml DI water (cobaltous ion concentration 32 ppm) to 216 mg DGW.

The supernatant was decanted, and the pellet (about 3 ml when cobaltous chloride was present in the DI water) was freeze-dried. Cobaltous ion was determined on both supernatant and freeze-dried pellet by atomic adsorption spectrophotometry. The ghosts were ashed 12 hr at 550 C in a muffle furnace, and the ash was dissolved in 4N HCl. A Perkin-Elmer Model 303 Atomic Absorption Spectrophotometer was used.

Adsorption of antioxidants and phytol was studied by solvent extraction of the freeze-dried ghosts followed by thin layer chromatography (TLC) and densitometry on Silica Gel G after controlled spraying with Emmerie-Engel reagents or concentrated H_2SO_4 using appropriate standards at roughly the same concentration. Densitometry standard concentration-response curves were prepared and used for determination of unknowns. A Schoeffel Model

TABLE IV

Relative Effectiveness of Antioxidants in RBC Ghosts.
Perfusion Uptake Method, Compounds More Effective Than Tocopherol

Compound	Mean relative effectiveness (REFF)[a]	Mean[b] deviation
Topanol-354[c]	19	± 0.1
	60<REFF<90[d]	Indefinite
BHA	15	1.1
	10	1.2
TBHQ	4.4	0.3
	5.0	0.2
α-Tocopherol	3.6	0.8
	4.7	1.2

[a]Means and deviations are for two separate experiments per compound.
[b]n = 2 for each experiment.
[c]3,5-di-t-butyl-4-hydroxyanisole.
[d]Induction period ended during interruption in readings.

SD 3000 Spectrodensitometer with SDC 300 Density Computer was used and set at 520 nm. Acid-sprayed plates were charred on a hot plate under controlled conditions. Extraction was by the method of Kayden (27) for tocopherol, phytol, and BHT, using KOH saponification with excess ascorbic acid in an ethanol-isotonic saline mixture as protection against oxidation by hemoglobin and methemoglobin during extraction. After addition of water, the saponification medium was extracted with hexane. Hexane was removed at RP, and TLC of the residue in chloroform was carried out on Silica Gel G using hexane-absolute ethanol (9:1) for tocopherol and BHT. The solvent for phytol TLC was petroleum ether-diethyl ether-acetic acid (30:70:1) followed by concentrated H_2SO_4 spraying and controlled charring.

TBHQ (t-butyl hydroquinone) was determined by Soxhlet extraction of the ghosts with redistilled, diethyl ether previously treated with acidified ferrous sulfate. The residue after solvent removal was taken up in $CHCl_3$-MeOH (1:1) for TLC on Silica Gel G with hexane-absolute ethanol (7:3). BHA was Soxhlet-extracted into hexane, which was treated with 85% H_2SO_4 followed by a saturated sodium bicarbonate wash to remove cholesterol, with which it co-chromatographs. The solvent was removed at RP, the residue taken up in $CHCl_3$, and TLC was performed using hexane-ethanol (9:1) on Silica Gel G. For each of the methods, recovery of known amounts of antioxidant added just before extraction to the lyophilized ghosts was 95-100%.

RESULTS

Cobaltous Ion Adsorption

When 216 mg DGW were shaken under standard conditions in DI water with 6.5 mg cobaltous ion as cobaltous chloride hexahydrate (cobaltous ion concentration 32 ppm), 0.94 ± 0.06 mg cobaltous ion was found in the ashed freeze-dried pellet, or 4.4 ± 0.3 mg Co^{++}/g DGW. 4.5 ± 0.2 mg was found in the supernatant. Recovery was thus 84%. Based on the ratio of pellet to supernatant volume (3/205), the entrapped, nondecanted and possibly unadsorbed Co^{++} could be no more than 0.10 mg. Thus, the ghosts positively adsorbed at least 0.84 mg or 3.9 mg/g DGW. This is a real adsorption of at least 13% of total Co^{++} added.

Even at the low addition level of 1 mg cobaltous ion/g DGW (1/25 of the standard amount), adsorption of about 150 ppm Co^{++} (DGW basis) could be expected, at least ten times that needed for activity in pure lipid systems. (Since 150 ppm DGW basis is about 830 ppm on a fatty acid basis.)

Antioxidant Uptake

Under the standard uptake conditions, 1 hr shake at 20 C, using 400 mg DGW in 400 ml 20 imOsm phosphate buffer with 10 mg of antioxidant, Table III shows the uptake of four important antioxidants and phytol.

The uptake of tocopherol, as Tinberg and Barber found with liver microsomes (19), is much greater than that of other antioxidants. Phytol shows an even greater uptake. In some cases, as for TBHQ, it is possible that antioxidant adsorbed from buffer cannot be extracted, whereas when added to the dried ghosts just before extraction, it is not so firmly bound, as in the test recoveries, which were virtually complete. Alternatively, some batches of ghosts with higher than normal hemoglobin or methemoglobin content may destroy some of

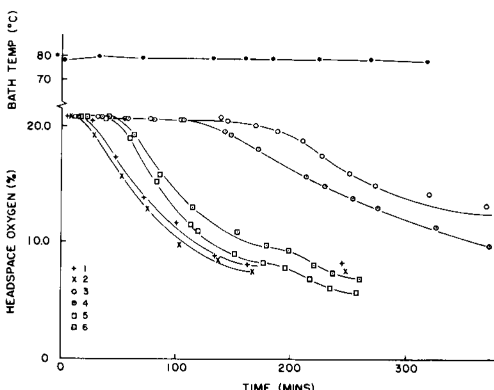

FIG. 1. Autoxidation of freeze-dried ghosts with BHA and propyl gallate. Standard methods of ghost preparation and uptake of antioxidants. Control, 1, 2; BHA, 3, 4; propyl gallate, 5, 6. Antioxidants added by perfusion uptake at 25 μg/ml of shake suspension.

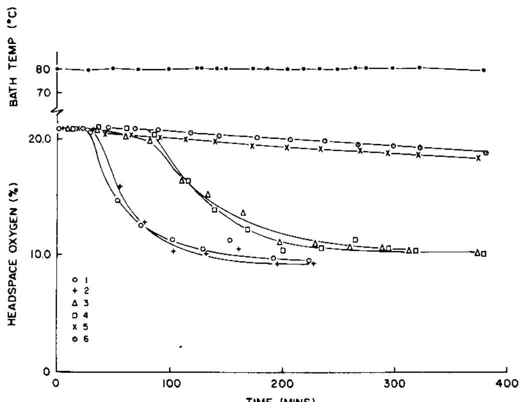

FIG. 3. Autoxidation of freeze-dried ghosts prepared by standard method with d-α-tocopherol. Standard methods of ghost preparation and uptake of antioxidants. Control, 1, 2; tocopherol added at 2.5 μg/ml, 3, 4; 25 μg/ml, 5, 6.

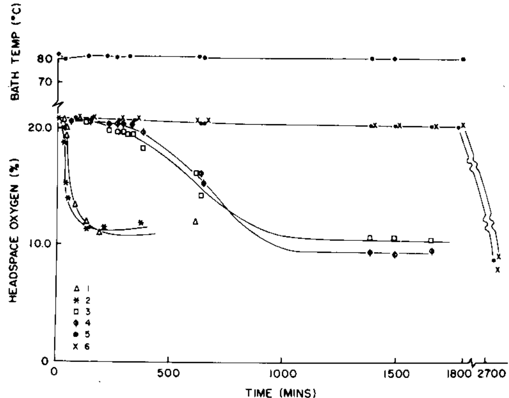

FIG. 2. Autoxidation of freeze dried ghosts with BHA and Topanol-354. Standard methods of ghost preparation and uptake of antioxidants. Control, 1,2; BHA, 3,4; Topanol-354, 5, 6. Antioxidants added by perfusion uptake at 25 μg/ml of shake suspension.

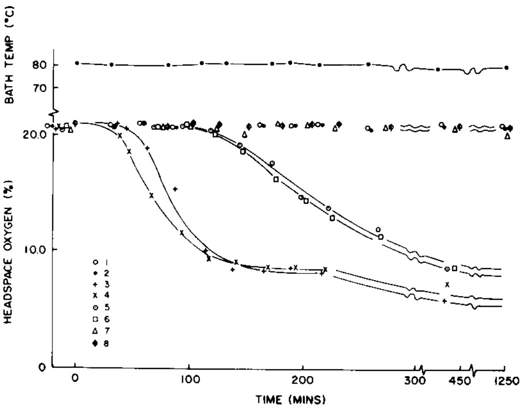

FIG. 4. Autoxidation of freeze-dried ghosts as a function of added cobaltous ion. Standard method of preparation. Cobaltous ion added – 1, 2, none; 3, 4, 83 mg/g DGW; 5, 6, 10 mg/g DGW; 7, 8, 1 mg/g DGW. See text for percentage adsorption.

the added antioxidant in the shake suspension.

Uptake of Oxygen without Antioxidant

Ghosts prepared by the standard method without antioxidant, where 25 mg cobalt ion have been added per g DGW, show little oxygen uptake for a brief period of 20-30 min at 80 C (Figs. 1, 2, 3, curves for no antioxidant). Then, a rapid, initially nearly linear uptake begins, which decreases at roughly an exponential rate near the end of the uptake. This uptake behavior is very reproducible. The short induction period observed is presumably due to the usual tocopherol content of most ghosts (measured by us at about 130 μg/g DGW, or about 0.1 moles percent of the phospholipid).

For the silica monolayer and RBC ghost experiments, induction period is defined as time to reach a headspace oxygen content of 20.0% from the normal 20.9%.

Total oxygen uptake under the standard conditions is about the same for each run and is about 2/3 of available flask oxygen or 57 μmoles. This is the molar equivalent of 16 mg linoleic acid/100 mg DGW, the flask charge, or 160 mg/g DGW. Maximum uptake is thus quite sharply limited and reproducible. The lyophilized membrane protein (the ghosts having at most 4000 ppm hemoglobin contamination) seems to have little or no oxygen uptake, when no cobalt is added (Fig. 4), similar to Chipault's results on freeze-dried chicken (5) and those of

TABLE V

Relative Effectiveness of Antioxidants in RBC Ghosts.
Perfusion Uptake Method, Compounds Less Effective Than Tocopherol

Compound	Mean relative effectiveness[a]	Mean deviation[b]
Propyl gallate	2.2	±0.1
	4.2	0.9
Hydroquinone	2.7	0.0
	1.8	0.1
BHT	2.1	0.0
	2.1	0.2
α-Tocopherol (1/10 of standard concentration)	1.3	0.05
	2.6	0.2
Caffeic acid	1.4	0.1
	0.9	0.2
Trolox C[c]	0.8	0.2
	0.4	0.02

[a]Means and deviations for two separate experiments per compound.
[b]n = 2 for each experiment.
[c]6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid.

Martinez and Labuza (23) on freeze-dried salmon. Thus, the fatty acids appear to have a total oxygen uptake compatible with an equivalent composition of 89% linoleic acid, 11% saturated fatty acid. Witting's data (4) show, however, that only 61% of the fatty acids in the usual RBC are unsaturated and only 42% polyunsaturated, so it appears that polyperoxidation or else rapid secondary reactions are occurring.

Uptake of Oxygen with Antioxidants Present (Figs. 1-3)

When ghosts have been perfused with antioxidant solutions, washed in unbuffered DI water or 20 imOsm saline, and freeze-dried after cobalt addition, rapid phase oxidation occurs finally at 80 C, but a reproducible and sometimes very great extension of the induction period occurs for some, but not all, antioxidants. Reduction of the ensuing rapid phase rate also occurs for some antioxidants, although maximum uptake remains nearly the same. Table IV and V show our current results, using perfusion uptake.

Relative effectiveness (REFF) is defined as the ratio:

$$\frac{\text{induction period with antioxidant}}{\text{induction period of untreated ghosts}}$$

where induction period is time to reach headspace oxygen of 20.0%.

Of the synthetic antioxidants previously studied, Topanol-354 (3,5-di-t-butyl-4-hydroxy anisole), BHA, TBHQ, and d-α-tocopherol at the standard concentration give the greatest extensions of induction period. However, the course of oxygen uptake at the standard concentration of tocopherol is unique so far (Fig. 3). It is a nearly linear, slow uptake, with no rapid phase up to 24 hr at 80 C, uptake being only about 30% of maximum possible at this point. Such behavior was observed on silica (13,14) with either linoleic acid or lecithin at high levels of tocopherol (5-6 moles percent of fatty acid). At the standard concentration level in ghosts, tocopherol is 5.8 weight percent and 3.8 moles percent of fatty acid. The lower levels of tocopherol (1/10 of standard concentration) showed no such effect, either in the silica or ghost systems, the usual rapid phase being observed after an extended induction period. This seems to be a case of "pro-oxidant" action of antioxidants at high concentration which Privett ascribes to chain initiating peroxide decomposition in competition with the antioxidant action of peroxide radical reduction and chain stopping (28).

Table VI shows results with vapor phase uptake. Although it might be claimed that BHT and BHA are liquid at the oxidation bath temperature (80 C), certainly the other compounds must have sublimed to the membranes without any "wicking" action. Topanol-354 and BHT are conspicuously effective, and the other compounds tested have much less effect. In general, in vapor phase uptake on RBC ghosts, multiple chain-branching by methyl groups, as with two t-butyl groups, coupled with low melting point, appears to result in great effectiveness.

TABLE VI

Relative Effectiveness of Antioxidants in TBC Ghosts. Vapor Diffusion Uptake Method

Compound	MP[a]	Mean relative effectiveness (REFF)	Mean deviation[b]
Topanol-354[c]	102-3	$20 < \text{REFF} < 110$[d]	± Indefinite
BHT	70-71	12	1.5
Hydroquinone	171-172	2.7	0.3
TBHQ	127-128	2.1	0.4
BHA	56-57	1.9	0.5
Caffeic acid	194-195	1.0	0.1
Propyl gallate	147-148	1.0	0.1

[a]Data of this laboratory.

[b]n = 2

[c]3,5-di-t-butyl-4-hydroxy anisole

[d]Induction period ended during interruption in readings.

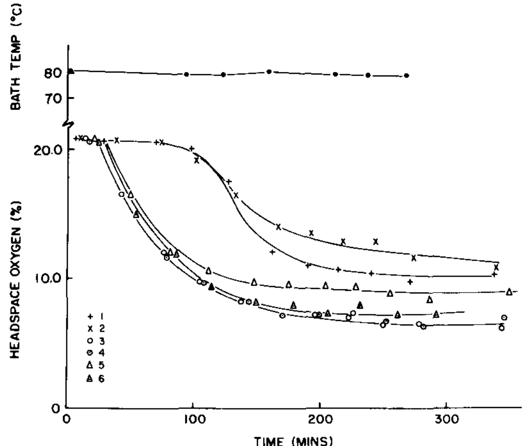

FIG. 5. Autoxidation of freeze-dried ghosts with cobalt activation – effects of preparative methods. 1, 2, standard buffer wash procedure followed by two washes with DI, addition of cobalt, freeze-drying; 3,4, standard buffer wash and freeze-drying procedure, rehydrate DI, two DI washes, addition of cobalt, *second* freeze-drying; 5, 6, standard buffer wash and freeze-drying procedure, rehydrate 20 imOsm phosphate buffer, two DI washes, addition of cobalt, and freeze-drying.

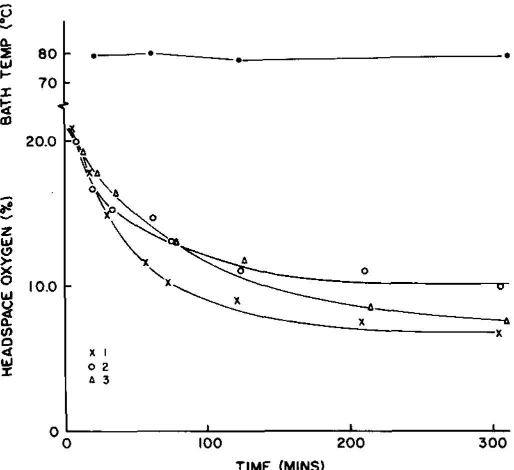

FIG. 6. Autoxidation of freeze-dried ghosts with cobalt activation – effects of preparative methods. 1, standard buffer wash and freeze-drying procedure, rehydrate 20 imOsm saline, two DI washes, cobalt addition, freeze-drying; 2, 3, standard buffer wash and freeze-drying procedure, rehydrate 20 imOsm saline, two washes with 20 imOsm saline, cobalt addition, freeze-drying.

Resistance to Oxidation

It was found, after repeated failures to oxidize ghosts under many combinations of stress, that reproducible rate and maximum of oxygen uptake by ghosts depends in part on addition of a suitable peroxide-decomposing metal catalyst, in this case cobaltous ion, which is a reducing species and without effect on antioxidants (α-tocopherol was recovered undiminished after 24 hr at 80 C in the presence of standard cobaltous ion concentration). When this is added in very large concentration and total amount to the thoroughly washed and buffer-free ghost suspension prior to lyophilization, dependable oxygen uptake occurs (Fig. 4). For example, 10 mg Co^{++}/g DGW (40% of the standard method concentration) give a relatively short induction period and maximum oxygen uptake, but 1 mg/g gave essentially no uptake in 20 hr. This is equal to about 5,000 ppm on a fatty acid basis, or about 2.5 moles percent. Even if the adsorption were only 13% at this lower concentration (as was found for a concentration 25 times that large), there still remains a very large cobalt to fatty acid ratio.

Cobaltous ion was chosen because of its

stable divalent state, lack of effect on antioxidants, and the fact that Marcuse (29) found it did not have antioxidant activity at high concentration and low oxygen pressure, as did cupric ion.

It seems plain that almost no active free metal ions remain after conventional washing and that the hemoglobin remaining [the ghosts are often faint pink and the hemoglobin content often 4,000 ppm (26)] functions poorly as a catalyst in the nearly intact dry ghost membrane, in contrast to its effect in wet, damaged erythrocytes (30).

However, cobalt is necessary, but not sufficient. It functions quite well with only one freeze-dry cycle if, prior to cobalt addition, in this case before the first freeze-drying, the ghosts are washed two times with five volumes of DI (Fig. 5). DI produces after four washes a progressive loosening and expansion of the ghost pellet and finally a wispy, poorly bounded pellet, with a foamy supernatant. If ghosts are rehydrated and washed two times after the first freeze-drying (Fig. 5), in either 20 imOsm saline or DI, cobalt will promote reproducible oxidation, as it will if rehydration is in 20 imOsm phosphate, followed by two DI washes before a second freeze-drying. Figure 5 also shows that a second freeze-dry cycle gives a pronouncedly shorter induction period and more rapid rate, given the same two DI washes, than one freeze-drying.

Figure 6 demonstrates that after rehydration in 20 imOsm saline, there is very little difference in oxidation rate between DI-washed and saline washed ghosts. Hence, ionic strength is of little consequence, at this level.

In all cases, the ionic washes produce a tight, well-bounded pellet after rehydration, the DI a progressive loosening after three washes as ionic strength is reduced. As summarized above in Table II, for standardized test, we settled on rehydration of 400 mg DGW in 400 ml 20 imOsm phosphate, with or without antioxidant, plus two washes of the resulting 15-30 ml packed ghosts in 150 ml DI, followed by cobalt addition and a second freeze-drying. This gives a very dependable induction period rate and limit.

The important fact is that the ghosts must be washed free of chelating buffers before cobalt activation and that rehydration and a second freeze-dry cycle promote more rapid oxidation. Thus, both cobalt and washing are required.

Supporting the hypothesis of autoxidation resistance of the membrane, we have found that freeze-dried, deoxygenated red blood cells will take up oxygen very rapidly at 80 C with no discernible induction period to levels more than three times that of the uptake expected from the associated lipid. In the process, hemoglobin completely changes to methemoblobin as spectrophotometry of the lysed cell supernatant reveals. The powdery cells are chocolate brown rather than the original rust red color.

In spite of this uptake, no rancidity is detectable in oxidized freeze-dried RBC, unlike oxidized ghosts, which have a very acrid, unmistakably rancid odor. Attempts to extract and characterize the lipid of these oxidized cells are not yet successful presumably because of the strong oxidizing action of methemoglobin on extracted lipid emulsions. It is suggested, however, that the hemoglobin may all be oxidized to the ferric form without much concurrent lipid oxidation in the intact freeze-dried cell. Alternatively, the hemoglobin at such high concentration may act as a lipid antioxidant (31-34).

DISCUSSION

The ghost membrane, washed with DI or hypotonic saline, a very pure, nearly undamaged tissue, seems in our system to be quite resistant to autoxidation after freeze-drying unless activated by a peroxide-splitting metal ion. It is possible that uncooked, unmacerated freeze-dried membranes, in general, providing that they are free of other lipid-containing cell organelles like fat depots or oil droplets and are previously washed free of catalytic metal ions, may be more resistant to autoxidation than has been thought. When prepared in the presence of chelating buffers, little to no oxidation occurs, even in the presence of high metal concentrations.

In summary, the dry, bilayered, relatively anchored lipid in the lyophilized ghost system permits testing of uptake and effectiveness of antioxidants and of pro-oxidants like metal ions in a dry membrane. The method also can be used to study in a dry membrane system the effects of: (a) metal chelators, the so-called acid synergists; (b) water activity, by controlled humidification; (c) temperature; (d) partial pressure of triplet or singlet oxygen; (e) surface concentration of polyunsaturated lipid; (f) possible steric inhibition of oxidation by adsorbed saturated lipids; and (g) the trapped, nonvolatile products of lipid autoxidation and lipid-antioxidant reactions, which otherwise might be lost to the ambient liquids of physiological composition in which oxidizing membranes are normally suspended.

The homogeneity of this oxidizing membrane should also lend itself to definitive

measurements of lipofuscin fluorescence (35,36), oxidative chemiluminescence (37), and, with suitable modification, electron paramagnetic resonance of free radicals which the dry state tends to stabilize (38).

The ghost membrane has been considered by Brunner as typical of such membranes as the milk fat globule (39). The composition of the polar lipid fatty acids is also somewhat similar to that of freeze-dried beef (6). The RBC ghost autoxidation system here described may, therefore, have a more general applicability in testing of antioxidants and synergists in dry membranes (19-21).

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Determination of Peroxide Value by the Colorimetric Iodine Method with Protection of Iodide as Cadmium Complex

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ABSTRACT

Improved procedures of the Swoboda and Lea method for the determination of peroxide values (POV) of fats and lipids are presented. After oxidation of iodide to iodine with the sample for 5 min under an inert atmosphere, an excess of the iodide ion is immediately converted to cadmium complex for protection from atmospheric oxygen. The iodine is measured colorimetrically at 358 or 410 nm, and POV is calculated from the absorbance. This method permits the rapid determination of POV with a small amount of sample at a moderate cost using usual glasswares. For the analysis of lipids in biological materials or food products, the chloroform solution obtained by the Bligh and Dyer method is directly subjected to this procedure without evaporation of the solvent. Conversions between POV obtained by the different methods are discussed.

INTRODUCTION

The method most widely used to determine peroxide value (POV) is the volumetric analysis of the iodine liberated from KI by oxidation with the peroxide at room temperature in acetic acid-chloroform medium (1). Modifications to colorimetric procedures have been occasionally reported, but there is not a routine colorimetric method which is widely used. A main factor which has heretofore prevented the wide employment of the colorimetric method seems to be the deviation of the absorbance data due to air oxidation of the excess iodide. In the volumetric method, deaeration by the bubbling or purging with inert gas is often used to prevent oxidation of the iodide (2-4). However, deaeration is not necessarily required. For example, the AOCS Official Method Cd 8-53 for determination of POV (5) does not include deaeration. In the colorimetric method, the higher ratio of iodine liberated by air oxidation to total iodine results in great deviation in the measurement. To protect iodide from air oxidation in the colorimetric measurement, the use of specially constructed cells was presented (4,6). However, they are somewhat troublesome and not readily available.

In this study, the necessary protection of the iodide ion from air oxidation is afforded by purging with CO_2 and shielding from light during the oxidation with peroxide, and by complexing the iodide ion with cadmium ion during measurement of the absorbance according to Swoboda and Lea (7). Complete color development is found to be attained in 5 min in our experiment while the reaction period for oxidation of KI was 1 hr (7) or 30 min (8) in the previous paper under standard condition. Shortening of the analytical time in the improved method permits the rapid determination of POV of many samples at the same time. The rapid colorimetric determinations of malonaldehyde by TBA reagent and total carbonyl compounds by dinitrophenylhydrazine have been used for the evaluation of autoxidation in the analysis of lipids. The method presented in this paper was found to be useful for the same purpose. The disadvantages of the method is the hazard in handling of the poisonous cadmium salt reagent and in the care necessary for discarding of the waste solution.

MATERIALS AND METHODS

Reagent and Apparatus

$Cd(CH_3COO)_2 \cdot 2H_2O$, KI, $K_2Cr_2O_7$ and acetic acid were of analytical-reagent grade obtained from Nakarai Chemicals, Ltd., Kyoto, Japan. Chloroform and methanol were prepared by distillation of the reagent grade products. All spectrophotometric measurements were made with a Hitachi 124 double beam recording spectrophotometer with 1-cm cells. The CO_2 flow was used after washing with $NaHCO_3$ solution and drying with concentrated H_2SO_4.

Colorimetric Determination of POV of Fats and Oils

An aliquot of sample oil (commercial salad oil, mixture of soybean and rapeseed oils autoxidized, usually 50 mg) is weighed into a 25 ml volumetric flask and then 5 ml of chloroform and 10 ml of acetic acid are pipetted into it. After deaeration by purging with CO_2 for 2 min, 1 ml of 50% w/v aqueous KI solution freshly prepared, is added into the flask by purging with CO_2 during 1 min. After stoppering, shaking, and allowing to stand in darkness for 5 min, the reaction mixture is diluted to the mark of the flask with 2% aqueous $Cd(CH_3COO)_2 \cdot 2H_2O$ solution. After shaking, the solu-

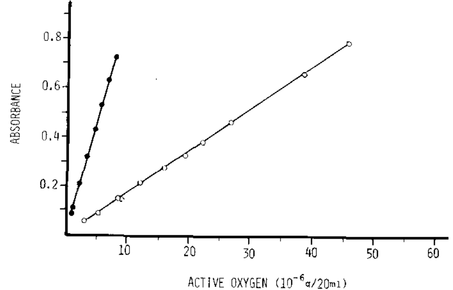

FIG. 1. Calibration curve of active oxygen determined at 358 nm (•) and 410 nm (○).

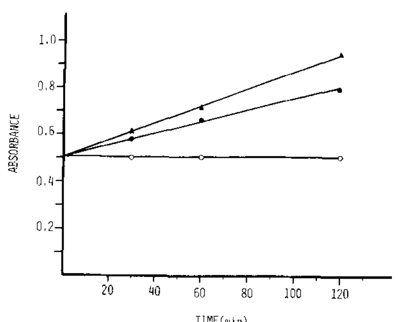

FIG. 2. The effect of cadmium on air oxidation of excessive I^-. Concentrations of $Cd(CH_3COO)_2.2H_2O$ are 0% (▲), 1% (•), and 2% (○).

tion is placed in darkness until the two phases are clearly separated. If necessary, the solution is warmed to about 40 C or centrifuged in a test tube at 4000 rpm for separation. The absorbance of the supernatant aqueous phase is measured spectrophotometrically after decanting into a cell against distilled water in the reference cell. The blank solution is prepared in the same way without sample.

A calibration curve is obtained by addition of 0.5 ml of 12 M HCl, 1 ml of 50% aqueous KI solution, 10 ml of water, and a known amount of $K_2Cr_2O_7$ solution to the 25 ml volumetric flask followed by the same procedures for the measurement of absorbance of the iodine liberated.

Plotting of the active oxygen content against the absorbance (Fig. 1) gave the following relation by the least-squares method.

$$\text{Active Oxygen Content (AO)} (\mu g) = (11.1\ A_{358} + 0.04) \text{ or } (60.14\ A_{410} + 0.69)$$

The correlation coefficients were 0.999 and 0.992, respectively.

The POV is calculated by the following equation.

$$\text{POV (meq/kg)} = (\text{AO}/8\text{W}) \times 1000$$

where A_λ is the absorbance at λ nm, and W is the weight of the sample (mg). The absorbance values for both the high and low range calibration curves are shown below.

Wave-length (nm)	Absorbance range	Active oxygen (μg/20 ml)	POV (sample 45.00 mg)
358	0.080-0.722	0.94-8.28	2.6-23.0
410	0.057-0.781	3.37-46.49	9.4-129.1

POV of Fats and Lipids in Biological Materials and Foods

An aliquot of the sample (usually 5 g) is blended with 10 ml methanol and 5 ml chloroform for 2 min by the homogenizer which consists of a glass tube and stainless-steel blades rotated by a motor. After addition of 5 ml each of chloroform and water, the mixture is blended for 30 sec each, respectively. The test tube is centrifuged at about 4000 rpm for 10 min. The lower layer is taken by a syringe and filtered through a glass fiber filter. An aliquot of the filtrate (usually 1 ml) is pipetted off, and the solvent is evaporated under reduced pressure. The weighing of the residue gives the content of the lipids. Another aliquot of the sample (usually 5 ml) is pipetted out, and after addition of the iodide solution, the liberated iodine is determined colorimetrically as described before. The POV of the sample is calculated from the lipid content, and the amount of iodine is liberated. The procedures described are carried out under inert atmosphere.

When the sample contains only little amount of water, the simple procedure can be taken to prepare the chloroform solution of fats for the determination of POV. A lot of the sample ground to powder or cut to fine pieces is extracted by addition of an appropriate volume of chloroform at room temperature under the inert atmosphere. The filtrate is subjected to the colorimetric analysis of iodine as described above.

Titrimetric Determination of POV of Fats

POV of the same samples were determined with various methods, the improved colorimetric method, the AOCS Official Method Cd 8-53 (9), and the standard method for the analysis of fats by the Japanese Oil Chemists' Society (JOCS method) (10). The outline of the JOCS method follows.

Weigh the sample (10. 10-5, 5-1, and 1-0.5 g for the samples of POV below 1, 1-10, 10-50, and above 50, respectively) into a glass-stoppered Erlenmeyer flask and then add 35 ml of chloroform-acetic acid (2:3, v/v) solution. Swirl the flask until the sample is completely dissolved. Substitute air in the flask with N_2 (or

CO_2) and then add 1 ml of saturated KI aqueous solution under purging of inert gas. After stopping the inert gas current, stopper the flask immediately. Allow the solution to stand in the dark for 5 min at room temperature after shaking for 1 min. Shake vigorously, titrate with 0.01 M $Na_2S_2O_3$ until the blue color by the starch indicator has just disappeared.

RESULTS AND DISCUSSION

The influence of the concentration of $Cd(CH_3COO)_2$ on the color development by the oxidation of iodide ion with air is shown in Figure 2. Practically complete inhibition of the iodine formation from the iodide was observed when the equivalent ratio of cadmium ion to the iodide was 1:4 by the addition of 2% $Cd(CH_3COO)_2 \cdot 2H_2O$ solution. The ratio is consistent with the formation of cadmium tetraiodide complex, $[CdI_4]^{2-}$ (9). Since the equilibrium constant of complex formation is very large and 10^6 by the literature (9), most of the iodide ion will be present as the complex in the solution. On the other hand, the concentration of iodide used in this study is the minimum required to get the rapid development of the color and reproducible results.

The development of color by the oxidation of iodide with peroxides is shown in Figure 3. In oxidation of iodide with hydroperoxides present in the fat, complete color development is attained in 5 min while the reaction period required for the stable peroxides such as di-tert-butyl and dicumyl peroxides is too long for them to be analyzed by the iodometric analysis at room temperature.

An example of the relations between absorbance and sample size is shown in Figure 4. The linear relationship between the absorbance and sample size is observed with sample size taken from 10 to 110 mg in agreement with Beer's law. The upper limit of POV measurement is ca.

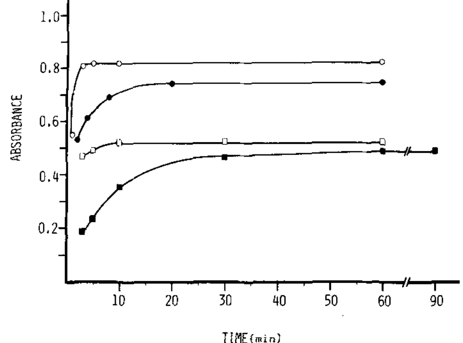

FIG. 3. Rate of color development by oxidation of iodide with various peroxides. Active oxygen contents in the mixture of rapeseed oil and soybean oil (○), *tert*-butyl hydroperoxide solution (●), benzoyl peroxide solution (■) 4.85, 4.27, 3.39, and 2.95 x 10^{-5}g/20 ml, respectively.

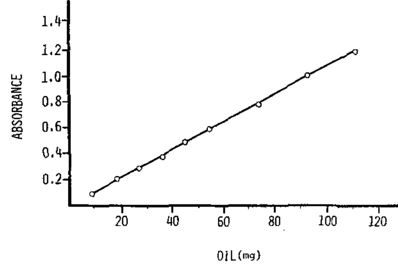

FIG. 4. Relationship between absorbance and sample size. Sample is autoxidized commercial food oil, POV = 58.9.

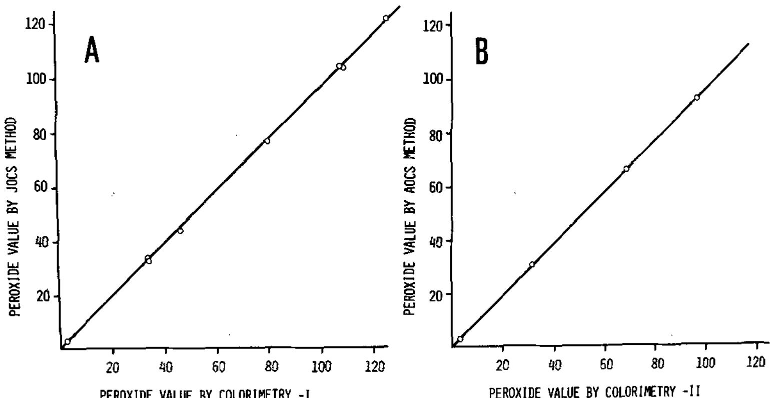

FIG. 5. Comparisons between JOCS method and colorimetry I (A), AOCS method and colorimetry II (B). The former reaction time is 5 min under CO_2, and the latter is 1 min in air. Each plot is an average of triplicate analyses.

TABLE I

Comparison of POV Obtained with Titrimetric and Colorimetric Methods[a]

Fats	Titration[b]		Colorimetry[b,c]	
	JOCS	AOCS	I	II
1	2.7 ± 0.6	2.8 ± 0.2	2.7 ± 0.1	3.0 ± 0.2
2	34.4 ± 0.7	30.9 ± 0.2	33.7 ± 1.1	31.3 ± 1.3
3	77.0 ± 1.5	66.7 ± 0.4	80.5 ±3.9	67.3 ± 2.1
4	105.4 ± 1.1	93.7 ± 0.3	106.8 ± 2.9	95.0 ± 2.1

[a]Average of triplicate analyses. All results expressed in POV ± standard deviation.
[b]Average of JOCS/AOCS and I/II are both 1.13 in samples 2-4.
[c]Reaction periods, I: 5 min under carbon dioxide atmosphere. II: 1 min in air. Sample taken, 45 mg.

TABLE II

POV of Lipids in Marine Products

Sample	Lipid content, %	Sample mg	POV average	Number of sample	Coefficient of variation,%
Salmon[a]					
flesh	3.5	56-73	12.0	4	3.6
dark meat	17.8	51-63	39.9	2	1.6
skin	13.8	57-76	73.9	2	0.2
Smoked salmon	56.6	269-282	14.3	2	2.8
Smoked cod	6.6	28-36	4.8	2	4.2
Whale bacon	21.0	81-129	4.2	2	2.4
Dried herring	26.3	108-111	65.6	2	0.5

[a]Stored at about -20 C for a long period.

600 when the sample taken is 10 mg. The POV measurement can be made to two decimal places when 400 mg of the sample is taken and the POV of sample is below 10.

Good agreement between the POV obtained by the colorimetric and titrimetric methods is shown in Figure 5. The results indicate that the values obtained by the colorimetric methods can be used without correction, while empirical calibration factors are necessary for the calculation of POV from data obtained by other colorimetric methods, e.g., ferric thiocyanate method (11) and titanium ion-hydroperoxide complex method (12). The periods for the oxidation of iodide ion with peroxides are 1 and 5 min in the AOCS and JOCS methods, respectively. The POV values obtained by the AOCS, JOCS, and colorimetric methods are compared in Table I. The POV by the AOCS method was generally about 13% lower than that by the JOCS method. The results obtained by the colorimetric methods with the reaction periods of 1 min in air and 5 min under the inert atmosphere agreed fairly well with those by the AOCS and JOCS methods, respectively. The equations for the exact conversions between POV obtained by different methods are shown as follows:

POV (JOCS) = 0.975 POV (colorimetry I) (1)

POV (AOCS) = 0.989 POV (colorimetry II) (2)

POV (JOCS) = 1.145 POV (AOCS) - 1.18 (3)

In Table I, the standard deviation in the data obtained by the colorimetric method is generally higher than those obtained by the titrimetric method. However, the increase of the sample taken in the former reduces the standard deviation easily to less than that in the latter. The colorimetric 5 min method (colorimetry I) is more convenient than the 1 min method (colorimetry II), since the latter strictly requires keeping the reaction period 1 min.

In the determination of POV with extracts by the Bligh and Dyer method (13), the aqueous iodide solution has shown the reliable colorimetric data regardless of the amounts and properties of the sample. It is attributable to removal of the nonpolar materials into the chloroform layer. Direct colorimetric analysis of the solution without evaporation of solvent eliminates the deviation caused by decomposition of the peroxides. The reproducibility of the method is shown by the coefficients of variation in Table II. In the low POV samples, the coefficients are relatively larger but not higher than 5%.

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The Metabolism of Lithocholic Acid-3α-sulfate by Human Intestinal Microflora [1]

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ABSTRACT

Lithocholic acid-3α-sulfate is metabolized by human intestinal microflora to nonpolar metabolites which have been partially purified by Sephadex LH-20 chromatography. These metabolites were characterized by thin layer and gas liquid chromatography as well as combined gas liquid chromatography-mass spectrometry. The chromatographic properties of one of the metabolites are consistent with those described for a Δ^2- or Δ^3-cholenate. The formation of cholenates by the microflora may represent a retoxification of the sulfate ester of lithocholic acid.

INTRODUCTION

Bile acids which are produced in the liver as end-products of cholesterol metabolism are excreted into the intestine. They are then either reabsorbed and further metabolized by the liver or eliminated in the feces. Metabolism of the primary bile acid, chenodeoxycholic acid, by intestinal microorganisms results in the formation of lithocholic acid which is highly toxic to animals and possibly to humans (1). Furthermore, the recent use of chenodeoxycholic acid therapy to dissolve gallstones in humans has stimulated interest in monitoring the increased production and excretion of this potentially hepatoxic compound (2).

The role of bile acids in promoting intestinal carcinogenesis has been reported by several workers using rat model systems (3,4). Lithocholic acid, specifically, has promoted the development of chemically induced tumors in the liver (5) and colon (6) of rats. Bacterial mutagenesis studies performed in our laboratory have shown that only lithocholic acid enhances the mutagenicity of suboptimal levels of 2-aminoanthracene (7) and benzo(a)pyrene (8) indicating that this bile acid may activate carcinogens having different chemical structures. Major bile acids such as cholic, chenodeoxycholic, and deoxycholic acids were not effective in stimulating the mutagenicity of these compounds.

As a continuation of our studies of lithocholic acid metabolism by human and rodent intestinal microflora (9-11), we became interested in the biotransformation of lithocholic acid-3α-sulfate by human microorganisms. One reason for this intesest is that the sulfate ester is a more water-soluble substrate and may be more extensively metabolized to possible mutagenic derivatives than either lithocholic or taurolithocholic acids.

Palmer (1) was the first investigator to describe the formation of bile acid sulfate esters of lithocholic acid and its glycine and taurine conjugates in humans and animals. It is believed that sulfation occurs in the liver and is another detoxification process which favors excretion of the bile salt (12). In addition, elevated levels of bile acid sulfates are excreted in urine, serum, and bile of patients with hepatobiliary diseases (13-15).

Studies of lithocholic acid metabolism in humans have shown that the bile acid is conjugated with glycine and taurine, and approximately half of these conjugates is sulfated (2,16,17). However, with continued enterohepatic cycling, any free bile acid would become sulfated (16) but would then be partially desulfated by bacterial sulfatases in the intestine prior to fecal excretion (18).

Palmer observed that labeled lithocholic acid fed to humans is extensively converted to isolithocholic acid, sulfate esters of conjugated lithocholic acid (1), and an unsaturated derivative in bile and feces (19). This unsaturated compound was slightly more polar than 5β-cholanic acid on thin layer chromagraphic plates and was tentatively identified as a Δ^2- or Δ^3-cholenate (20). Incubation of lithocholic

[1] The following names and abbreviations for chemicals and methods have been used throughout the text: lithocholic acid (LA) = 3α-hydroxy-5β-cholan-24-oic acid; isolithocholic acid (ILA) = 3β-hydroxy-5β-cholan-24-oic acid; 3-keto = 3-keto-5β-cholan-24-oic acid; 5β = 5β-cholan-24-oic acid; Δ^2-cholenate = 5β-chol-2-en-24-oate; Δ^3-cholenate = 5β-chol-3-en-24-oate; $LASO_4$ = lithocholic acid-3α-sulfate; methyl lithocholate (MLA) = methyl-3α-hydroxy-5β-cholan-24-oate; methyl isolithocholate (MIL) = methyl-3β-hydroxy-5β-cholan-24-oate; methyl-3-keto (Me-3-keto) = methyl-3-keto-5β-cholan-24-oate; methyl-5β (Me-5β) = methyl-5β-cholan-24-oate; methyl deoxycholate (MEDOXY) = methyl-3α12α-dihydroxy-5β-cholan-24-oate; GLC = gas liquid chromatography; GLC-MS = gas liquid chromatography-mass spectrometry; GFP = glass fiber paper; ITLC-SG = instant thin layer chromatography-silica gel; ITLC-SA = instant thin layer chromatography-silicic acid; BHI = brain heart infusion; MFC = mixed fecal culture.

acid-3α-sulfate with fecal microorganisms gave a product with an R_f similar to those found for cholenate(s) on thin layer plates, but the identity of these metabolites was not proven conclusively. Although cholenates were identified in human bile and feces, Palmer suggested that they may have been formed from alkaline hydrolysis procedures used in the extraction method (20). Alternatively, he suggests that unsaturated derivatives may be produced by intestinal microorganisms.

In order to clarify whether or not cholenates can be produced by microfloral metabolism of lithocholic acid-3α-sulfate, metabolites were isolated and characterized by thin layer, column, and gas liquid chromatography as well as chemical ionization mass spectrometry. This report describes the isolation of nonpolar metabolites which have been tentatively identified as cholenates.

EXPERIMENTAL PROCEDURES

Chemicals

Lithocholic acid (LA), isolithocholic acid (ILA), 3-keto-5β-cholanic acid (3-keto), and deoxycholic (deoxy) acids were obtained from Omni Research Inc., Caribbean Research Laboratories (Mayaguez, Puerto Rico). The 5β-cholanic acid (5β) was purchased from Schwartz/Mann (Orangeburg, NY). These compounds had a purity of at least 95% as judged by TLC and GLC (9,24). Methyl esters were prepared using diazomethane generated from Diazald (Aldrich Chemical Co., Inc., Milwaukee, WI) according to the manufacturer's directions based on the work of deBoer and Backer (21).

Preparation of cholenic acids was performed using a modification of the method described by Palmer (22). Lithocholic acid-3α-sulfate [($LASO_4$), 154 mg] was refluxed with 10 ml of 5N sodium hydroxide for 19 hr. After cooling the reaction mixture to room temperature, the product was extracted from a neutral solution with chloroform-methanol (2:1), and the solvent was removed in vacuo. The residue was methylated with diazomethane and analyzed by GLC and combined GLC-MS as described below.

[^{14}COOH]-LA used for the synthesis of labeled lithocholic acid-3α-sulfate ($LASO_4$) was obtained from Mallinckrodt Chemical Co. (St. Louis, MO) and had a specific activity of 5.36 mCi/mmole. We currently purchase this product (specific activity = 59 mCi/mmole) from Amersham Searle (Arlington Hts., IL). The [^{14}COOH]-LA was judged to be 98% pure by Gelman (Ann Arbor, MI) ITLC-SG sheets (see Methods). A 10 μCi (0.7 mg) aliquot was diluted with 51.2 mg of LA (purified by Sephadex LH-20 chromatography – see Methods) for the preparation of [^{14}COOH]-$LASO_4$ according to the method of Palmer (22). The final product had a specific activity of 0.06 mCi/mmole and was found to be 99% pure on ITLC-SG sheets which were developed in two solvent systems: isooctane-diisopropyl ether-acetic acid (75:30:1) and isooctane-isopropanol-ammonium hydroxide (20:80:0.5). All solvent systems described are expressed as ratios by volume.

The scintillation cocktail used for all radiological measurements was Aquasol (New England Nuclear, Boston, MA). All radioactive measurements were obtained with an Isocap/300 Liquid Scintillation System (Nuclear Chicago, Des Plaines, IL).

Reagent-grade solvents were purchased from either Burdick-Jackson (Muskegon, MI) or Fisher Scientific (Pittsburgh, PA).

Incubation Conditions

Approximately 2.5 g (wet wt) of freshly collected human feces was added to 180 ml of brain heart infusion (BHI) medium which was prepared and maintained under anaerobic conditions as described in the V.P.I. Anaerobe Laboratory Manual (23). An aliquot of 30 ml of this cell suspension was used for metabolism studies using 20 μmoles of [^{14}COOH]-$LASO_4$, and the incubation was conducted for 48 hr at 37 C under anaerobic conditions. In order to conserve substrate, a control containing only 2-3 μmoles of labeled $LASO_4$ in medium was incubated under the identical conditions and was analyzed as described below.

Extraction of Metabolites

After the designated incubation period, the cell suspensions were extracted as described previously (9,10), and the recovery of labeled metabolites in the organic layer varied from 80-90% of the total activity added. The recovery of unmetabolized [^{14}COOH]-$LASO_4$ from medium was only 27% due to the water solubility of the substrate.

Glass Fiber Paper (GFP) and Gas Liquid Chromatography (GLC) Analyses

Analyses of labeled metabolites by GFP and GLC have been adequately described (24). Gelman ITLC-SA sheets were occasionally used to supplement GFP analyses performed with ITLC-SG using the same solvent system: isooctane-diisopropyl ether-acetic acid (75:30:0.5).

	% DPM LASO$_4$ + MFC	% DPM LASO$_4$ + BHI	
5β	56.7	0.1	5β
	22.3	0.3	
	6.1	0.4	
3-Keto	0.9	0.1	3-Keto
	0.9	0.0	
ILA	1.0	0.3	ILA
	0.6	0.1	
LA	0.9	1.5	LA
	0.4	0.3	
	1.0	0.4	
LASO$_4$ Mix	9.2	96.8	LASO$_4$ Mix
Total DPM	20,300	950	

FIG. 1. Analysis of an extract from the incubation of [^{14}COOH]-LASO$_4$ with MFC and medium (BHI) by GFP (ITLC-SG) chromatography. The numbers represent % of total radioactivity associated with each zone, and the metabolites are designated as follows: 5β(5β-cholanic acid), 3-keto (3-keto-5β-cholanic acid), ILA (isolithocholic acid), LA (lithocholic acid), LASO$_4$ (lithocholic acid-3α-sulfate).

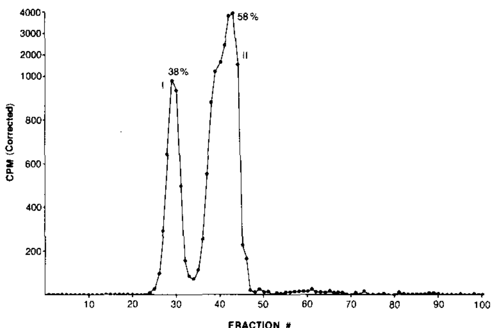


FIG. 2. Sephadex LH-20 chromagographic purification of metabolites isolated following the incubation of [^{14}COOH]-LASO$_4$ with human intestinal flora. A solvent mixture of isooctane-chloroform-methanol (2:1:1) was used to elute the metabolites from the column which had a bed volume of 147 ml. The percentages shown indicate the average % column volume that each fraction required for elution from the column. An aliquot of 20 μl from the 2 ml fractions collected was used for liquid scintillation counting.

Radioactive measurements of the ITLC-SG or ITLC-SA sheets were performed by cutting out the zones corresponding to the mobilities of reference standards which were spotted on the sides of the sheets and counting the activity by liquid scintillation methods described above.

Sephadex LH-20 Column Chromatography

Sephadex LH-20 (Pharmacia Fine Chemicals, Piscataway, NJ) columns were prepared and run as described previously (10) with 2 ml fractions collected and assayed by GFP and liquid scintillation counting.

	% DPM I	% DPM II	
	0.2	0.0	
Me-5β	0.9	62.4	Me-5β
	24.2	0.2	
	51.0	2.5	
	0.6	15.8	
5β	1.5	14.0	5β
	13.6	0.3	
Me-3-Keto	2.9	2.8	Me-3-Keto
MLA MIL	2.6	0.8	MLA MIL
LASO$_4$	2.6	1.2	LASO$_4$
Acid Methyl Mix Mix			Methyl Acid Mix Mix
Total DPM	1460	7260	

FIG. 3. Analysis of Sephadex LH-20 purified metabolites (see Fig. 2) by ITLC-SA glass fiber sheets. The fractions were methylated prior to analysis. Abbreviations used for the metabolites included Me-5β (methyl-5β-cholanate), Me-3-keto (methyl-3-keto-5β-cholanate), MIL (methyl isolithocholate), MLA (methyl lithocholate), and 5β (5β-cholanic acid).

Gas Liquid Chromatography-Mass Spectrometry (GLC-MS)

A Varian Aerograph (Varian Associates, Palo Alto, CA) GLC equipped with a 6-ft column of 1% QF-1 coated on Gas Chrom Q (100-200 mesh) maintained at 240 C was interfaced with a Finnigan Model 1015 (Finnigan Corp., Sunnyvale, CA) mass spectrometer. Methane gas was used both as a carrier (25 ml/min) and chemical ionization (CI) reagent gas (100 C, 150 eV, 500 mA emission current). Data acquisition was obtained with a Finnigan 6000 Data System.

RESULTS

Analysis of the extracts by GFP (ITLC-SG) shown in Figure 1 demonstrates that the majority of radioactivity from the LASO$_4$ + human mixed fecal culture (MFC) incubation was associated with the 5β or nonpolar region. A small amount of activity was found in the LA and ILA regions suggesting that the MFC contains "sulfatase" activity which would convert the sulfate ester into the free hydroxyl group. Incubation of [^{14}COOH]–LASO$_4$ in BHI medium under identical experimental conditions resulted in low counts in the organic extract, and the majority of activity was recovered as unmetabolized LASO$_4$ which would remain at the baseline.

Sephadex LH-20 chromatography of the crude extract from the MFC incubation gave two major peaks of radioactivity as seen in Figure 2. The peaks eluted within column volumes of 38 and 58%, respectively, indicated

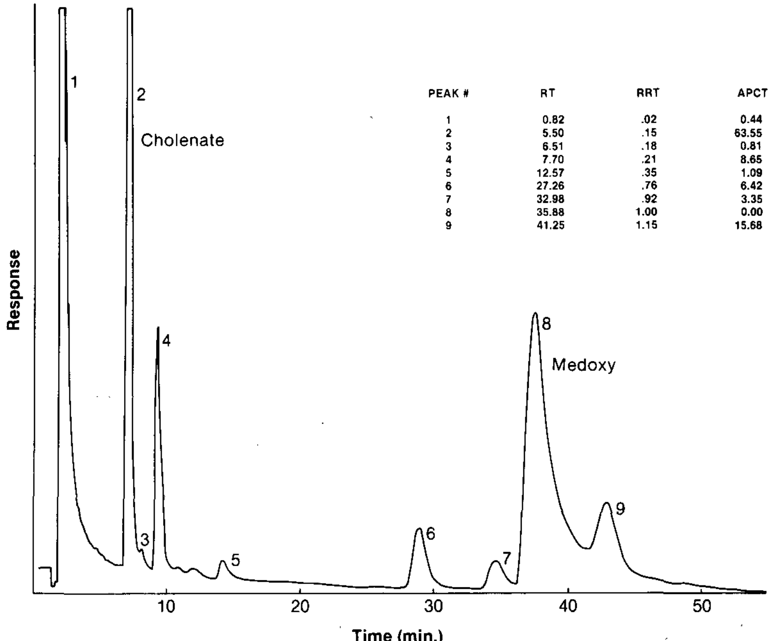


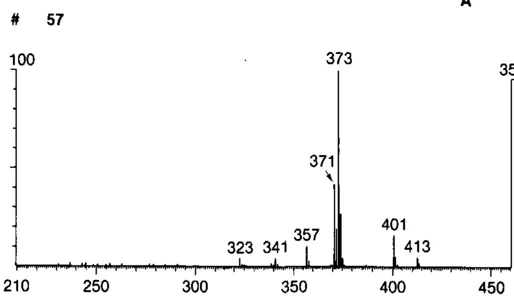
FIG. 4. Analysis of methylated peak II (Fig. 2) by GLC on a 1% QF-1 column. A 182 cm x 0.3 cm ID, silanized glass column packed with1% QF-1 coated on Gas Chrom Q (100-120 mesh) was obtained from Supelco (Bellefonte, PA). Operating conditions included oven and injection port temperatures of 210 C and detector oven at 280 C. Helium gas was used as a carrier at a flow of ca. 60 ml/min. Medoxy was used as an external standard to calculated relative retention times. Areas and retention times were determined with a Hewlett Packard (Avondale, PA) 3352A laboratory data system linked through a Hewlett Packard 1865 A/D converter. Abbreviations used include retention time (RT), relative retention time (RRT) and area percent (APCT).

that the metabolites were fairly nonpolar (10,11). Unmetabolized $LASO_4$ would not be eluted from the LH-20 column in this region using the conditions described for Figure 2 since more polar solvents such as chloroform-methanol (1:1) or methanol are necessary to remove this compound. The fractions corresponding to each of the peaks were pooled and reassayed by GFP chromatography using the system described in Figure 1. Again the majority of the radioactivity was associated with the 5β-region. Methylation of these two fractions followed by analysis of ITLC-SA GFP sheets gave radioactive profiles shown in Figure 3, and the ITLC-SA sheets appeared to be a good system for separation of the nonpolar metabolites as their methyl esters. Fraction I contained the bulk of its activity in a region slightly more polar than Me-5β, whereas Fraction II counts were located mainly in the Me-5β zone.

GLC of Fractions I and II from the LH-20 column was performed with columns of 1% QF-1 and 3% OV-17. The QF-1 profile of Fraction II (Fig. 4) contained several peaks which had retention times similar to Me-5β (peak No. 2), coprostanol (peak No. 4), unknown (peak No. 5), impurity in MEDOXY standard (peak No. 6), 3-keto (peak No. 7), and an unknown peak (peak No. 9). However, when Fraction II was chromatographed on a 3% OV-17 column, only the Me-5β region agreed with the previous GLC data, whereas the other peaks did not have retention times identical with any of the reference compounds tested.

Fraction II was also characterized by GLC-MS (Fig. 5A) and spectrum number 57 from the reconstructed GLC chromatogram gave peaks at m/e=413 $(M + C_3H_5)^+$, 401 $(M + C_2H_5)^+$, 373 $(M + H)^+$, 371 $(M - H)^+$ 357 [(M + H) - 16], and 341 $[(M + H) - 32]^+$. These fragmentation patterns were identical with CI spectra obtained for unpurified methyl cholenates produced by alkaline hydrolysis of $LASO_4$, as suggested by Palmer (20).

Fraction I contained one peak which also had a fragmentation pattern similar to the cholenate above and some additional peaks which appeared to be due to the presence of fecal sterols. However, the cholenate peak observed by GLC-MS was not found during GLC analyses described in the Experimental



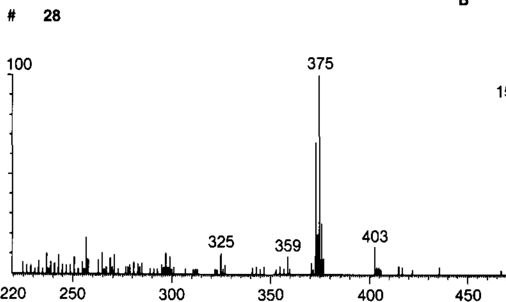


FIG. 5A. Chemical ionization GLC-MS fragmentation of methylated peak II (Fig. 2). Conditions are given in the text. 5B. Mass spectrum of 5β-cholanic acid (methyl ester) obtained from a Sephadex LH-20 purified extract of an incubation of [^{14}COOH]-taurolithocholate with human intestinal microflora.

Section above possibly due to the fact that a more concentrated sample was used for the GLC-MS determination.

DISCUSSION

Palmer has suggested that cholenates may be produced by the action of intestinal microorganisms on $LASO_4$ (20), and this report describes in more detail the formation and partial characterization of these metabolites. Although most of the metabolites produced by MFC metabolism of $LASO_4$ were cholenates, some low activity was also found in the LA and ILA regions (Fig. 1). These results suggest that the human MFC does contain "sulfatase" activity and forms metabolites containing a free hydroxyl group. ILA was presumably formed by oxidation of the LA to the 3-keto derivative followed by reduction to the 3β-ol (19). The mechanism of formation of the cholenates is not clear since an elimination of the sulfate ester group may be involved as is the case for chemical elimination reactions (25).

Sephadex LH-20 profiles of the extract of the [^{14}COOH]-$LASO_4$ incubation with MFC are reproducible since subsequent experiments with $LASO_4$ and freshly prepared microflora from the same donor gave similar results. The peaks are eluted in a column volume which is similar to that for nonpolar compounds, but endogenous fecal sterols are also eluted from the column with these cholenates as suggested by the GLC profiles of sterol standards. Recent unpublished data obtained in our laboratory indicate that further purification of the LH-20 fractions by ITLC-SA sheets can effectively eliminate interference by these sterols.

Combined GLC and CI MS provided evidence that the cholenate(s) produced could be separated from the sterols and characterized. The CI fragmentation pattern (Fig. 5A) contained quasi molecular ions at m/e = 413 and 401 and $[M + H]^+$ ion at m/e = 373, which are consistent with the molecular ion at m/e = 372 obtained by electron impact MS (20). However, the remaining two fragments were due merely to loss of methane (m/e = 357) and methanol (m/e = 341). In addition, the fragmentation and GLC retention times were identical with those obtained for cholenates produced in a reaction mixture obtained by alkaline hydrolysis of $LASO_4$. Since this reaction produces many side-products, better synthetic routes are being developed for preparing and purifying authentic cholenates. These reference standards will facilitate further characterization of the microbial metabolites isolated and will also be properly tested in several in vitro systems for their potential carcinogenicity.

It is interesting to note that substrate specificity will alter the metabolic profile produced in incubations of LA derivatives with human MFC. A nonpolar fraction isolated from the incubation of [^{14}COOH]-taurolithocholate (TL) with MFC gave a fragmentation pattern identical with authentic Me-5β as seen in Figure 5B, and the peaks are shifted 2 amu upward from the cholenate spectrum (5A). The TL was also metabolized to LA, 3-keto, ILA, and other unidentified nonpolar compounds (11).

Since a mixture of sulfated and unsulfated LA and its conjugates are excreted in bile, a variety of metabolites should be produced by the action of the intestinal flora. The more water-soluble $LASO_4$ is less toxic than LA and is presumably formed to promote excretion. However, one encounters differences in species since mice, rats, and humans are capable of sulfating LA (26), whereas the Rhesus monkey cannot (27). The microflora of these species may also have different enzymatic capabilities in metabolizing the sulfate ester. Recently, we have found different levels of cholenate metabolites produced by human intestinal flora obtained from donors on different diets (Kelsey

and Hwang, unpublished observations).

Bremmelgaard (28) and Palmer (11) found that fecal bacteria can hydrolyze the sulfate ester of LA, whereas they were unable to find individual strains of organisms that could metabolize $LASO_4$. Recently, Imperato et al. (29) found that *Pseudomonas aeruginosa* isolated from human feces could hydrolyze the $LASO_4$ under aerobic conditions using minimal support medium. LA was the only metabolite identified, indicating that the sulfatase activity of this organism may be a useful method for detecting sulfate esters in feces. Alkaline hydrolysis procedures which are used currently in extraction procedures may produce artifacts in the analysis of fecal bile acids (20,22), and could be replaced by an enzymatic procedure using the above microorganism.

Our results now confirm the earlier proposal of Palmer (20) that intestinal microorganisms can form cholenates from $LASO_4$. Since this compound is a major bile salt in the colon, the microbial metabolism of this substrate may result in the formation of more toxic and/or mutagenic products in "high risk" (for contracting colon cancer) individuals. The investigation of this hypothesis is now in progress.

ACKNOWLEDGMENTS

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COMMUNICATIONS

Hydrolysis of Biliary Phospholipids in the Upper Small Intestine of the Chick

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ABSTRACT

Chick endogenous phospholipids were doubly labeled by an intravenous injection of [^{32}P] phosphate and [1-^{14}C] oleic acid, and the free fatty acid and phospholipid fraction of gall bladder bile and in contents of upper small intestine were analyzed 4 days later. There was evidence of hydrolysis of biliary phosphatidylcholine to lysophosphatidylcholine in the duodenum and jejunum, but this did not account for the pronounced increase in the ^{14}C radioactivity of the free fatty acids relative to the ^{32}P phospholipid radioactivity between bile and upper intestinal segments. It is suggested that phosphatidylcholine is largely absorbed in the duodenum of the chick while the remainder is progressively hydrolyzed and absorbed.

INTRODUCTION

Chicken bile, like bile of other species, contains phospholipids, whose role in lipid digestion is not clear. Scow et al. (1) showed that in the rat, phosphatidylcholine (PC) is hydrolyzed in part to lysophophatidylcholine (LPC) before absorption, and Leat and Harrison (2) observed a similar pattern in sheep. It was assumed (1,2) that both phospholipids play a role in bringing dietary lipids into micellar solution before absorption. More recent work with rats by Boucrot (3,4) and Nalbone et al. (5,6) suggests that native biliary PC, unlike exogenous PC, is resistant to hydrolysis and appears to be absorbed intact.

In the present study, an attempt was made to gain information on the hydrolysis of phospholipids in the chicks' upper intestine by labeling both the phophorus and fatty acid moieties of endogenous phospholipids and analyzing the lipids of bile and contents of upper intestinal segments.

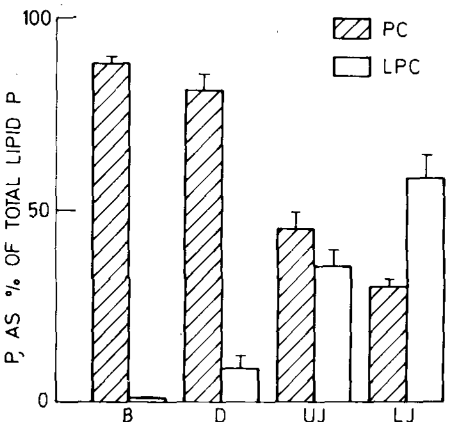

FIG. 1. Contribution of phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) to total phospholipids in bile and contents of upper small intestine. Each bar represents the mean of six chicks with one SEM shown on top of each bar. B, bile; D, duodenum; UJ and LJ, upper and lower jejunum, respectively.

METHODS

Twenty-one-day-old Leghorn x New Hampshire male chicks were fed a semisynthetic diet based on defatted soybean meal and glucose (7). The diet contained 22% protein and 3% added soybean oil. On the second day after the start of the experimental diet, the chicks received intravenously 0.5 ml of a solution containing 70 μCi [^{32}P] orthophosphoric acid (Nuclear Research Center, Beer Sheva), 15 μCi [1-^{14}C] oleic acid (Radiochemical Research Centre, Amersham), and enough sodium bicarbonate to bring the pH to 7.4. Chicks were killed on the sixth day with an overdose of sodium pentobarbital. Bile was obtained from the gall bladder and frozen immediately. Contents of the duodenum and upper and lower jejunum were extruded, homogenized, and frozen, as described previously (8).

Lipids were extracted from bile and from aliquots of homogenized digesta, using chloroform-methanol (2:1 by vol). After evaporation of the solvents under reduced pressure, the lipids were taken up in chloroform. Free fatty acids (FFA) were separated from the other lipids by thin layer chromatography (TLC) on 0.5 mm thick layers of Silica Gel G, using as a developing solvent petroleum ether-diethyl ether-acetic acid (70:30:1 by vol). Heptadecanoic acid (Sigma Chemical Co. Inc., St. Louis, MO) was added as an internal free fatty acid standard before chromatography. In the above system, the phospholipids remain at the origin. After localization of the fractions by spraying with 2′,7′-dichlorofluorescein and observation under UV light, and identification of the FFA fraction by comparison with an oleic

acid standard, the FFA were extracted with chloroform and the phospholipids with methanol. Separation of PC and LPC was done on plates prepared as above but using as a developing solvent chloroform-methanol-water (65:30:5 by vol). The two fractions were visualized as above, located by comparison with authentic standards (Sigma Chemical Co., Inc.), and extracted with methanol.

A portion of the FFA fraction was methylated and submitted to gas chromatography as described previously (8), and the total amount of FFA was calculated by means of the internal C_{17} standard. Phosphorus was determined in the total phospholipids and in portions of the PC and LPC fractions by the method of Ames and Dubin (9). Additional portions of total phospholipids, PC and LPC, were taken for scintillation counting of ^{32}P and ^{14}C. Corrections for quenching and for the counts of one isotope in the channel of the other were applied using internal standards of both isotopes.

RESULTS AND DISCUSSION

The present approach, involving radioactivity measurements in the contents of the upper intestinal segments, is valid only if the specific radioactivities of lipid phosphorus and endogenous fatty acids on the final day of the experiment are constant during one time of passage of the digesta through the small intestine (ca. 2 hr). The specific activity of plasma unesterified fatty acids, which is the lipid fraction with the fastest turnover rate, decreased by less than 0.5%/hr at the end of the experimental feeding, and a check of the changes in ^{32}P and ^{14}C specific activities of the plasma phospholipid fraction revealed even smaller rates of decrease. Therefore, any changes in specific activity of lipid P and ^{14}C-fatty acids in the digesta along the small intestine must have been due to digestive and absorptive processes.

Figure 1 shows that intact PC made up the bulk of the bile phospholipids, whereas the phospholipids in successive segments of the upper small intestine contained an increasing proportion of LPC. Thus, progressive deacylation undoubtedly takes place in the proximal part of the small intestine, but since changes in lipid composition are the net result of digestive and absorptive processes, the above results do not, by themselves, provide any indication of the extent to which phosphoglycerides are hydrolyzed before absorption.

Additional information on the fate of biliary phospholipids is presented in Table I. There were only slight changes in specific activity of lipid phosphorus between bile and intestinal

TABLE I

Relative Amounts and Radioactivities of Phospholipids and Free Fatty Acids (FFA) in Gall Bladder Bile and Contents of the Upper Small Intestine of Chicks, 4 Days after an Intravenous Injection of [^{32}P]Phosphate and [1-^{14}C]Oleic Acid[a]

	Phospholipid fraction			FFA	Lipid P/FFA	
	P spec. activity (cpm/mg P)	Wt ratio P/FA (mg/g)	$^{32}P/^{14}C$ (cpm/10^3cpm)	specific activity (cpm/mg)	Wt ratio (mg/g)	Isotope ratio (cpm/10^2cpm)
Bile	545 ± 31a	50.5 ± 7.8a	490 ± 13a	18.9 ± 3.4a	125.1 ± 7.2a	368 ± 8a
Duodenum	492 ± 40a	57.8 ± 7.3ab	508 ± 21a	8.3 ± 0.9b	9.0 ± 0.9b	86 ± 7b
Upper jejunum	513 ± 57a	64.4 ± 8.5bc	669 ± 36b	10.5 ± 2.7ab	5.8 ± 0.7bc	29 ± 4c
Lower jejunum	523 ± 73a	74.4 ± 10.1c	680 ± 45b	12.6 ± 2.9ab	4.3 ± 0.4c	18 ± 3c

[a]Results are means ± SEM for six chicks. Values in columns not followed by a common suffix letter differ significantly ($P < 0.05$).

segments, and these did not achieve statistical significance at the 95% probability level. Thus, the phospholipids present in the upper intestine are essentially of endogenous origin and are only very slightly diluted by exogenous PL. This is also indicated by the following facts: previous work in which $^{91}YCl_3$ was used as a nonabsorbable reference substance (7,10) had established that net secretion of lipid phosphorus into the duodenum of chicks of this size amounts to roughly 20-40 mg/day. In the present experiment, the feed assayed 50.1 μg lipid P/g diet, so that at a feed intake of 25 g/day, the daily lipid phosphorus provided by the diet amounted to 1.25 mg/day, or 3-6% of the amount secreted. Thus, dilution of endogenous ^{32}P radioactivity by dietary phospholipids was very small.

On the other hand, the pronounced drop in specific activity of the FFA fraction, seen in Table I, may be ascribed to dilution by exogenous fat digestion products. We have previously reported (10) that dietary oil is largely hydrolyzed already in the chicks' duodenum. The dilution of endogenous FFA radioactivity by exogenous fatty acids far exceeds any increase in radioactivity that might be caused by partial hydrolysis of ^{14}C-labeled phospholipids.

Table I also shows that both the weight ratio and isotope ratio of phosphorus to fatty acids *within the phospholipid fraction* increased between bile and successive intestinal segments. This is an additional indication of the hydrolysis taking place in the phospholipids, as already seen from the data in Figure 1. On the other hand, the ratio of lipid P to FFA, both by weight and by radioactivity, dropped precipitously between bile and upper small intestine. While a drop in weight ratio would be expected from the influx of exogenous fatty acids already referred to, the decrease in isotope ratio, although smaller than that of the weight ratio, is not as readily explained. Even assuming equal distribution of the fatty acid radioactivity between the C-1 and C-2 positions of the PC molecules and complete conversion of lecithin to lysolecithin, the calculated decrease in isotope ratio of lipid P to FFA, based on the relative amounts and radioactivities of biliary phospholipids and FFA, would be far less than the actual drop shown in the last column of Table I. Thus, the observed results cannot be ascribed to a simple hydrolysis of biliary phospholipids.

A plausible explanation of the present results could be that intact biliary PC is partly absorbed in the duodenum and to a lesser extent in the jejunum, and that small amounts of nonabsorbed PC are progressively hydrolyzed to LPC and FFA, which are in turn taken up by the mucosal cells. Our previous data on lipid absorption in the chick have indicated that absorption of lipid P is essentially complete at the level of the lower jejunum and that FFA are absorbed more slowly than the glyceride residues (10). The above explanation would account, qualitatively at least, for the observed drop in isotopic lipid P/FFA ratio between bile and upper intestine.

The results of the present experiment are consistent with the concept recently advanced by Boucrot (3,4) and Nalbone et al. (5,6) according to which native biliary PC are partly absorbed in their unhydrolyzed state in the rat upper intestine.

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Inhibition of Diacylglycerol:CDPcholine Cholinephosphotransferase Activity by Dimethylaminoethyl *p*-Chlorophenoxyacetate

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ABSTRACT

Cholinephosphotransferase [EC 2.7.8.2] activity of rat liver microsomes, with 1,2-di-*O*-[^{3}H]acyl glycerol or 1-*O*-hexadecanoyl [U-^{14}C]ethanediol as substrate, was inhibited by *N,N*-dimethylaminoethyl *p*-chlorophenoxyacetate (centrophenoxine). Inhibition progressed in a linear fashion with increasing drug levels and was complete at 30 mM concentration. It appears that the microsomal enzyme was largely affected by the drug itself because the hydrolysis products of centrophenoxine, viz., *N,N*-dimethylaminoethanol and *p*-chlorophenoxyacetic acid, were less inhibitory.

INTRODUCTION

De novo synthesis of 1,2-diacyl *sn*-glycero-3-phosphocholine from 1,2-diacyl glycerol and cytidine 5′-diphosphocholine (CDPcholine) (1) is catalyzed by a membrane-bound choline-phosphotransferase [EC 2.7.8.2] (2,3). We have recently shown (4) that rat liver microsomes also catalyze cholinephosphate transfer from CDPcholine to *O*-acyl ethanediol to form acyl diol phosphocholines. In order to determine whether glycerol- and diol-derived (5-7) cholinephospholipids are produced by the same enzyme system, a number of potential inhibitors were tested.

In the course of these studies, we found that centrophenoxine (Helfergin®, Promonta), a drug that is used for its neuroanabolic and psychoanaleptic effects (8) and which is known to inhibit lipofuscin formation in central nervous tissue (9), most effectively and rather specifically inhibited phosphocholine transfer from CDPcholine to diglyceride or glycol monoester. The pharmacological activity of centrophenoxine could well be related to the apparent effectiveness of the drug in inhibiting one of the key enzymes of phospholipid synthesis which would result in altered membrane permeability.

MATERIALS AND METHODS

1,2-[^{3}H]Diacyl *sn*-glycerol was prepared by phospholipase C hydrolysis (7) of catalytically tritiated (New England Nuclear, Boston, MA) rat liver phosphatidylcholine and was diluted to a specific activity of 13.6 mCi/mmol. 1-*O*-Hexadecanoyl [U-^{14}C]ethanediol was synthesized as described previously (4) and was used at a specific activity of 1.8 mCi/mmol. CDPcholine was purchased from Sigma Chemical Co., St. Louis, MO. Centrophenoxine (Helfergin®) was obtained from Chemische Fabrik Promonta GmbH, Hamburg, Germany. Sodium *p*-chlorophenoxyacetate was purchased from Eastman Organic Chemicals, Rochester, NY; *N,N*-dimethylaminoethanol was from Aldrich Chemical Co., Milwaukee, WI.

Rough rat liver microsomes were isolated as outlined by Fleischer and Kervina (10). The livers from two male rats (200-250 g; Sprague-Dawley, Madison, WI) were homogenized in five volumes of 0.25 M sucrose in a 50 ml Potter-Elvehjem type glass homogenizer at 1000-1100 rpm, first with a loose pestle (0.026 in. clearance), and then with a tight pestle (0.012 in. clearance). The homogenate was filtered through nylon cloth, and the filtered homogenate was centrifuged at 960 x g for 10 min. The supernatant was decanted and centrifuged at 25,000 x g for 10 min to remove the mitochondria, lysosomes, and peroxisomes. The resulting supernatant was then centrifuged at 34,000 x g for 30 min to sediment the heavy microsomes. The supernatant containing the light microsomes was centrifuged at 40,000 x g for 57 min (using a Spinco Ti60 rotor), and the pellet was resuspended in 0.25 M sucrose and saved. The heavy microsomes were gently resuspended in 10 ml of 52% sucrose in 0.1 M phosphate buffer (pH 7.1), and the sucrose concentration was adjusted to 43.7%. This fraction was placed in a SW 25.2 rotor centrifuge tube and overlayered sequentially with 11-15 ml, 10 ml, 10 ml, and 12 ml of 38.7%, 36%, 33%, and 29% sucrose, respectively, and was centrifuged at 25,000 rpm for 53 min. The Golgi fraction was removed from the 29% and 33% interface, and the heavy microsomes were recovered from the bottom of the gradient. Equal volumes of the suspended heavy and light microsomes were combined and diluted with 28 ml of 0.25 M sucrose. One M CsCl was added to bring the concentration to 0.015 M. This suspension was

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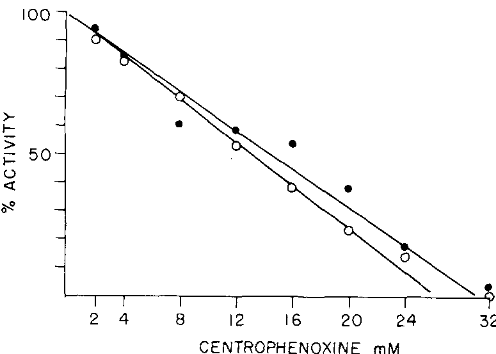

FIG. 1. Effect of centrophenoxine on cholinephosphotransferase activity. Percent activities are averages of at least two incubations and are based on the amounts of 1,2-di-*O*-[^{3}H]acyl *sn*-glycero-3-phosphocholine (•) and 1-*O*-hexadecanoyl [U-^{14}C]-ethanediol 2-phosphocholine (○) formed from the respective diglyceride and glycol monoester used as substrates. For the complete incubation system see Materials and Methods and Table I.

layered into two tubes, each containing 20 ml of 1.3 M sucrose/0.015 M CsCl and was centrifuged in the Ti60 rotor at 58,000 rpm for 110 min. The rough microsomal pellet was collected, resuspended in 0.25 M sucrose, and stored in small aliquots at -70 C. Only freshly thawed microsomes were used for each set of incubations. Protein was determined by the method of Lowry et al. (11) using crystalline bovine serum albumin as standard.

The complete incubation system contained the microsomes (0.2 mg protein) in 0.6 ml of 0.25 M sucrose; CDPcholine, 4 μmol; $MgCl_2$, 20 μmol; 1-*O*-hexadecanoyl [U-^{14}C]ethanediol, 2 x 10^6 dpm/500 nmol or 1,2-[^{3}H]diacyl glycerol, 15 x 10^6 dpm/500 nmol; and 0.5 M Tris buffer (pH 7.2) to make a total volume of 2 ml. Incubations were carried out at 37 C in 20 ml screw cap vials. The microsomal suspension and cofactors were preincubated for 2 min, and then the lipid substrate was added as sonicated dispersion. Incubations were terminated after 10 min by addition of 8 ml of chloroform-methanol (2:1, v/v).

Lipid extraction and analysis: After termination of an incubation with chloroform-methanol, the lower organic phase was removed, and the upper phase was reextracted with two 5 ml portions of chloroform. The combined chloroform extracts were dried, 5 mg of either rat liver phosphatidylcholine or synthetic hexadecanoyl ethanediol phosphocholine (7) were added as carriers, and the respective cholinephospholipids were purified twice by chromatography on 0.3 mm layers of Silica Gel H (Merck) using first chloroform-methanol-acetic acid (30:70:4 v/v/v), then chloroform-methanol-2N ammonium hydroxide (20:80:6, v/v/v) as developing solvents. Fractions were eluted from the adsorbent with chloroform-methanol-water (50:40:10, v/v/v). The solvent was removed in vacuo, and radioactivities were determined by liquid scintillation counting (scintillation liquid was prepared from toluene, 2000 ml; Triton X-100, 1000 ml; Omnifluor, New England Nuclear, 12 g). Counting efficiency was 81% for ^{14}C and 48% for ^{3}H.

RESULTS AND DISCUSSION

The effect of increasing concentrations of centrophenoxine on the formation of 1,2-di-*O*-[^{3}H]acyl *sn*-glycerophosphocholine and 1-*O*-hexadecanoyl [U-^{14}C]ethanediol phosphocholine from diglyceride and glycol monoester, respectively, was studied using rat liver microsomes. As is evident from Figure 1, inhibition of cholinephosphate transfer from CDPcholine to 1,2-diacylglycerol (50% inhibition at 15 mM) or acyl ethanediol (I_{50} at 13 mM) was directly proportional to the centrophenoxine level in the system. At 30 mM centrophenoxine concentration, inhibition with both substrates was complete.

Because centrophenoxine is readily hydrolyzed in solution at physiological pH and temperature (12), leading to the formation of *p*-chlorophenoxyacetic acid and dimethylaminoethanol, cholinephosphotransferase inhibition due to the products of hydrolysis, or due to pH fluctuation, was not excluded a priori. In fact, initial experimentals had shown that 30 mM centrophenoxine in the incubation system using 0.1 M Tris buffer resulted in an eventual decrease in pH by almost two units. Therefore, in the present experiments, an optimum pH of 7.2 was maintained throughout the entire incubation period by use of 0.5 M buffer. The higher buffer concentration lowered apparent enzyme activity, in absence of the drug, by less than 20%.

When either of the hydrolysis products of centrophenoxine, namely sodium *p*-chlorophenoxyacetate or dimethylaminoethanol, were added to the incubation system (Table I), cholinephosphotransferase activity was inhibited by 56-77% at 30 mM concentration. Also, simultaneous addition of both hydrolysis products, each at 30 mM concentration, affected formation of diacyl glycerophosphocholine (65% inhibition) and acyl glycol phosphocholine (62% inhibition) to a lesser extent than did centrophenoxine per se (100% inhibition at 30 mM), thus supporting a mechanism of

TABLE I

Effect of Centrophenoxine and Its Hydrolysis Products on Cholinephosphotransferase Activity of Rat Liver Microsomes

Incubation conditions	Percent activity based on formation of	
	[^{3}H]Diacyl glycero-phosphocholine	Acyl [U-^{14}C]-ethanediol phosphocholine
Complete system[a]	100	100
+ 15 mM Centrophenoxine[b]	51	43
+ 30 mM Centrophenoxine[b]	1	0
+ 15 mM Dimethylaminoethanol[b]	57	69
+ 30 mM Dimethylaminoethanol[b]	31	40
+ 15 mM Na *p*-chlorophenoxyacetate[b]	37	65
+ 30 mM Na *p*-chlorophenoxyacetate[b]	23	44
+ 30 mM Dimethylaminoethanol + 30 mM Na *p*-chlorophenoxyacetate[b]	35	38
+ 30 mM Glutathione + 30 mM centrophenoxine[b]	20	11
Centrophenoxine preincubation[c]	8	3

[a]The complete system (see Materials and Methods) produced 1.4 nmol of 1,2-di-*O*-[^{3}H]acyl glycerophosphocholine or 1.9 nmol of *O*-hexadecanoyl [U-^{14}C]ethanediol phosphocholine per hour per mg protein. Values given are averages of at least two incubations.

[b]Additives were freshly dissolved in ice-cold 0.5 M Tris buffer and were preincubated in the complete system (less lipid substrate) for 2 min.

[c]Protein, 2 mg, was preincubated with 30 mM centrophenoxine for 2 min, then a 10% aliquot of the preincubated protein/centrophenoxine mixture was transferred to the incubation medium.

inhibition that would foremost involve centrophenoxine, rather than the products of hydrolysis.

When ten parts of microsomes (2 mg protein) were preincubated with 30 mM centrophenoxine, followed by tenfold dilution of enzyme and inhibitor by transferring a 10% aliquot into the incubation medium, cholinephosphotransferase activity was still not detected (Table I). Hence, it appears that the cholinephosphotransferase system of rat liver microsomes is rather directly and irreversibly affected by centrophenoxine, possibly by altering the active site of the enzyme. Glutathione provided only minimal protection against the drug (Table I). On the other hand, neither glucose 6-phosphatase (13) activity (1.5 μmol/min/mg protein) nor the enzymatic hydrolysis of glycol monoester in our system, was influenced at all by centrophenoxine, indicating that the drug may act quite specifically on the cholinephosphotransferase.

Our finding that centrophenoxine effectively interferes with a major pathway of cholinephospholipid synthesis in mammalian systems could shed light on the molecular mechanisms that govern stimulation of the central nervous system by the drug (8) and that control lipofuscin levels (9). Impaired phosphatidylcholine synthesis and concurrent changes in membrane integrity and permeability could also be an underlying cause of the potentiation by centrophenoxine of the cytotoxic action of "activated" cyclophosphamide and other antitumor agents (14-17).

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LETTER TO THE EDITOR

Preparation of Choline and Ethanolamine Plasmalogens by Enzymatic Hydrolysis of the Accompanying Diacyl Analogs

Sir: Alk-1-enyl acyl glycerophospholipids (plasmalogens) occur only in association with their diacyl and alkyl acyl analogs, and the three species are not separable. Procedures have been devised to remove the diacyl glycerophospholipids by selective enzymatic or chemical hydrolysis, but no method is available to separate alkyl acyl from alk-1-enyl acyl glycerophospholipids. Phospholipase A_2 from *Crotalus atrox* (1), phospholipase C from *Clostridium perfringens* (2), and phospholipase D from cabbage (3) hydrolyze diacyl phosphatidylcholine more rapidly than the corresponding alk-1-enyl acyl and alkyl acyl glycerophosphocholines. By alkaline hydrolysis under very mild conditions (4), diacyl glycerophospholipids are deacylated more rapidly than the alkyl acyl or alk-1-enyl acyl analogs. Since all these methods make use of gradual differences in the reactivitity of ether ester and diester glycerophospholipids, a certain amount of the desired plasmalogen will also be hydrolyzed, and the final yield will be reduced. Furthermore, the enzymatic methods are applicable only to choline glycerophospholipids.

Woelk et al. (5) described the isolation of choline and ethanolamine plasmalogens from the corresponding phospholipid fractions obtained from bovine heart and brain, respectively, employing highly purified lipase from porcine pancreas, which cleaves specifically primary acyl ester bonds of diacyl glycerophospholipids. Alkyl acyl and alk-1-enyl acyl glycerophospholipids are not attacked by this enzyme. Choline and ethanolamine plasmalogens were thus obtained in high yield (73% and 74%, respectively) and purity (98%). This method has considerable advantages, but its general application appears to be limited because highly purified pancreatic lipase is not commercially available. This led us to propose the use of lipase from *Rhizopus arrhizus,* which has the same specificity for primary acyl ester groups as has pancreatic lipase (6); it can be purchased in highly purified form.

Ethanolamine and choline glycerolipids were isolated from calf brain and beef heart lipids, respectively, by a combination of column chromatography and thin layer chromatography (TLC), employing established methods. The plasmalogen content (52% in the ethanolamine phospholipids and 43% in the choline phospholipids) was measured by the iodine method (7), alkyl acyl glycerophospholipids were quantitated as alkali and acid stable lipids (8), and phospholipid phosphorus was determined by the method of Bartlett (9).

Identical procedures were followed for the treatment of both the choline and ethanolamine glycerophospholipid fractions with lipase. The lipid (100 mg) was dispersed in 20 ml of buffer [40 mM Tris-HCl, pH 7.6, 0.1 M NaCl, 3 mg/ml Na-deoxycholate (Merck, Darmstadt), 4.5 mg/ml bovine serum albumin (Calbiochem), 5 mM $CaCl_2$] by shaking for 10 min, followed by sonication for 20 sec at 0-5 C (Braun Sonic 300 S, 50 W). After addition of 100 μl (1 mg, 6000 I.E.) of lipase from *Rhizopus arrhizus* (Boehringer, Mannheim), the mixture was incubated with shaking at 25 C for 2 hr. After incubation, the lipids were extracted by the addition of 100 ml chloroform-methanol, 2:1. The lower phase was evaporated to dryness, and the residue was dissolved in chloroform-methanol, 2:1. Plasmalogens (together with accompanying alkyl acyl glycerophospholipids) were isolated by preparative TLC on Silica Gel H, with chloroform-methanol-water, 65:25:4, as developing solvent.

Chromatographically pure ethanolamine and choline plasmalogens were obtained with a yield of 80%, based on the amount of plasmalogen present in the ethanolamine and choline phospholipid fractions. The aldehyde:P ratio was 0.97 and 0.93, respectively. Deviation from unity was due to the presence of alkyl acyl glycerophospholipids (3% in ethanolamine plasmalogen and 7% in choline plasmalogen) which were identified and quantitated after alkaline and acidic hydrolysis (8) of the plasmalogen fraction. The ratio alk-1-enyl:alkyl groups was the same in the respective substrates and final products, indicating that there is no selective loss of one of the two types of ether phospholipids during the isolation procedure.

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Progressive Gross Changes in Renal Medullary Composition in Pregnant Rats[1]

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ABSTRACT

The biochemical composition of the renal medulla and the blood pressures of pregnant rats and nonpregnant controls were compared on days 15, 18, 20, and 22 of pregnancy. Deoxyribonucleic acid and protein content of the renal medulla changed together with the tissue weight (wet weight) during gestation except on day 20 when a slight increase in protein was observed. However, the glycerophospholipids (phosphatidyl ethanolamine, phosphatidyl choline, and phosphatidyl serine) and sphingomyelin showed significant increases throughout the latter stages of pregnancy with the maxima in most cases occurring on day 20 of the gestation period. Medullary hypertrophy and increased lipid content per cell coexist with the decrease in blood pressure and may indicate peak activity in production of antihypertensive renal lipids.

INTRODUCTION

Intracellular lipid granules were observed in frozen tissue sections of the kidney before any functional indication for these materials existed. In early histochemical studies, kidney lipids were observed as osmophilic droplets found both in the interstitial tissue and in the epithelial tissue of the tubules (1). Smith (2) found lipid droplets in the kidney of cat, dog, rat, and rabbit. MacNider (3) showed that the fat infiltration into the kidney increased with age in dogs. In studies on young and adult cats, Modell (4) and Modell and Travell (5) confirmed the increase in kidney lipid content with age but noted that the most prominent accumulation occurred in the kidneys of pregnant cats.

Few studies have appeared on the chemical composition of the renal lipid material. Morgan et al. (6) analyzed the lipid composition of rabbit kidney cortex and medulla and reported a difference in lipid content and composition. Recently Soula et al. (7) analyzed lipid material from human kidney and reported differences in composition for cortex and medulla in normal subjects.

Lobban (8), using histochemical methods, correlated tubular lipid accumulation with the estrous cycle of the cat and suggested that the accumulated lipid was similar to estrogen, but Helmy and Logley (9), who extracted and identified medullary lipids in young and adult cats and dogs using thin layer chromotography, could not find steroids. However, no reports have appeared on the composition of the lipid material which histochemically was shown to have accumulated most abundantly in the kidneys of pregnant animals.

Changes in kidney composition were also reported to occur during changing renal functional load. A close relationship between post-salt hypertension and the renal lipid droplet level was observed histochemically by Tobian and Azar (10) when high dietary salt was used to produce the renal functional load. Postnov and Fedina (11) reported a small increase of renal lipids together with hypertrophy and hyperplasia of the kidney when chronic saline ingestion was used to produce the renal functional load. Both renal hypertrophy (12) and renal lipid level (10) were shown to be related to changes in blood pressure of the animals. However, unphysiological conditions such as uninephrectomy (12) and sodium loading (10,11) were used in developing this information. Extrapolation of these findings to normal animals is questionable.

Pregnancy is a normal physiological adjustment. During pregnancy, decreased systolic blood pressure is observed in women (13,14) and rats (15). The relationship of abundant stainable lipid granules and kidney composition to the lowered blood pressure in pregnancy has not been investigated.

In this study, therefore, the renal compo-

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sition of pregnant rats at different stages of pregnancy was examined to determine: (a) whether it differs from nonpregnant tissue in weight, deoxyribonucleic acid (DNA), protein and major components of the lipid fraction; and (b) whether any differences observed relate to the changes in blood pressure that occur in pregnancy.

MATERIALS AND METHODS

Animal Management

Five groups of female Sprague-Dawley rats (8-9 animals per group) were used. All animals were fed a semi-purified ration established in this laboratory as adequate for pregnancy for five days prior to day 1 of the experiment. The experimental diet contained: (in %) casein, 26; sucrose and vitamin mixture, 18.95; cornstarch, 34; hydrogenated fat, 10; corn oil, 5; agar, 2; l-cystine, 0.15; choline, 0.4; Na-free salt mix, GBI (modified Williams-Briggs, containing as g/kg of total mix: $CaCO_3$, 207.141; $CaHPO_4$, 494.289; KCl, 208.571; $MgSO_4$, 65.714; $MnSO_4 \cdot H_2O$, 4.40; $ZnCO_3$, 1.514; $CuSO_4$, 1.428; ferric citrate, USP, 16.857; KIO_3, 0.086) 3.5; NaCl, 1.76. The vitamin mixture contained: (mg/100 g diet) thiamin. HCl, 2; riboflavin, 2; p-aminobenzoic acid, 200; nicotinic acid, 10; pyridoxine.HCl, 0.8; Ca-pantothenate, 8; biotin, 0.04; i-inositol, 400; b_{12}-mannitol triturate (0.1%), 4; folic acid, 0.4; naphthoquinone, 1. Fat-soluble vitamins were mixed in corn oil to contain 450 IU Vitamin A acetate, 400 IU vitamin D_3 and 10 mg dl-alpha tocopheryl acetate in two drops administered by mouth every three days. One group of animals was continued on the diet for 22 more days and served as the nonpregnant controls; the other four groups of animals were mated. Animals in one of the four pregnant groups were killed on day 15 of gestation; the other three groups were killed on days 18, 20, and 22. A mating schedule was planned with the aim that one animal from each group would have completed the experimental period on the same day. On the day of autopsy, one nonpregnant rat and pregnant rats from each group were decapitated following an injection of succinylcholine chloride (Burroughs Wellcome & Co., Tuchahoe, NY) and the tissues were analyzed simultaneously.

Biochemical Analysis of the Kidney Medulla

Following decapitation, blood was drained, and both kidneys from each animal were removed and weighed. Kidneys were decapsulated, quartered, and medulla-papilla tissue was obtained by cutting through the medulla-cortex junction. The remaining cortex tissue was weighed, and weight of medulla-papilla sample was obtained by difference.

Medulla-papilla tissue from both kidneys of each individual animal was homogenized in 1 ml physiological saline at 0 C with an ice-jacketed Potter-Elvehjem tissue grinder for 10 min. The homogenate was poured into a 15 ml graduated centrifuge tube using three 0.5 ml rinses of cold saline to complete the transfer. The homogenate was adjusted to 3.5 ml with the cold saline after the final rinse.

Schneider's method (16) for DNA determination was used on 0.9 ml of the homogenate with freshly hydrolyzed beef thymus DNA as the standard. Protein was determined by the Lowry method (17) using 0.1 ml of homogenate and bovine serum albumin (General Biochemicals, Chagrin Falls, OH) as the standard.

The homogenate (2.5 ml) was extracted three times with 4 ml ethyl ether in a graduated centrifuge tube. After each extraction and centrifugation at 1,000 x g for 10 min, the packed volume of the interphase fluff layer was recorded. The aqueous and fluff layers were extracted three times with 4 ml of a chloroform-methanol mixture (2:1, v/v) (18). The combined ether and chloroform-methanol extracts were washed with 1 ml distilled water and kept at room temperature for 3 hr. The water layer was discarded. Lipid extracts, after washing (% recovery of components measured: cholesterol 90 ± 2.5, fatty acids 87.7 ± 2.4, triglycerides 89.6 ± 1.9, phosphatidyl serine 74 ± 2.0, sphingomyelin 86.1 ± 2.4, phosphatidyl choline 83.4 ± 2.7, phosphatidyl ethanolamine 85.4 ± 2.9. These values were used in calculating values of samples), were dried over anhydrous sodium sulfate and evaporated to dryness under a stream of nitrogen. The residue was redissolved in 100 μl of the chloroform-methanol mixture, and portions of this concentrated lipid extract were used for the measurement of lipid components.

Seven to 10 μl of the concentrated lipid extract were spotted on a 20 x 20 cm glass plate coated with 0.25 mm Silica Gel G (Prekotes, Applied Science Laboratories, State College, PA). A set of standard mixtures containing 2, 10, 20, 30, and 40 μg of cholesterol, oleic acid, and triolein (Sigma Chemical Co., St. Louis, MO) were spotted

TABLE I

Kidney and Mudulla Weights of Nonpregnant Rats and Pregnant Rats at Different Stages of Gestation

Terminal day of gestation	Kidney (wet weight, pair) (g)	Medulla (wet weight, pair) (mg)
Nonpregnant (9)[a]	1.4977 ± 0.0308[b]	254.6 ± 5.5
Day 15 (8)	1.6762 ± 0.0258[c]	304.2 ± 8.7[d]
Day 18 (8)	1.7748 ± 0.0262[d]	337.2 ± 8.0[d]
Day 20 (8)	1.7094 ± 0.0343[d]	337.7 ± 11.4[d]
Day 22 (8)	1.6735 ± 0.0299[c]	363.6 ± 11.6[d]

[a]Number of animals in each group.
[b]Mean ± SE.
[c]Significantly different from the nonpregnant group: $P < 0.01$.
[d]Significantly different from the nonpregnant group: $P < 0.001$.

on the same plate. The plate was developed by the solvent system n-hexane-ether-acetic acid-methanol (90:20:2:4, by vol) for lipid class separation using nanograde or freshly redistilled analytical grade solvents (19). Two to 3 μl of the concentrated lipid extract were spotted on another Silica Gel G coated plate with a set of standard mixtures (Supelco Co., Bellefonte, PA) containing 12.5, 25, 37.5, 50, and 62.5 μg of phosphatidyl ethanolamine, phosphatidyl choline, phosphatidyl serine, and sphingomyelin, respectively. The plate was developed by the solvent system chloroform-methanol-NH_4OH (75:25:4, v/v/v) for separation into the different phospholipid components (20) using nanograde or freshly redistilled analytical grade solvents.

After development, the plates were charred at 200 C after spraying with 6% sulfuric acid in 50% ethanol (21). Quantitative measurements of standards and sample were made by using a densitometer. Comparisons of sample and standards were made by the quantitative paper weighing method of the peaks recorded on the chart paper (22).

Blood Pressure Measurements

Blood pressures were obtained with a programmed electrosphygmomanometer (Narco Biosystems, Inc. Houston, TX) using a tail cuff. After the animals became accustomed to the procedure and the blood pressure stabilized, three consecutive readings were averaged as one measurement. At least three such measurements obtained on three different days established the baseline blood pressure prior to the experimental period.

Blood pressure measurements were taken the afternoon before mating and on the day before the terminal day. In every case, three consecutive measurements were obtained and averaged. The premating blood pressure measurements were compared with the previously obtained baseline blood pressures and no differences were found between the two values.

Statistical Method

Means and differences between means of the nonpregnant group and each pregnant group were examined by t-test using the formula for small samples (23).

RESULTS AND DISCUSSION

The kidney weight, medullary weight (Table I), blood pressure measurements (Table II) and medullary contents of DNA, protein, and different classes of lipids per mg medulla weight (Table III) in the nonpregnant rats and rats at different stages of pregnancy were compared. The relationships among increases of each of the cellular components at different stages of pregnancy were studied by comparing the ratios of each component to DNA, to protein, and to cholesterol content of the tissue (Tables IV, V, VI).

When DNA and protein concentrations were calculated as μg/mg wet medullary tissue (Table III), no significant differences could be found at different stages of pregnancy. However, since the total weight of medulla increased continuously during gestation (Table I), the increase in tissue weights must have been due to hyperplasia. At gestation day 20, the slight decrease in DNA concentration accompanied by a small increase in protein concentration indicate that hypertrophy also occurred.

Hypertrophy was not caused by protein alone since some proteins and other material such as lipids appeared as the interphase

TABLE II

Blood Pressure of Nonpregnant Rats and Pregnant Rats at Different Stages of Gestation

Time of blood pressure measurement	Nonpregnant (9)[a] (blood pressure in mmHg)	Terminal day of gestation (blood pressure in mmHg)			
		Day 15 (8)	Day 18 (8)	Day 20 (8)	Day 22 (8)
Premating	110 ± 2.4[b]	111 ± 3.0	114 ± 2.4	106 ± 3.0	106 ± 2.1
Day before sacrifice	108 ± 1.9	103 ± 3.5	104 ± 1.2[c]	96 ± 0.8[c]	85 ± 3.0[d]

a Number of animals in each group.
[b]Mean ± SE.
[c]Significantly different from premating values: $P < 0.01$.
[d]Significantly different from premating values: $P < 0.001$.

TABLE III

Medullary Composition of Nonpregnant Rats and Pregnant Rats at Different Stages of Gestation

Component (per mg wet medulla)	Nonpregnant (9)[a]	Terminal day of gestation			
		Day 15 (8)	Day 18 (8)	Day 20 (8)	Day 22 (8)
Deoxyribonucleic acid (μg)	8.3 ± 0.5[b]	7.8 ± 0.4	8.1 ± 0.2	6.9 ± 0.2	8.1 ± 0.4
Protein (μg)	122 ± 4	117 ± 3	120 ± 2	126 ± 2	123 ± 3
Cholesterol (μg)	1.9 ± 0.1	1.9 ± 0.1	2.4 ± 0.1[c]	2.3 ± 0.1	2.4 ± 0.1[c]
Triglycerides (μg)	0.61 ± 0.09	0.55 ± 0.08	0.60 ± 0.08	1.16 ± 0.12[c]	1.07 ± 0.04[d]
Nonesterified fatty acids (μg)	0.11 ± 0.03	0.09 ± 0.02	0.14 ± 0.04	0.38 ± 0.08[c]	0.25 ± 0.07
Phosphatidyl ethanolamine (μg)	7.1 ± 0.3	7.2 ± 0.4	9.9 ± 0.5[d]	10.1 ± 0.5[d]	9.8 ± 0.5[d]
Phosphatidyl choline (μg)	8.4 ± 0.4	9.9 ± 0.6	11.7 ± 0.6[d]	12.8 ± 0.4[d]	11.9 ± 0.5[d]
Phosphatidyl serine (μg)	2.5 ± 0.3	3.3 ± 0.4	5.1 ± 0.4[d]	4.7 ± 0.2[d]	4.8 ± 0.3[d]
Sphingomyelin (μg)	1.0 ± 0.2	1.9 ± 0.3	2.9 ± 0.2[d]	2.5 ± 0.3[d]	2.8 ± 0.4[c]
Interphase fluff layer packed volume (ml)[e]	3.0 ± 0.2	3.9 ± 0.1	4.3 ± 0.3	4.7 ±0.3[d]	3.9 ± 0.3

[a]Number of animals in each group.
[b]Mean ± SE.
[c]Significantly different from the nonpregnant group: $P < 0.01$.
[d]Significantly different from the nonpregnant group: $P < 0.001$.
[e]Measurements of packed volumes after the second ether extraction. Packed volumes of fluff layers after the first and last ether extractions were similar in all groups. Interphase material has not been defined but is believed to be a mixture of protein and lipids.

TABLE IV

Medullary Components per μg DNA of Nonpregnant Rats and Pregnant Rats at Different Stages of Gestation[a]

Component	Nonpregnant (9)[b] (μg/μg DNA)	Terminal day of gestation (μg/μg DNA)			
		Day 15 (8)	Day 18 (8)	Day 20 (8)	Day 22 (8)
Protein	15.1 ± 1.0[c]	15.2 ± 0.9	14.9 ± 0.3	18.4 ± 0.7	15.4 ± 0.6
Cholesterol	0.23 ± 0.03	0.24 ± 0.01	0.29 ± 0.01	0.34 ± 0.02[d]	0.30 ± 0.02
Nonesterified fatty acids	0.013 ± 0.004	0.012 ± 0.002	0.017 ± 0.004	0.056 ± 0.012[d]	0.032 ± 0.004[d]
Triglyceride	0.07 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.17 ± 0.02[e]	0.14 ± 0.01[d]
Phosphatidyl ethanolamine	0.87 ± 0.05	0.94 ± 0.07	1.23 ± 0.04[e]	1.47 ± 0.07[e]	1.24 ± 0.09[d]
Phosphatidyl choline	1.04 ± 0.10	1.31 ± 0.10	1.45 ± 0.07[d]	1.87 ± 0.08[e]	1.50 ± 0.10[d]
Phosphatidyl serine	0.32 ± 0.05	0.44 ± 0.06	0.64 ± 0.04[e]	0.68 ± 0.03[e]	0.59 ± 0.03[e]
Sphingomyelin	0.12 ± 0.02	0.26 ± 0.05	0.36 ± 0.03[e]	0.37 ± 0.03[e]	0.34 ± 0.03[e]

[a]Mean ratios ± SE calculated from ratios of values obtained for each individual animal.
[b]Animals in each group.
[c]Mean ± SE.
[d]Significantly different from the nonpregnant group: $P < 0.01$.
[e]Significantly different from the nonpregnant group: $P < 0.001$.

TABLE V

Medullary Lipids per mg of Protein of Nonpregnant Rats and Pregnant Rats at Different Stages of Gestation[a]

Component	Nonpregnant (9)[b] μg/mg protein	Terminal day of gestation (μg/mg protein)			
		Day 15 (8)	Day 18 (8)	Day 20 (8)	Day 22 (8)
Cholesterol	15.3 ± 1.3[c]	16.1 ± 0.8	19.7 ± 0.9	18.5 ± 0.8	19.9 ± 1.4
Nonesterified fatty acid	0.9 ± 0.3	0.8 ±0.2	1.2 ± 0.3	3.0 ± 0.6[d]	2.1 ± 0.5
Triglyceride	5.0 ± 0.7	4.7 ± 0.7	5.0 ± 0.7	9.2 ± 1.0[d]	8.8 ± 0.5[e]
Phosphatidyl ethanolamine	58.3 ± 2.0	61.7 ± 4.0	81.9 ± 4.0[e]	80.5 ± 4.0[e]	79.9 ± 4.4[e]
Phosphatidyl choline	69.5 ± 4.4	85.6 ± 5.4	97.5 ± 4.4[e]	102.6 ± 5.6[e]	96.8 ± 3.6[e]
Phosphatidyl serine	20.5 ± 2.3	28.2 ± 3.5	42.8 ± 2.8[e]	37.3 ± 1.8[e]	38.5 ± 2.0[e]
Sphingomyelin	7.7 ± 1.4	16.3 ± 2.5[d]	24.1 ± 1.8[e]	20.2 ± 2.0[e]	22.1 ± 2.5[e]

[a]Mean ratios ± SE calculated from ratios of values obtained for each individual animal.
[b]Animals in each group.
[c]Mean ± SE.
[d]Significantly different from the nonpregnant group: $P < 0.01$.
[e]Significantly different from the nonpregnant group: $P < 0.001$.

TABLE VI

Medullary Lipids per μg Cholesterol of Nonpregnant Rats and Pregnant Rats at Different Stages of Gestation[a]

Component	Nonpregnant (9)[b] μg/μg cholesterol	Terminal day of gestation (μg/μg cholesterol)			
		Day 15 (8)	Day 18 (8)	Day 20 (8)	Day 22 (8)
Nonesterified fatty acids	0.06 ± 0.03[c]	0.05 ± 0.01	0.06 ± 0.02	0.16 ± 0.02	0.11 ± 0.03
Triglycerides	0.34 ± 0.06	0.29 ± 0.03	0.26 ± 0.04	0.50 ± 0.05	0.45 ± 0.03
Phosphatidyl ethanolamine	4.02 ± 0.34	3.90 ± 0.31	4.23 ± 0.17	4.43 ± 0.31	4.15 ± 0.34
Phosphatidyl choline	4.72 ± 0.39	5.44 ± 0.46	5.02 ± 0.31	5.62 ± 0.37	5.05 ± 0.42
Phosphatidyl serine	1.37 ± 0.17	1.81 ± 0.27	2.19 ± 0.15[d]	2.04 ± 0.12[d]	2.01 ± 0.18
Sphingomyelin	0.56 ± 0.14	1.05 ± 0.20	1.26 ± 0.15[d]	1.10 ± 0.10[d]	1.16 ± 0.16

[a]Mean ratios ± SE calculated from ratios of values obtained for each individual animal.

[b]Animals in each group.

[c]Mean ± SE.

[d]Significantly different from the nonpregnant group: $P < 0.01$.

material during lipid extraction. The estimated level of this fluff layer as packed volume per mg medulla (Table III) increased significantly at day 20. These data suggest that hypertrophy resulted from a combined increase of protein and lipids.

All fractions of lipid material measured increased slightly as pregnancy progressed (Table III). Highly significant increases in concentrations per cell, particularly of phospholipids and triglycerides (Table IV), occurred at gestation day 20, which support the concept of cellular hypertrophy at that time.

The increased ratio of different fractions of phospholipids to protein (Table V) clearly indicates a net increase of phospholipids over protein. Net increases of phospholipids suggest a possible increase of high phospholipid-containing structures such as the mitochondria. The number of mitochondria in the cell usually parallels the work load. In uninephrectomized rats, the work load of the remaining kidney is elevated, and Johnson and Amendolar (24) found that the number of mitochondria per tubular section in the remaining kidney increased by 50% two days after uninephrectomy along with an increase of the dry weight of the tissue. In pregnant rats, the work load of medulla increases when the reabsorptive and regulatory functions increase in late pregnancy. Therefore, the net increase of phospholipids may be related to an increase in mitochondria number. How much of this increase in medullary phospholipids is contributed by an increase in mitochondria number is currently being investigated through cytological study. Preliminary data indicate an increase in the number of mitochondria, often in condensed conformation, in pregnant rats on day 20, and fewer mitochondria no longer in condensed conformation on day 22.

The increase in triglyceride content per cell (Table IV) also indicates adaptation of cellular metabolism to the increased work load and energy need of the cells. The regulatory mechanism that lead to kidney growth and increased triglyceride content per cell may also be responsible for the lipid droplets found in histochemical (1) and ultrastructural studies (unpublished data) of pregnant tissues.

The quantity of structural phospholipids is related to the cholesterol content of the cell. At gestation day 20, the ratio of cholesterol to protein was higher than the nonpregnant level but not significantly so; however, ratios of phospholipids to protein were significantly

different (Table V). The increase in phospholipids was greater than the increase in cholesterol and is observed in the phospholipids to cholesterol ratios (Table VI) which are slightly higher in pregnant than nonpregnant rats.

Medullary phospholipids are thought to be involved in transporting the fatty acid precursor for renal prostaglandin synthesis (25). It was not possible to elucidate in this study which of the phospholipid fractions contain the esterified fatty acid precursor for prostaglandin production. However, the data indicate decreased blood pressure (Table II) in day 20 pregnant animals along with cellular hypertrophy and high phospholipid content in the renal medulla. These data suggest that gestation day 20 may be the period of maximum activity in the production of renal antihypertensive lipid, presumably prostaglandin (26).

One problem in studying prostaglandins in vivo is the extremely minute quantity formed by the tissue under ordinary conditions. The use of pregnant tissue to obtain maximum conversion of precursors to prostaglandins could overcome some of the limitations in quantitative assays.

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Atherogenesis in Swine Fed Several Types of Lipid-Cholesterol Diets

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ABSTRACT

Eighteen-month-old Nebraska strain minipigs were fed diets containing 2% cholesterol and 20% corn oil, lard, or coconut oil for 12 to 18 months. Concentrations of serum total lipid, total cholesterol, and total phospholipid increased 200 to 300% with each diet. Changes in serum concentrations of S_f 20+ and S_f 0-20 lipoproteins varied with diets fed. Serum concentration of high density lipoprotein was increased in all cases. Intima concentration of S_f 0-20 lipoprotein fraction was elevated by feeding the corn oil diet. There was no development of atherosclerosis as a result of feeding the corn oil-cholesterol diet, but there was an increase in atherosclerosis as a result of feeding the lard or coconut oil diet. There were no correlations between fatty acid patterns of several lipid fractions from serum and corresponding lipid fractions from aortic intima of corn oil fed animals.

INTRODUCTION

It has been shown that the pig develops spontaneously a vascular disease similar to atherosclerosis in man and that this disease can be produced experimentally by feeding pigs diets rich in lipid (1-4). Feeding of atherogenic diets usually results in hyperlipidemia and changes in serum lipid and lipoprotein patterns (5-7). Distribution and severity of atherosclerosis which develops are influenced by factors such as type of diet fed (8), type of husbandry practiced (1), and amount of exercise (9).

Lipoproteins, resembling the lipoproteins of serum, can be found in the walls of large vessels (10,11). Therefore, atherosclerosis may occur as a result of lipoprotein transfer from serum into intima.

The studies described in this work were carried out to determine if correlations existed among type of diet fed, concentration of blood lipids and lipoproteins, concentration of lipoproteins in the intima of large vessels, and degree of atherosclerosis developed.

MATERIALS AND METHODS

Minipigs were obtained from the Veterinary Science Department of the College of Agriculture of the University of Nebraska, Lincoln, Nebraska (12). They were about 18 months of age and weighed between 50 and 90 kg.

The animals were divided into six groups of five to ten animals each. Three groups were kept as controls and three groups were fed the high lipid diets. Diets were prepared from a custom mixture of the following composition (%): middlings, 12.8; corn, 54.2; dry alfalfa, 3.8; soybean meal, 18.9; cane molasses, 5.1; meat scraps, 2.6; salt, 0.6; dicalcium phosphate, 1.3; minerals, vitamins, and fish meal mixture, 0.9. The diet for control animals was made by adding 10 kg of corn to 35.4 kg of the custom mixture. The diet for experimental animals was prepared by adding 9.1 kg of corn oil, coconut oil, or lard and 0.9 kg cholesterol to 35.4 kg of custom mixture. The diet for control animals contained 15.4% protein, and the diet for experimental animals contained 13.4% protein. Animals fed the corn oil diet and their controls were fed for 18 months; those fed the lard or coconut oil diets and their controls were fed for 12 months. All animals were fed ad libitum and consumed 1 to 1.5 kg of food per day.

In a separate experiment three pigs, three months old and weighing about 18 kg each, were fed the lard-cholesterol diet for 6 months.

Blood was taken from the jugular vein of each pig after an overnight fast at the time experimental feeding was begun and at 6-month intervals thereafter. Samples were allowed to clot and serum separated. An aliquot of each sample of serum was assayed for total cholesterol (13). Another aliquot was extracted for total lipid according to the method of Sperry and Brand (14). Total lipid was separated into three fractions by silicic acid column chromatography (15). These fractions were: A, cholesterol esters; B, triacylglycerols, free fatty acids, and free cholesterol; and C, phospholipids. The B fraction was separated into B-1 (free cholesterol and triacylglycerol) and B-2 (free fatty acid) by hexane extraction of neutral lipid from an alkaline alcoholic solution of B fraction.

A third sample of serum was centrifuged at 20,000 x g for 20 min to remove chylomicrons.

[1] Deceased.

TABLE I

Serum Lipids of Pigs Fed Control Diets and Pigs Fed Atherogenic Diets for 12 or 18 Months

Lipid Fraction	Corn oil diet (18 months) Control	Corn oil diet (18 months) Lipid fed	Lard diet (12 months) Control	Lard diet (12 months) Lipid fed	Coconut oil diet (12 months) Control	Coconut oil diet (12 months) Lipid fed
	(mg/dl)		(mg/dl)		(mg/dl)	
Total lipids	161 ± 11[a] (6)[b]	301 ± 41[c] (6)	232 ± 21 (5)	528 ± 55[c] (6)	212 ± 14 (5)	487 ± 40[c] (7)
Total cholesterol	53 ± 5 (6)	137 ±24[c] (6)	72 ± 5 (5)	222 ± 28[c] (6)	71 ± 6 (5)	207 ± 20[c] (7)
Esterified cholesterol	63 ± 9 (6)	200 ± 24[c] (4)	72 ± 20 (5)	252 ± 58[c] (6)	60 ± 13 (5)	131 ± 30[c] (7)
Phospholipid	56 ± 8 (6)	156 ± 20[c] (4)	101 ± 13 (5)	243 ± 27[c] (6)	101 ± 8 (5)	195 ± 10[c] (7)
Free fatty acids	9 ± 2 (5)	11 ± 6 (3)	22 ± 5 (4)	22 ± 5 (6)	17 ± 2 (4)	36 ± 4[c] (5)
S_f20+ lipoprotein fraction	18 ± 2 (5)	11 ± 1[c] (4)	19 ± 6 (5)	39 ± 9 (6)	13 ± 4 (5)	36 ± 7[c] (7)
S_f0-20 lipoprotein fraction	64 ± 22 (3)	68 ± 14 (5)	47 ± 3 (5)	130 ± 13[c] (5)	49 ± 3 (5)	161 ± 29[c] (7)
High density lipo protein fraction	85 ± 3 (6)	167 ± 16[c] (6)	81 ± 4 (3)	239 ± 50[c] (6)	134 ± 20 (5)	272 ± 27[c] (7)
Sediment lipoprotein fraction	24 ± 1 (6)	38 ± 4[c] (5)	26 ± 1 (5)	42 ± 6[c] (6)	----	----

[a]Standard error of mean.
[b]Number of animals.
[c]$P<0.05$ when control and lipid fed values are compared.

TABLE II

Aortic Lesions in Control Pigs and in Pigs Fed Atherogenic Diets for 12 or 18 Months

Groups	Number of pigs	Relative amount of atherosclerosis in aorta[a]			
		Arch	Thoracic	Abdominal	Total
Control	9				1.3 ± 0.3[b]
Corn oil	8				2.0 ± 0.4
Control	5	1.2 ± 0.3	0.0	0.6 ± 0.4[c]	1.8 ± 0.6[c]
Lard	6	1.2 ± 0.4	0.3 ± 0.3	2.3 ± 0.4	3.8 ± 0.3
Control	5	1.3 ± 0.3[c]	0.0	1.0 ± 0.4	2.3 ± 0.4[c]
Coconut oil	7	2.5 ± 0.1	0.6 ± 0.3	1.6 ± 0.3	4.7 ± 0.5

[a]Numerical values calculated as described in text.
[b]Standard error of the mean.
[c]$P<0.02$ for control vs. fed animals.

These usually were absent or present in very small amounts and were discarded. Serum was centrifuged at 114,000 x g for 24 hr, and the floating lipid was removed as the S_f 20+ (VLD) lipoprotein fraction. An insoluble gelatinous residue was removed as the "sediment" fraction. The remainder of the serum sample was brought to a density of 1.063 with NaCl and centrifuged at 75,000 x g for 16 hr. The floating lipid was removed as the S_f 0-20 (LD) lipoprotein fraction. The lipid in the infranatant was recovered as the high density (HD) lipoprotein fraction.

Lipoprotein fractions were extracted for total lipid, and the lipid was separated into fractions A, B-1, B-2, and C by silicic acid fractionation. Silicic acid fractions were methylated by treatment with methanolic HCl followed by microsublimination by the method of Stoffel et al. (16). Methyl esters were separated by gas chromatography in an F and M Model 810 gas chromatograph with flame ionization detector. This chromatograph had stainless-steel tubing (1.8 m long, 0.63 cm diameter) packed with 10% diethylene glycol succinate on Chromosorb W. Nitrogen gas flow was 70 ml/min, hydrogen gas 55 ml/min, and air 600 ml/min. The oven temperature was 190 to 200 C, port injection temperature 270 C, and detector temperature 273 C. About 5 λ of fatty acid methyl esters in hexane were injected.

At the end of each feeding period, animals were stunned by electric shock, exsanguinated, and necropsied. Heart and aorta, including some of the iliac arteries, were removed. Unstained tissues were examined visually for atherosclerotic plaques and fatty deposits by two sets of investigators. The "atherosclerosis profile" method of Roberts et al. (17) was used for obtaining a relative numerical value of the amount of atherosclerosis present. The relative amount of atherosclerosis was calculated for the arch, thoracic, and abdominal segments of each aorta of lard and coconut oil fed animals and their controls. Numerical values for the three parts of each aorta were added together to give a total value for each aorta. In the case of corn oil fed animals and their controls, each aorta was evaluated only in its entirety.

The intima was removed from each aorta with as much atherosclerotic tissue as possible. This was minced in 0.9% NaCl, homogenized and centrifuged at 1700 x g for 20 min. The supernatant solution was centrifuged under the same conditions as mentioned for serum to give the S_f 20+, S_f 0-20, sediment, and high density lipoprotein fractions. Lipid in these fractions was isolated, fractionated by silicic chromatography, and assayed for fatty acids by the methods reported previously for serum lipoproteins.

Where possible and desirable, the various values obtained in this work are expressed as means ± standard errors. A meaningful difference between values was assumed to exist if a P value as determined by the standard t-test was <0.05. In certain cases, insufficient material was available from a single animal for evaluation and so corresponding fractions from groups of animals were combined for study. As time went by, some animals were necropsied and some died. The number of animals studied, therefore, was subject to change as the work progressed.

RESULTS

Three animals died during the course of the experiments but there was no indication that any of the deaths was related directly to experimental conditions.

Concentrations of total lipid and various fractions thereof in the sera of pigs fed the

TABLE III

Lipoproteins of Aortal Intima of Control Pigs and of Pigs Fed Atherogenic Diets for 12 or 18 months

Fraction	Corn oil-cholesterol diet		Lard-cholesterol diet		Coconut oil-cholesterol diet	
	Control	Lipid fed	Control	Lipid fed	Control	Lipid fed
	mg/100 g wet intima		mg/100 g wet intima		mg/100 g wet intima	
S_f20+	18 ± 6[a]	16 ± 4	13	21	10	14 ± 2
	(4)[b]	(8)	(3)	(c-7)[c]	(2)	(7)
S_f0-20	10 ± 1	49 ± 11[d]	20	27 ± 9	40	34 ± 9
	(4)	(8)	(2)	(5)	(2)	(6)
HD	79 ± 24	101 ± 20	---	76	147	63
	(4)	(8)		(c-7)	(2)	(c-7)
Sediment	259 ± 52	337 ± 49	166	183	260	239 ± 29
	(4)	(8)	(2)	(c-7)	(2)	(7)

[a]Standard error of the mean.
[b]Number of individual aortas studied.
[c]Number of intimas combined for study.
[d]$P<0.02$ for control vs. fed animals.

experimental diets for 12 or 18 months and of their corresponding controls are shown in Table I. In general agreement with the work of others (4,7,18), the feeding of atherogenic diets resulted in increases in the concentrations of many of the major lipid classes in blood. There was an increase of from 100 to 200% in total lipid, total cholesterol, and phospholipids as a result of feeding all three diets. Free fatty acid concentrations were elevated only in sera from coconut oil fed pigs. The increase in total cholesterol was accompanied by a general increase in esterified cholesterol. These serum concentrations were elevated in the first 6 months of feeding and continued to increase over the next 6 to 12 months. Other investigators have found similar increases in lipid values but in some cases these declined with time (18-20).

The corn oil diet caused a decrease in concentration of S_f 20+ fraction with no change in concentration of S_f 0-20 fraction. The lard diet produced no change in concentration of S_f 20+ fraction and increased S_f 0-20 fraction. The coconut oil diet resulted in an increase in concentration of both fractions. The high density and sediment fractions also were increased in all cases.

The relative degree of aortic atherosclerosis which developed upon lipid feeding is summarized in Table II. In all cases, the actual amount of atherosclerosis was small. There was no gross scarring or calcification of tissues, and there were no thrombi or atherosclerotic lesions in coronary arteries. There was no enhanced development of atherosclerosis at the trifurcation. The corn oil-cholesterol diet did not appear to increase the degree of atherosclerosis. Animals fed lard or coconut oil diets developed a greater amount of atherosclerosis than corresponding controls. The amount of atherosclerosis resulting from the lard diet was not different from that resulting from the coconut oil diet. There may have been more involvement of the arch in the case of coconut oil fed animals and of the abdominal aorta in the case of lard fed animals.

Determination of lipoprotein in the intima of atherosclerotic areas of the vessels was difficult because of the small amount of atherosclerosis present. Variations in values were wide, and, in many cases, samples had to be combined to obtain sufficient quantity of material for evaluation. Results obtained are shown in Table III. There was no protein-lipid complex corresponding to the chylomicrons. In only one case was there a difference between control and fed animals, and that was in the S_f 0-20 fraction for corn oil.

Fatty acid compositions of some serum lipid fractions from control animals and animals fed the corn oil diet are shown in Table IV. There were some differences in fatty acid composition between fractions from control and fed animals, but it was difficult to see an extensive pattern of change due to diet. Corn oil is characterized by a relatively large amount of linoleic acid and an even larger amount of oleic acid (21). Linoleic acid generally is increased in serum lipid fractions of animals fed the corn oil diet, but oleic acid is reduced in amount. The fatty acid patterns of the various serum lipid fractions from corn oil fed animals appeared to be developed by 6 months, and there were no consistent changes throughout an additional 6 to 12 months of feeding.

No detailed studies were made of the fatty acid composition of serum lipids from lard or coconut oil fed animals. However, in several

TABLE IV

Fatty Acid Composition of Several Serum and Aortal Lipid Fractions from Pigs Fed a Control Diet (C-Diet) and Pigs Fed a Corn Oil Cholesterol Diet (L-diet) for 12 or 18 Months

			Relative % of total fatty Acids															
			Serum lipids								Aortic intimal lipids							
Fraction[a]	Mo.[b]	Diet	<16	16:0	16:1	18:0	18:1	18:2	18:3	>18	<16	16:0	16:1	18:0	18:1	18:2	18:3	>18
									S_f 20 + fraction									
A	18	C	1.7	21.2	3.5	12.0	35.3	21.2	1.2	3.8	8.2	23.2	17.6	12.2	19.5	9.8	2.1	7.5
		L	1.8	16.9	3.1	12.1	30.0	31.5	1.1	3.6	7.5	21.3	12.5	11.4	21.3	16.4	1.4	8.2
B-1	12	C	2.0	21.7	6.9	4.4	35.4	17.2	1.5	10.7	6.6	27.8	5.9	14.9	22.6	4.4	0.9	16.9
		L	1.9	17.2	3.4	7.8	25.0	32.1	1.4	11.4	5.8	23.6	5.3	13.7	22.8	5.4	1.4	21.6
B-2	18	C	3.9	25.7	5.7	12.3	30.0	11.9	1.1	9.4	5.0	29.5	4.9	22.8	21.1	5.0	1.9	9.1
		L	3.7	26.4	4.9	13.5	26.9	11.0	1.2	12.4	8.1	28.1	8.1	14.9	22.3	2.8	1.6	14.0
C	18	C	2.1	21.8	2.2	28.0	17.2	21.0	1.0	6.6	3.5	26.0	4.1	27.3	17.7	5.1	2.5	14.0
		L	1.8	18.8	2.0	32.2	14.4	21.5	0.9	8.6	4.9	27.0	3.1	28.4	19.8	7.0	1.9	7.8
									S_f 0-20 fraction									
A	18	C	0.4	8.7	2.0	6.4	30.7	45.7	1.4	4.6	6.0	25.1	11.3	16.6	21.9	8.1	1.4	9.8
		L	0.3	6.5	1.5	5.0	25.9	55.5	1.6	3.5	5.8	24.0	11.0	15.0	23.5	11.0	1.0	9.0
B-1	18	C	1.7	21.8	4.5	9.1	38.2	11.1	1.1	12.7	4.3	29.9	4.2	21.3	23.3	4.5	1.2	11.2
		L	2.9	24.4	4.2	12.4	29.4	10.1	1.5	15.1	3.6	32.2	4.2	14.7	24.2	6.4	3.0	11.9
B-2	18	C	2.7	21.2	3.8	10.3	17.5	6.2	1.7	36.4	10.2	36.3	5.6	19.3	18.3	3.0	0.8	6.7
		L	4.3	21.5	3.2	15.5	13.6	6.1	2.0	33.7	7.5	31.5	4.7	17.4	24.2	7.1	1.3	6.1
C	18	C	0.8	23.3	2.0	35.1	14.8	15.1	1.6	7.4	2.1	20.6	3.1	18.6	18.8	8.6	2.7	- - -
		L	0.7	23.9	1.0	35.2	8.2	23.0	1.3	6.0	1.6	27.0	2.8	32.0	20.8	5.9	1.7	8.1
									High Density Fraction									
A	12	C	2.0	15.2	4.3	6.1	33.2	34.2	1.3	3.6	13.3	19.8	20.8	15.3	15.3	5.5	1.0	9.0
		L	3.7	17.0	3.7	4.4	28.1	40.8	0.6	1.9	12.0	23.5	17.9	12.7	21.4	8.0	0.9	4.1
B-1	18	C	6.0	27.9	6.0	12.1	24.5	5.6	1.7	16.2	6.5	25.1	6.0	12.1	26.5	5.1	1.3	12.4
		L	4.6	24.0	6.4	13.0	20.3	5.7	1.6	24.2	5.2	23.8	5.9	14.7	32.2	7.5	5.0	4.8
B-2	12	C	2.9	25.5	3.8	13.2	33.7	6.0	1.0	14.0	7.4	33.3	7.0	19.1	21.5	3.0	1.6	7.0
		L	4.0	28.4	2.8	17.3	15.0	4.2	1.7	26.7	7.0	32.4	7.2	16.5	22.3	5.2	1.9	7.6
C	18	C	1.5	22.4	2.4	35.1	15.8	13.9	1.5	7.2	3.9	25.7	4.2	26.0	20.4	7.0	1.2	11.7
		L	1.3	16.4	2.9	8.2	37.7	27.2	0.7	5.6	2.9	21.3	2.6	27.2	26.2	8.7	2.3	8.7

[a]Silicic acid column fraction. A=cholesterol esters; B-1=free cholesterol and triacylglycerols; B-2=free fatty acids; C=phospholipids.
[b]Months of feeding.

serum fractions which were analyzed, linoleic acid was reduced while oleic acid and saturated fatty acids varied in amounts. Both lard and coconut oil contain low quantities of linoleic acid (21).

Fatty acid compositions of several aortal lipid fractions from control animals and animals fed the corn oil diet are shown in Table IV. There were relatively small differences in fatty acid compositions of fractions from control animals and animals fed corn oil. There was an increase in linoleic acid in many fractions which probably was due to the relatively large amount of this fatty acid in serum.

The 3-month-old pigs fed the 20% lard-2% cholesterol diet developed within 1 month serum cholesterol levels of 500 mg/dl and serum total lipid levels of 1000 mg/dl. These values were twice as high as those developed in the older animals fed the same diet. These values persisted during 6 months of feeding without a further increase after the first month. When the young animals were necropsied at the end of 6 months, they had a degree of atherosclerosis comparable to that of the older pigs fed the lard-cholesterol diet for 12 months. Here again, the thoracic portion of the aorta was relatively free of atherosclerosis.

DISCUSSION

Development of atherosclerosis in minipigs was minimal in this work even though hyperlipemia was maintained in experimental groups for 6 to 12 months. Maximum serum lipid concentrations obtained (500 mg/dl for total lipid and 200 mg/dl for cholesterol), however, were not high when compared with serum lipid concentrations of some other species which develop atherosclerosis. Thus, the swine values fell only in the range of normal human values (22). Also, the concentration of S_f 0-20 fraction, which is associated with atherosclerosis in humans (23), rose only to 130 to 160 mg/dl serum. These values were lower than normal human values (24,25). It is possible, therefore, that the hyperlipidemia in these pigs was not in a critical range for extensive atherogenesis.

The greater development of atherosclerosis in animals fed lard or coconut oil diet than in animals fed the corn oil diet was in agreement with the common concept that polyunsaturated fatty acids reduce serum cholesterol concentrations and atherosclerosis (22). The fact that low density lipoprotein concentration was not elevated above control values in serum of corn oil fed animals while S_f 0-20 lipoprotein concentration was elevated in serums of pigs fed the other diets may explain in part the relative amounts of atherosclerosis found in the three experimental groups.

The enhanced hyperlipidemia and atherosclerosis in the very young animals (12 weeks of age) may represent the response of the rapidly growing animals to the high calorie to protein ratio of the diet as discussed by Barnes et al. (20,26). The animals in this work, however, did not exhibit overt signs of malnutrition.

Only the S_f 0-20 fraction was elevated in aortic intima lipids. There is evidence that low density lipoproteins can pass from blood into the walls of the aorta (23). However, if intact serum lipoproteins entered the vessel walls in corn oil fed pigs in this study, it was not evidenced by changes in fatty acid patterns of intimal lipids. Fatty acid patterns of fractions from intimal lipoproteins of corn oil fed animals resemble more closely fatty acid patterns of control animals than they do fatty acid patterns of corresponding serum lipid fractions of fed animals.

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The Circadian Cycles of Plasma Corticosterone and Adrenal Cholesteryl Esters in the Normal and EFA-deficient Female Rat[1]

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ABSTRACT

In female rats subjected to a 12 hr light-12 hr darkness schedule and fed a semipurified diet containing 10% corn oil, plasma corticosterone concentration showed a monophasic circadian cycle with minimum and maximum concentrations at the start of the light and dark periods, respectively. Adrenal total cholesteryl ester concentration was inversely related to plasma corticosterone, as were those of several of the individual esters; changes in cholesteryl ester concentration appeared to follow rather than precede changes in plasma corticosterone. There was preferential depletion of the cholesteryl esters of 18:1, 18:2ω6, and 20:4ω6 during glucocorticoid secretion. [Abbreviations: EFA, essential fatty acid (s); X:YωZ, fatty acid with X carbon atoms and Y olefinic bonds with the terminal double bond Z carbon atoms from the methyl group.] In female rats fed hydrogenated coconut oil (EFA-deficient), a monophasic cycle for plasma corticosterone was also observed, but the peak was much broader than that recorded for rats fed corn oil, although minima and maxima occurred at similar times for the two groups. No significant cycle of adrenal total cholesteryl esters was evident in the deficient rats, but the 20:3ω9 and 22:3ω9 esters did decrease significantly during the period of high plasma corticosterone concentration. Preferential net decreases in adrenal cholesteryl esters during corticosteroidogenesis were more apparent in normal than in EFA-deficient rats.

Many species exhibit circadian rhythmicity in pituitary adrenal function in the absence of applied stress (1-3). This is true of the rat, and several workers have demonstrated diurnal variation in corticosterone, the primary adrenal glucocorticoid in rat plasma (4-6). The circadian variation in plasma glucocorticoids is frequently associated with the normal alternation of light and darkness in the animals' quarters, with rats of both sexes having low levels of glucocorticoid in the early morning hours and a peak concentration just prior to the onset of darkness (7). However, the effect of the photoperiod may be an indirect one, and the major influence may reside with the feeding pattern of the animal (8), which is itself related to the photoperiod, rats tending to eat during the hours of darkness. In any event, the photoperiod may be employed to regulate plasma glucocorticoid concentration in this species.

Adrenal cholesteryl esters consisitute a reservoir of cholesterol and appear to be easily hydrolyzed in response to the demand for glucocorticoid synthesis in the rat (9). Moreover, specific esters are preferentially depleted from the adrenal during stress-induced corticosteroidogenesis (10-12). Among the preferentially utilized esters are those of linoleic and arachidonic acids, two of the essential fatty acids (11,12). Furthermore, glucocorticoid synthesis in response to stress was impaired in adrenals of essential fatty acid-deficient rats (10,13,14).

The current investigation was undertaken to test the hypotheses that adrenal cholesteryl esters would exhibit a circadian rhythm inversely related to the plasma corticosterone concentration, that specific cholesteryl esters would be preferentially depleted during increased glucocorticoid synthesis, and that essential fatty acid (EFA) deficiency would interfere with normal circadian cycles for both plasma corticosterone and adrenal cholesteryl esters in the rat. The experiments were conducted on female rats maintained on a fixed photoperiod to establish the plasma hormone cycle.

EXPERIMENTAL PROCEDURES

Sexually mature female rats of the Wistar strain (Woodlyn Laboratories, Guelph), weighing 175-200 g, were housed in individual stainless-steel cages and maintained on a fixed photoperiod of 12 hr light (0700-1900) and 12 hr darkness. They were fed ad libitum a casein-dextrose based, semipurified diet (15) containing 10% corn oil; the oil contained the following fatty acids (weight % of total fatty acids): 16:0, 11.1%; 18:0, 1.7%; 18:1, 24.5%; 18:2, 60.8%; and 18:3, 0.9%. Estrous cycles were verified after the second week of the experiment by examination of vaginal smears (16). After 3 weeks on the experimental diet, the animals were killed by intraperitoneal injection of sodium pentabarbital (40-50 mg/rat) on the third day of the estrous cycle, at 4 hr intervals over a continuous 24 hr period. Blood was collected in heparinized tubes by heart puncture and immediately centrifuged (within 20 min of collection) at 0 C and 8000 x g for 30 min. Plasma was removed, frozen, and stored at

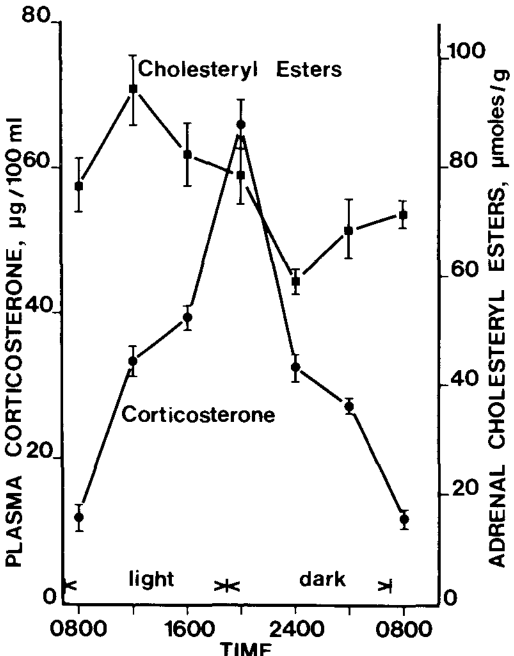

FIG. 1. Variations in plasma corticosterone and adrenal total cholesteryl esters with time for female rats fed corn oil. Mean values for 6 rats are plotted for each sample time except 1200 (8 rats) and 2000 (7 rats). Vertical bars are ± SEM.

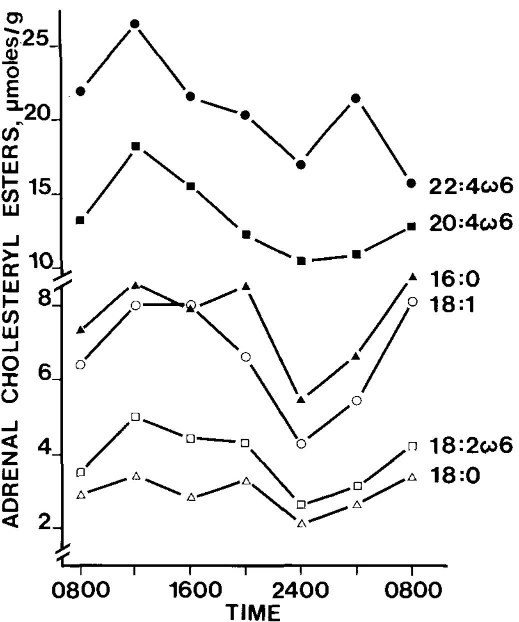

FIG. 2. Variations in adrenal major cholesteryl esters with time for female rats fed corn oil. Mean values for 6 rats are plotted for each sample time except 1200 (8 rats) and 2000 (7 rats).

-10 C until assayed for corticosterone. Adrenals were removed, transferred to a vial containing isotonic saline, immediately frozen in liquid nitrogen, and stored at -10 C until analyzed.

In the second phase of the experiment, female weanling rats, weighing 50-75 g, were fed the semipurified diet containing 10% hydrogenated coconut oil (Canada Packers, Toronto, Ontario) in place of corn oil. The coconut oil contained the following acids: 10:0, 4.2%; 12:0, 50.1%; 14:0, 20.7%, 16:0, 10.5%; 18.0, 12.6%; 18:1, 1.8%; and 18:2, 0.1%. After 3 months, when animals exhibited symptoms typical of EFA deficiency, estrous cycles were verified by examination of vaginal smears (16), and the animals were killed on the third day of the cycle at 4 hourly intervals as described above. Plasma and adrenals were obtained for corticosterone and cholesteryl ester analysis, respectively.

Plasma corticosterone was assayed fluorimetrically (17). Adrenals were dissected free of perirenal fat, weighed, and the lipids extracted in an all-glass apparatus (18) by the procedure of Folch et al. (19), with a water-wash to remove nonlipid impurities. After addition of a known weight of cholesteryl pentadecanoate as an internal standard, the lipids were fractionated by thin layer chromatography and the total cholesteryl esters were isolated (12). The esters were interesterified with boron fluoride-methanol (20), and the resulting methyl esters were subjected to gas liquid chromatography in a 3 m x 2 mm ID glass column packed with 3% EGSP-Z on 100-120 mesh Gaschrom Q (Applied Science Labs, Inc., State College, PA), and operated isothermally at 175 C. Relative peak areas were measured with an electronic digital integrator (Autolab System I, Spectra-physics, Corp., Santa Clara, CA), and peaks were identified by comparison of retention data with those of standard esters chromatographed under similar conditions. The concentrations of individual esters were determined by comparison of the peak areas with that of the internal standard. Data were subjected to analysis of variance and Scheffe's test (21). In order to determine whether specific cholesteryl esters were being preferentially depleted from the adrenal, selectivity indices were calculated as described by Gidez and Feller (11). The selectivity index for an ester (A) is the % of the decrease in total cholsteryl esters attributable ester A divided by the % of A in the total adrenal cholesteryl esters. A value in excess of unity is indicative of preferential utilization.

TABLE I

Concentrations and Selectivity Indices of the Adrenal Major Cholesteryl Esters from Rats Fed Corn Oil[a]

	Sampling time				
	1200 (n=8)		2400 (n=6)		
Ester	μmoles/g tissue	mole %	μmoles/g tissue	mole %	SI[2]
16:0	8.6±0.65	9.2±0.48	5.4±0.30	9.1±0.40	1.01
18:0	3.4±0.23	3.6±0.17	2.1±0.19	3.5±0.24	1.02
18:1	8.0±0.57	8.6±0.64	4.2±0.28	7.1±0.47	1.26
18:2	5.0±0.30	5.3±0.15	2.6±0.19	4.4±0.30	1.29
20:3ω6	3.0±0.14	3.2±0.09	1.7±0.07	2.9±0.17	1.16
20:4ω6	18.2±1.33	19.3±0.73	10.3±0.45	17.4±0.64	1.17
22:4ω6	26.5±2.26	27.9±0.77	17.0±1.21	28.6±1.09	0.93
22:5ω6	4.7±0.68	4.9±0.47	3.6±0.23	6.1±0.29	0.64
22:6ω3	2.8±0.27	2.9±0.15	2.0±0.33	3.3±0.50	0.75
Total esters	94.2±6.30	---	59.2±2.17	---	---

[a]Values represent the mean ± SEM. The number of animals (n) is given in parentheses after the sampling time. Only esters accounting for at least 3% of the total are included.
[b]SI_A=100 (Decrease in ester A/Decrease in total esters) / (mole % of A in 1200 sample).

RESULTS

In female rats fed corn oil, plasma corticosterone followed a monophasic circadian rhythm with the minimum concentration coinciding with the change from darkness to light and the maximum ca. 12 hr later, at the onset of darkness (Fig. 1). The effect of time on plasma corticosterone was significant (P<0.01). There was a 4.6-fold increase in the plasma hormone during the 12 hr light period. Adrenal total cholesteryl ester concentration also varied significantly (P<0.05) with time in rats fed corn oil (Fig. 1). The adrenal cholesteryl ester rhythm was inversely related to that of plasma corticosterone. There was, however, a phase difference of up to 4 hr between the two cycles, with the peak concentration of the esters at 1200, 4 hr after the trough in the corticosterone cycle, and the minimum at 2400, following the peak in glucocorticoid concentration. This implies that, during the cycle, the primary stimulus is on corticosterone synthesis which in turn creates a demand for free cholesterol, thereby triggering hydrolysis of adrenal cholesteryl esters. Adrenal cholesteryl esters declined by 37% during the increase in plasma glucocorticoid concentration.

Most major adrenal cholesteryl esters varied significantly with time over the 24 hr observation period, and rhythms were similar to that recorded for the total cholesteryl ester content of the tissue. Examples are shown in Figure 2. The esters of 22:5ω6 and 22:6ω3 did not vary significantly with time. The possibility that specific utilization of esters occurred in response to the increased output of corticosterone was tested by calculating the selectivity index (SI) for each ester, using the concentrations at the maximum (1200) and minimum (2400) in the daily cycle in this computation (Table I). The SI values for cholesteryl oleate, linoleate, eicosatrienoate (ω6), and arachidonate were in excess of unity, as were those of some of the minor components (e.g., 20:1) not listed in the table. Cholesteryl palmitate and stearate had SIs of approximately unity, whereas that of the major ester, 22:4ω6, and those of 22:5ω6 and 22:6ω3 were less than one. On the basis of the SI values, there was selective depletion of oleate, linoleate, eicosatrienoate (ω6), and arachidonate during the 12 hr period of decreasing adrenal total cholesteryl ester concentrations. The decrease in saturated esters was commensurate with their concentration in the adrenal; the utilization of the major 22:4ω6 ester was slightly less than expected, and of the 22:5ω6 and 22:6ω3 esters substantially less than expected on the basis of tissue concentrations. This pattern of utilization is also evident in the mole % composition of the adrenal cholesteryl esters at 1200 and 2400; the proportions of 16:0 and 18:0 were the same in the two samples, 18:1, 18:2, 20:3ω6 and 20:4ω6 constituted less of the 2400 samples, and 22:4ω6, 22:5ω6, and 22:6ω3 constituted proportionately more of the 2400 ester sample (Table I).

In female rats fed hydrogenated coconut oil, there was a significant (P<0.01) monophasic circadian rhythm for plasma corticosterone (Fig. 3). The minimum concentration occurred at the beginning of the light period (0800); the maximum occurred ca. 12 hr later, but, in

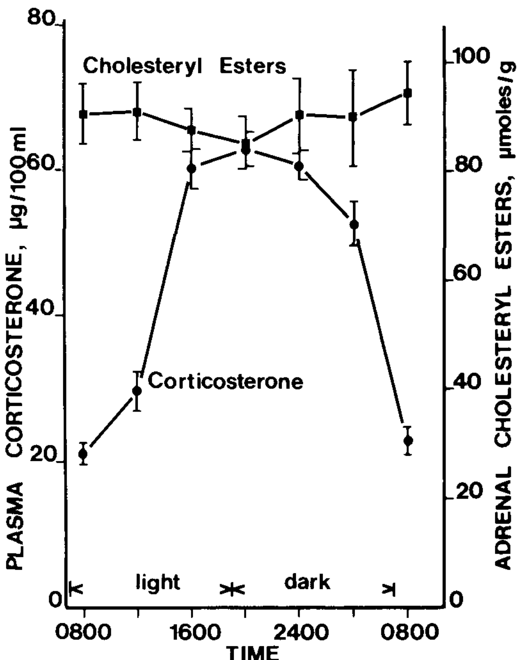

FIG. 3. Variations in plasma corticosterone and adrenal total cholesteryl esters for female rats fed hydrogenated coconut oil. Mean values for 7 rats are plotted for each sample time except 0800 and 1200 (6 rats). Vertical bars are ± SEM.

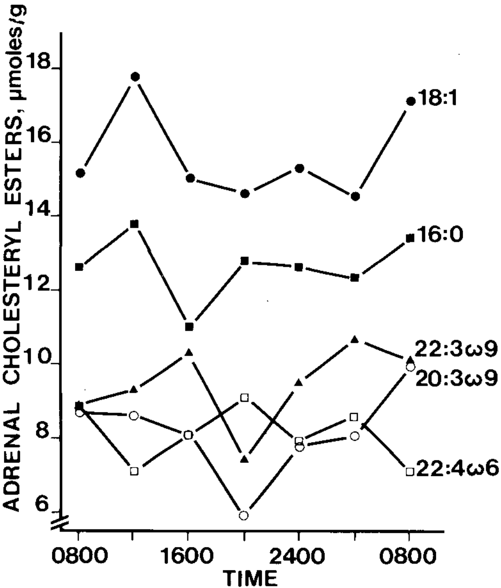

FIG. 4. Variations in adrenal major cholesteryl esters for female rats fed hydrogenated coconut oil. Mean values for 7 rats are plotted for each sample time except 0800 and 1200 (6 rats).

marked contrast to animals fed corn oil, the EFA-deficient animals showed a very broad maximum concentration of plasma corticosterone, extending over an 8-12 hr period. The minimum resting level of plasma corticosterone was greater in the EFA-deficient rats than in those fed corn oil, but there was no difference in the maximum concentrations for the two groups.

There was no significant effect (P>0.05) of time on the total adrenal cholesteryl esters of the rats fed hydrogenated coconut oil, although there was a tendency for the concentration to be slightly lower at 2000 than at other times (Fig. 3). The slight "through" corresponded with the peak plasma corticosterone concentration. This lack of a net decrease in adrenal cholesteryl esters was in marked contrast to the rhythm observed in rats fed corn oil (c.f. Fig 1). Although the total cholesteryl esters did not change substantially during the 24 hr glucocorticoid cycle, the concentrations of individual esters did change. The variations in the concentrations of five of the major esters, 16:0, 18:1, 20:3ω9, 22:3ω9, and 22:4ω6, are shown in Figure 4. The concentrations of cholesteryl stearate, linoleate, and arachidonate changed very little over the 24 hr observation period. There was a tendency for the concentration of four of the major esters to be high in the morning, when plasma corticosterone was low, and to be lower during the broad peak in the glucocorticoid cycle (i.e., in the 1600 to 0400 period). The pattern for 22:4ω6 ester was almost the reverse of this. Figure 4 does explain the relative lack of variation in the total adrenal cholesteryl ester concentration since maximal and minimal concentrations of different esters occurred at different times and tended to smooth out the curve for the total esters (c.f. Fig. 3). The absolute and relative differences in the maximum and minimum concentrations of these esters in the EFA-deficient rats were smaller than the differences recorded for the major esters in rats fed corn oil.

Since the total cholesteryl esters did not decline significantly during the glucocorticoid cycle, calculation of SI values was considered to be invalid for the EFA-deficient rats. However, the concentration of the 20:3ω9 was significantly lower at 2000 than at other sampling times and that of the 22:3ω9 ester was significantly lower at its minimum concentration (2000) than at its maximum concentration (0400), indicating possible selective depletion of these two esters in response to glucocorticoid synthesis and secretion. For none of the other esters were there significant differences in concentration at different sampling times.

DISCUSSION

The circadian variation in plasma corticosterone in rats fed corn oil in this investigation was similar in pattern, sharpness of the peak, and minimum and maximum steroid concentrations to that reported by Morimoto et al. (22) for rats on a 12 hr light-12 hr darkness regimen. Although other workers (7,23,24) have reported similar findings for adult female rats, rhythms of substantially smaller amplitude have also been reported for such animals (25-28). The reasons for the differences among these studies are not evident. Strain differences may account for some of the variability among the studies cited; indeed, attention has been drawn to differences in amplitude of the plasma corticosterone rhythm between rats of ostensibly the same strain obtained from two different sources (27). At least four different strains of rat were employed in the studies cited above, none of which was the Wistar strain used in the present investigation. Dietary differences among the various studies cannot be ruled out as potential sources of variability. In the current investigation, for example, essential fatty acid deficiency did modify the rhythm, producing a much broader corticosterone peak, extending over several hours. Little attention has been afforded the relationship between the nature of the diet and the circadian corticosterone cycle.

Although the maximum resting glucocorticoid concentrations were similar in normal and EFA-deficient female rats, the minimum concentration was significantly higher in the deficient than in the normal animal, suggesting that EFA deficiency acts as a nonspecific stress. This is in contrast to the data of Fukuda and his co-workers (14,29), who reported that male rats fed an EFA-deficient diet had lower resting plasma corticosterone levels than did controls fed sesame oil. Administration of ACTH increased plasma corticosterone more in control than EFA-deficient male rats (14,29), which again contrasts with the similarity in maximum plasma glucocorticoid levels associated with the natural cycles in the present study. Gonadal steroids do affect adrenal function in the female rat (7,23,28,30), which may account for some of the differences between the current and previous investigation. The current experiments were conducted on the third day of the estrous cycle to minimize variations resulting from the effects of gonadal steroids on adrenal function.

Although plasma corticosterone was regulated by maintaining a fixed photoperiod in this study, other factors, such as the sleep-alertness cycle or food intake pattern, may be the primary control agents for the cycle (8,31); meal eating (8), food and water restriction (32) do modify the corticosterone rhythm. None of these factors was investigated in this work, but the possibility that the broadening of the plasma hormone concentration peak resulsts from behavioral changes associated with ingestion of the EFA-deficient diet bears further study.

The inverse relationship, in rats fed corn oil, of adrenal total cholesteryl ester concentration and plasma corticosterone level is consistent with these esters acting as a source of cholesterol for corticosteroidogenesis (9-12). Davis (33) has postulated that provision of cholesterol for pregnenolone production, resulting from activation of adrenal cholesteryl ester hydrolase by ACTH (via cyclic AMP), should be the primary regulatory mechanism in steroidogenesis. Although cAMP does activate this hydrolase (34), the current investigation suggests that adrenal cholesteryl ester hydrolysis occurs in response to increased corticosteroid synthesis rather than preceding and thereby regulating this process. This is evident in the phase difference between the cholesteryl ester and corticosterone rhythms.

In EFA-deficient rats, the lack of significant variation in adrenal cholesteryl esters during the plasma glucocorticoid cycle implies that net hydrolysis of these esters is not obligatory for active glucocorticoid synthesis. However, individual esters did fluctuate during the cycle, sometimes significantly (e.g., 20:ω9). Moreover, only net changes in cholesteryl ester concentrations were measured; turnover of esters without a net change in concentration is possible. Both hydrolysis and synthesis of cholesteryl esters occur in the adrenal, and our data indicate more hydrolysis than synthesis in rats fed corn oil but a relative balance between these two processes in deficient animals. In a separate investigation, labeled esters did turnover without a net decrease in concentration during ACTH-induced steroidogenesis in deficient rats, and the activity of adrenal cholesteryl ester hydrolase was lower in those animals than in controls (Young and Walker, unpublished data).

Gidez and Feller (11) found that cholesteryl esters of 18:1, 18:2, and 20:4ω6 were preferentially depleted during stress-induced steroidogenesis; similar results were subsequently reported for rats fed a normal diet by Walker and Carney (12). In rats fed corn oil, selective depletion of these esters occurred during the circadian cycle, but there appeared to be more uniform utilization of esters, with saturated

esters having SI values of approximately unity, higher than reported in the aforementioned stress studies (11,12). Lack of a significant decrease in adrenal cholesteryl esters during the cycle in the deficient rats precluded calculation of selectivity indices, but significant changes were recorded for the esters of 20:3ω9 and 22:3ω9, which were also well utilized in EFA-deficient male rats subjected to cold stress (12). Unlike this earlier study in which 20:4ω6 and 22:4ω6 esters were preferentially depleted during cold stress (12), there was no evidence for preferential utilization of these esters during the circadian cycle in EFA-deficient female rats. On the basis of the current and previous studies (11,12), hydrolysis of specific esters during steroidogenesis does occur and is modified by diet; it is not a function of the essentiality of the esters esterified to cholesterol.

This investigation has confirmed the existence in the female rat of a circadian cycle of plasma corticosterone and has demonstrated that this cycle is modified in EFA deficiency. As was postulated, in control animals, total and individual adrenal cholesteryl esters were inversely related to plasma glucocorticoid concentration; the relationship was not well defined in EFA-deficient rats and was restricted to individual esters. Preferential depletion of specific esters during steroid biosynthesis and secretion was demonstrated. The existence of circadian cycles for tissue components and their dependence on dietary and other environmental conditions may seriously affect results obtained in biochemical investigations. For example, the higher concentration of cholesteryl esters reported in the adrenals of EFA-deficient male rats in a previous study (11) may have been a direct consequence of the stage in the corticosterone cycle at which the rats were killed. Because of differences between the ester cycles for normal and EFA-deficient rats (c.f. Fig. 1 and 3), the relative concentrations of adrenal cholesteryl esters in rats from the two dietary groups is very dependent on sampling time. As has been pointed out by Nelson et al. (8), the success of biochemical investigations requires careful standardization of all environmental conditions including both the nature of the diet and the mode and timing of its presentation to the experimental animals.

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Equilibrium of Taurodeoxycholate between Mixed Micellar and Aqueous Phases: Effect of Amphiphile

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ABSTRACT

The equilibration of taurodeoxycholate between mixed micellar and aqueous phases has been studied by equilibrium dialysis. The presence of amphiphiles in the form of lecithin, long chain monoglyceride, and fatty acid in the bile salt solution will greatly decrease the bile salt concentration in the aqueous (intermicellar) phase. At high amphiphile concentration relative to bile salt, the concentration of bile salt in the aqueous phase will be below the critical micellar concentration (CMC) of the pure bile salt solution. Under these conditions, few simple micelles will be present and no binding of bile salts to protein takes place as indicated by experiments with colipase. The lowering of the concentration of bile salt in the aqueous phase by the presence of amphiphile may be a physiological mechanism to regulate bile salt absorption during the digestive phase of fat absorption.

INTRODUCTION

The mixed micellar critical micelle concentration (CMC) should theoretically be below the CMC of the pure solubilizer (1). This effect may be of interest for the situation in the small intestinal content to minimize bile salt absorption as long as fat absorption takes place. Intermicellar bile salt concentration (monomeric bile salt + simple micelles) from which bile salt absorption takes place can be expected to be low as long as amphiphiles are provided by bile and hydrolysis of dietary lipids.

The present work was undertaken to actually measure in vitro the effect of amphiphiles on the intramicellar concentration of taurodeoxycholate using equilibrium dialysis (2). The CMC for mixed bile salt/lecithin micelles has recently been determined by this method by measuring the intermicellar bile salt concentration of serially diluted bile salt lecithin dispersions and extrapolating to zero mixed micellar bile salt (3). In the present study, the aqueous (intermicellar) concentration of taurodeoxycholate (TDC) in equilibrium with different concentrations of lecithin and with monoglyceride and fatty acid has been measured. As an indication of the activity of the bile salt in the aqueous phase, its binding to pancreatic colipase was determined.

MATERIALS AND METHODS

[^{3}H]TDC was synthesized in this laboratory and was better than 98% pure by thin layer chromatography (TLC) (4). Lecithin was prepared from fresh eggs and was pure on TLC (5). 1-Monoolein, oleic aicd, and triolein were commercial products. TDC concentrations were obtained from radioactivity. Test for lipids in the dialyzate was done by TLC after extraction. No more than traces of lipids were seen in any case in the dialyzates.

Equilibrium dialysis was performed using a three compartment dialysis cell as previously described (6). The compartments were separated by Spectrapor 1 membrane (Spectrum Medical Industries Inc., Los Angeles, CA) with approximate molecular weight exclusion of 6-8000. Dialysis was performed for 5 days at 25 C, the buffers used contained 0.02% sodium azide.

Two different procedures were followed: (a) the labeled bile salt was introduced in the middle compartment (B), with buffer (A) and buffer plus colipase (C) in the lateral compartments; or (b) all three compartments contained the same concentration of bile salt from the start, other conditions being similar. The lipids were introduced into compartment B as a sonicated dispersion.

Pancreatic colipase was prepared as previously described and was electrophoretically pure (7). Colipase was almost completely retained by the dialysis membrane as measured by its biological activity (7).

RESULTS AND DISCUSSION

TDC alone in the concentrations used (6-12 mM) equilibrated over the membrane completely in 24 hr (Table I). The CMC for TDC under the conditions of the experiments was 0.8-1.3 mM (8), and thus monomeric as well as micellar bile salt equilibrate over the membrane either due to dialysis of intact simple micelles or to the secondary formation of simple micelles in the dialyzate. In the presence of mixed micelles, the equilibration time was longer and dialysis was performed for 5 days.

TABLE I

Effect of Different Lipids on the Equilibration of TDC between Aqueous and Mixed Micellar Phases and the Binding of TDC to Pancreatic Lipase[a]

A. Effect of lecithin.

Starting conditions:

A	B	C
Buffer	12 mM [^{3}H]TDC ± Lecithin	0.13 mM colipase

Concentrations after dialysis:

Lecithin in B mM	mM [^{3}H]TDC				
	A	B	C	C-A	v[b]
0	3.06	3.31	4.88	1.82	14.0
2	1.56	7.71	2.05	0.49	3.8
5	1.18	8.85	1.29	0.11	0.8
10	0.86	9.51	0.90	0.04	0.3
20	0.55	10.4	0.57	0.02	0.2

B. Effect of various lipids.

Starting conditions:

A	B	C
6 mM [^{3}H]TDC	6 mM [^{3}H]TDC ± lipids	6 mM [^{3}H]TDC 0.13 mM colipase

Concentrations after dialysis:

Lipids in B	mM [^{3}H]TDC				
	A	B	C	C-A	v
0	5.67	5.41	7.10	1.43	11.0
Monoolein, 10 mM	1.30	14.8	1.44	0.14	1.1
Oleic acid, 10 mM	1.80	15.9	2.11	0.31	2.4
Triolein, 10 mM	5.56	5.56	6.94	1.38	10.6
Monoolein, 3.3 mM Oleic acid, 3.3 mM Triolein, 3.3 mM	1.75	13.3	2.04	0.29	2.2

[a]A three compartment dialysis cell (A-C) was used separated by Spectrapor 1 dialysis membranes. Dialysis was continued for 5 days. The buffer used was 2 mM Tris-maleate pH 7.0, 1 mM $CaCl_2$ and 150 mM NaCl with 0.02% sodium azide. The figures are means from two experiments.

[b]v moles TDC bound per moles of colipase.

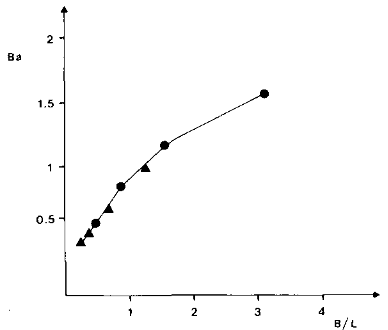

FIG. 1. Aqueous phase concentration of TDC (Ba) as a function of the ratio of bile salt/lecithin in mixed micelles (B/L). The figures were obtained by equilibrium dialysis using a three compartment dialysis cell. The micellar bile salt concentration was calculated from the bile salt in the retinate minus the concentration in the aqueous phase. The most concentrated solutions are to the left and were turbid at the equilibrium. The values are from the figures in Table I and one additional experiment in which the 6 mM TDC was in compartment B together with varying concentrations of lecithin (0-20 mM) at the start of the experiment.

With no lipid in B and colipase in compartment C, bile salt concentration in the latter compartment was higher due to the binding of TDC to colipase to an extent of 11 to 14 moles per mole colipase depending on bile salt concentration as previously shown (9). The presence of lecithin leads to a retention of bile salt, and the concentration of TDC in the dialyzate decreases with increase in lecithin concentration to well below the CMC for the pure salt solution. Already at low concentrations of lecithin, the bile salt concentration in the aqueous phase becomes too low to allow the binding of bile salt to colipase. This indicates that the bile salt concentration in the aqueous phase is too low for aggregation to occur on the protein or to micelles, and the presence of lecithin, therefore, most probably prevents the formation of simple micelles.

Amphiphiles other than lecithin which are known to form mixed micelles with a ratio amphiphile/detergent of $\simeq 1.0$, i.e. monoglyceride and oleic acid, also decrease the bile salt concentration in the aqueous phase in a similar way. A water-insoluble polar lipid (10) as triolein, on the other hand, has no effect on the bile salt partition and, therefore, does not interact with the bile salts to any appreciable extent. In this case, probably only a monolayer of bile salt covers the oil phase. An equimolar mixture of monoolein, oleic acid, and triolein retains bile salt and comes into equilibrium with a bile salt solution whose concentration is slightly above the CMC for the pure bile salt solution. The above findings can also be expressed so that bile salt molecules for thermodynamic reasons tend to aggregate to form mixed amphiphile micelles rather than to aggregate to protein or to form pure detergent micelles.

When the data for the aqueous phase bile salt concentration (Ba) are plotted against the

ratio of micellar bile salt/lecithin (B/L) as done by Duane (3) (Fig. 1), it appears that the aqueous concentration of bile salt is well above the CMC for bile salt alone when the ratio B/L is high, and well below when this ratio is low. When the B/L ratio is below $\simeq 0.5$, the solutions become turbid due to the formation of another phase with a lamellar or hexagonal structure (11). The designs of the experiments are different than those of Duane (3) because he serially diluted the samples; in the present experiments, the total bile salt concentration of the system is kept constant. The conclusions which can be reached regarding the equilibration of bile salt between the aqueous and mixed micellar phases are, however, rather similar.

The results obtained in the present experiments may have some implications for the situation in the small intestinal content during digestion. In the luminal content, emulsified triglycerides are mixed with bile containing mixed lecithin/bile salt micelles and pancreatic juice containing lipase, colipase, and prophospholipase A_2 (rapidly activated to phospholipase A_2). Lipase can adsorb to the triglyceride emulsion in the presence of colipase and catalyze the formation of amphiphilic fatty acids and monoglycerides which go into mixed micelles (12). The presence of lecithin and the amphiphile formed during digestion keeps the concentration of bile salt in the aqueous phase low, presumably below the CMC for the pure bile salt and little simple bile salt micelles will be present. As the bile (and dietary) phospholipids are hydrolyzed to lysocompounds and these are adsorbed together with the hydrolytic products of the dietary fat, the concentration of bile salt in the aqueous phase will increase and favor bile salt adsorption. This interpretation explains the recent results published by Saunders and Sillery (13) that lecithin inhibits bile salt adsorption from the rat small intestine in vivo. It is also in line with the finding that addition of lecithin reversed the secretory effect of glycodeoxycholate in perfusion studies in man (14).

Colipase, a peptide of intestinal contents which has been found to bind bile salts when these are present in concentrations above the critical micellar concentration (9), has been used in this investigation as a model for bile salt binding to protein in the intestinal contents. The results indicate that the occurrence of amphiphile in the intestinal contents may prevent such binding by decreasing the concentration of free bile salts.

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Mass Spectrometric Identification of C_{20} Fatty Acids in Bovine Lens Using the Pyrrolidide Derivative

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ABSTRACT

Unsaturated C_{20} fatty acids from bovine lens have been examined via gas chromatography-mass spectrometry of their pyrrolidide derivatives. The presence of arachidonic acid and 5,8,11-20:3 in bovine lens has been confirmed by this study. In addition, the 10-, 11-, 12-, and 13-isomers of 20:1 and the 7,13-, 8,14-, and 12,15-isomers of 20:2 were identified.

INTRODUCTION

During an earlier study of the fatty acids of bovine lens phospholipids (Chapman, Mizuno, and Chipault, unpublished results), gas chromatography indicated a number of components with equivalent chain lengths (ECL 18.7-23.0) which did not correspond to fatty acids commonly presumed to be constituents of lens tissue (1). Based on electron impact mass spectrometry of methyl esters of fatty acids, Broekhuyse and Soeting (2) reported a C_{20} acid containing four double bonds in the phospholipid fraction of calf lens and suggested that this acid was arachidonic (20:4ω6). The presence of other unsaturated acids was noted also, but except for a triunsaturated C_{20} acid, they were not characterized.

Because the lens is isolated from the vascular system and has been assumed to be essential fatty acid (EFA) deficient, the possibility existed that lens lipids contain isomers of essential fatty acids. Although mass spectrometry of methyl esters is not capable of distinguishing isomeric unsaturated acids (3-6), these isomers can be identified via mass spectra of the pyrrolidide derivatives (7,8). Therefore, several fractions of fatty acid esters from bovine lens lipids were isolated by gas chromatography (GC), converted to their pyrrolidide derivatives and analyzed by gas chromatography-mass spectrometry (GC-MS) to identify their structures.

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EXPERIMENTAL PROCEDURES

Eyes were obtained from freshly killed cattle at a local slughterhouse, and lenses were dissected within 2 to 4 hr. Total lipids were extracted according to the method of Broekhuyse (9), special care being taken to make sure that the tightly bound lipids were completely freed from the proteolipids (10). The total lipids were reextracted by the Folch procedure (11), and the methyl esters

TABLE I

Unsaturated C_{20} Fatty Acids from Bovine Lens

Initial fraction	ECL range	Purified fraction	Tentative identity from GC[a]	Identity of fatty acids by GC-MS[b]	Wt % total fatty acids
1	20.20-20.78	A	20:1	Δ11-20:1(ω9)	6.4
				Δ13-20:1(ω7)	3.5
		B	20:1	Δ13-20:1(ω7)	
				Δ11-20:1(ω9)	
				Δ10-20:1(ω10)	Trace
				Δ12-20:1(ω8)	Trace
2	20.78-21.13	C	20:2	Δ7,13-20:2	1.3
				Δ8,14-20:2	0.6
				Δ12-15-20:2(ω5)	Trace
3	21.13-21.44	D	20:3	Δ5,8,11-20:3(ω9)	3.8
4	21.81-22.18	E	20:4	Δ5,8,11,14-20:4(ω6)	2.0

[a]Number of carbons in fatty acyl moiety:number of double bonds.

[b]In each case, the first fatty acid is the major component.

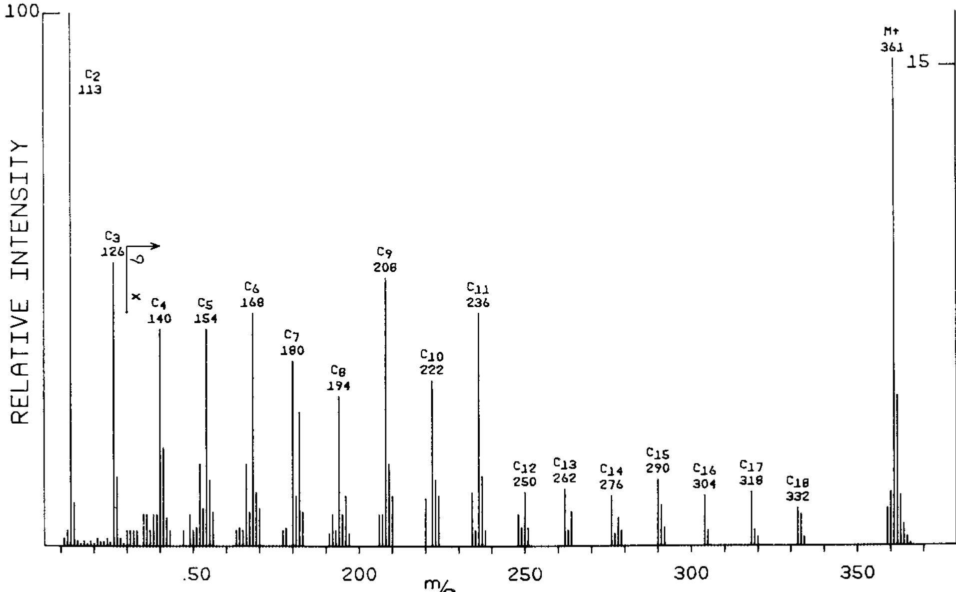

FIG. 1. Mass spectrum of eicosa-7,13-dienoylpyrrolidine.

were prepared from the purified lipids using 6% H_2SO_4 in methanol at 90 C for 4 hr in sealed tubes. The methyl esters were fractionated on a 1/4 in. OD x 6 ft aluminum column packed with 10% EGSS-X on 100/120 Gas Chrom P (Applied Science Laboratories, State College, PA) at 180 C with helium as the carrier gas.

The peaks of uncertain identity in the chromatogram were collected in glass tubes inserted in the exit port and are listed as Initial Fractions in Table I. Each fraction was further purified by rechromatographing on the same column and collecting only the center portion of the peak. These are listed as Purified Fractions in Table I.

The pyrrolidides of the purified fractions were quantitatively prepared on a micro scale from the methyl esters by the procedure of Andersson and Holman (7). Mass spectra were obtained on an LKB 9000 single focusing mass spectrometer operating at an ionization potential of 70 eV and equipped with a 10 in. x 1/8 in. column packed with 3% OV-1 on 80/100 mesh Chromosorb W (HP) (Applied Science Laboratories, State College, PA) and programmed from 150 to 300 C at 4 C/min with a flow rate of 20 ml He/min. The mass spectrometer was interfaced to a PDP 8-e laboratory computer for spectral acquisition, and scans were taken throughout the elution of a peak.

Methyl arachidonate was obtained from the Lipids Preparation Laboratory, The Hormel Institute, University of Minnesota, Austin, MN.

RESULTS AND DISCUSSION

The pyrrolidide derivative was chosen for this study because it offers several advantages for the identification of unsaturated fatty acids by mass spectrometry. The derivative is capable of supporting the positive charge induced by electron impact on the amide linkage, and thus the fragmentation pattern produced is much simpler than that provided by the analogous methyl ester. In an MS investigation of the location of the double bond in isomeric monounsaturated fatty acids (7), the spectra of the pyrrolidides were comprised principally of fragments derived from the polar end of the molecule. The structurally informative portion of the spectrum begins with the base peak of each spectrum, m/e 113 produced by a McLafferty rearrangement, analogous to m/e 74 in the MS of methyl esters. This is followed by the C_3 fragment, m/e 126. If one then observes the most intense fragment in each 14 atomic mass unit (amu) cluster of fragments, an interval of 14 amu is encountered for each $-CH_2-$ group. A double bond at C_n produces an interval of 12 amu between fragments corresponding to C_{n-1} and C_n. This pehnomenon was seen consistently in fatty

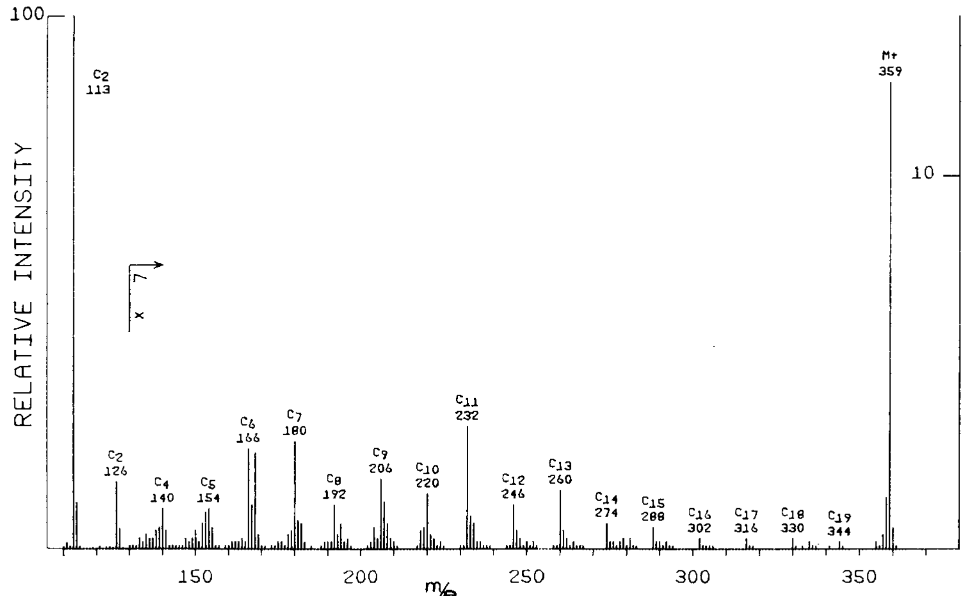

FIG. 2. Mass spectrum of eicosa-5,8,11-trienoylpyrrolidine.

pyrrolidides possessing an unsaturation between Δ5 and ω4 and the remaining isomers yielded characteristic spectra.

To illustrate the use of the pyrrolidide derivatives in the structural analysis of fatty acids, Figure 1 shows the mass spectrum of the pyrrolidide of the major fatty acid in fraction C. The intense molecular ion, m/e 361, indicated a diunsaturated C_{20} acid. In common with most mass spectra of pyrrolidides, the base peak in the spectrum was m/e 113, followed by m/e 126 (C_3), m/e 140 (C_4), m/e 154 (C_5), and m/e 168 (C_6) – all 14 amu intervals. The most intense peak of the next cluster occurred at m/e 180 (C_7), an interval of 12 amu between C_6 and C_7, indicating a double bond at C_7. The other interval of 12 amu was found between m/e 250 (C_{12}) and m/e 262 (C_{13}), thus the second double bond occurred at C_{13}. The fatty acid was thus 7,13-20:2, and not a methylene-interrupted fatty acid. The acid which followed it immediately was identified as 8,14-20:2 by these rules. Only a trace amount of a methylene-interrupted fatty acid was found in this fraction, and it was identified as 12,15-20:2. The detection and identification of two major and two minor isomers of 20:1 are shown in Table I.

The limits of the rule for the location of double bonds are Δ5 for mono- and dienoic fatty acids and Δ6 for those containing three or more double bonds. In Figure 2, the spectrum of the pyrrolidide of a 20:3 acid is shown, and the diagnostic intervals of 12 amu locate double bonds at C_8 (m/e 180 and m/e 192) and at C_{11} (m/e 220 and m/e 232). The other 12 amu interval (m/e 154 and m/e 166) suggests a double bond at Δ6. However, the spectrum of an authentic Δ5,8,11-20:3 pyrrolidide shows the same deviation from the rule (A.J. Valicenti and R.T. Holman, unpublished data) and the Δ5 unsaturation of the compound represented in Figure 2 was established by direct comparison with this standard. The 154 m/e peak in the C_5 cluster is probably due to rearrangement of this double bond under electron impact (12) to a conjugated system (5,8 → 6,8) causing an increase in the intensity of the 5:0 fragment (m/e 154) at the expense of the 5:1 fragment (m/e 152).

Since the mass spectra of isomeric pyrrolidides are all different, even fatty acids possessing unsaturation at Δ2-5 may be identified by comparison with reference compounds as was done above and as shown in Figure 3. The component assumed to be arachidonate was converted to its pyrrolidide derivative, and its mass spectrum was compared with that of the pyrrolidide of authentic arachidonic acid. The spectra are virtually superimposable, except for minor statistical variations in ion collection. If several scans are acquired from the unknown and averaged, the averaged spectra become virtually

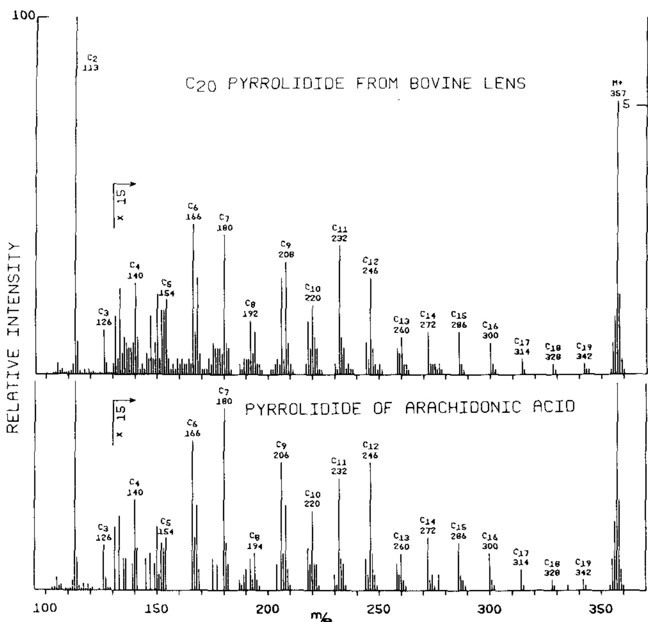

FIG. 3. Above: Mass spectrum of the 20:4 pyrrolidide from bovine lens. Below: Mass spectrum of the pyrrolidide derivative of authentic arachidonic acid.

TABLE II

Diagnostic Fragments in the Mass Spectra of C_{20} Acyl Pyrrolidines from Bovine Lens

Fatty acid	C no; mass of ion (relative intensity)	Molecular ion (relative intensity)
11-20:1	10,224(2.0); 11,236(1.9); 12,250(2.3)	363 (28.6)
13-20:1	12,252(1.3); 13,264(2.1); 14,278(2.6)	363 (21.0)
7,13-20:2	6,168(7.3); 7,180(5.8); 8,194(4.7); 12,250(1.7); 13,262(1.8); 14,276(1.6)	361 (15.2)
8,14-20:2	7,182(4.5); 8,194(4.5); 9,208(6.0); 13,264(4.5); 14,276(1.5); 15,290(1.5)	361 (17.9)
5,8,11-20:3	4,140(1.1); 5,154(1.1); 6,166(2.7); 7,180(2.9); 8,192(1.2); 9,206(1.9); 10,220(1.5); 11,232 (3.3); 12,246(1.2)	359(12.5)
5,8,11,14-20:4	4,140(1.7); 5,154(1.4); 6,166(2.8); 7,180(2.6); 8,192(1.0); 9,206(1.8); 10,220(1.3); 11,232(2.4); 12,246(1.8); 13,260(0.7); 14,272(0.8); 15,286(0.8)	357 (5.1)

identical with that of the known. The only non-amide-containing fragment of importance in these spectra is m/e 150, $C_{11}H_{18}$. This fragment is produced in greater than 50% relative intensity in the EI mass spectrum of methyl arachidonate (13). Key fragments in the single-scan mass spectra of the C_{20} fatty acids in this study are summarized in Table II.

Two ω7 C_{20} fatty acids were examined and found to be Δ13-20:1 and Δ7,13-20:2. Vaccenic acid (*trans*-11-18:1) (ω7) is a natural fatty acid in ruminants, and it is conceivable that elongation and desaturation of vaccenic acid could produce these fatty acids in the small amounts encountered in the lens. Alternatively, the may be of dietary origin. The ω7 acids have been shown to be constituents of menhaden oil (14,15), and the tetramethylene-interrupted acids are found in marine animals (16) and in seed oils (17). Incorporation of 7,13-20:2 into phospholipids has been demonstrated in the rat (18), but this acid was found to be resistant to further desaturation. This acid, as well as several other ω7 acids, has been

identified in the liver of the fat-starved rat (19), and their presence may be an indicator of essential fatty acid deficiency. The purpose of these unusual fatty acids in lens phospholipids is, at present, unknown.

Although the essential fatty acid, arachidonic acid, has been identified as a constituent of lens lipids, the presence of the ω9 acids, 11-20:1 and 5,8,11-20:3, characteristic of essential fatty acid deficient tissues suggests that the amount of EFA in the lens is suboptimal, and the lens attempts to compensate in the usual manner by desaturation and elongation of oleic acid. That the lens is moderately EFA deficient is indicated also by the ratio of 20:3ω9/20:4ω6 which equals 1.9.

In humans, the lens becomes isolated from the circulatory system very early, when the embryo is at the 7 mm stage (20). It is not known when this occurs in the bovine, but it is unlikely that the EFAs found in the lens represent residues from the amounts present before the lens became separated from the vascular system. More likely, these enter the lens from the surrounding aqueous and vitreous humors. Both these tissues also contain small amounts of arachidonic acid (Chapman and Chipault, unpublished results).

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Neutral Lipids from the Temporal Gland of the African Elephant, *Loxodonta africana*

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ABSTRACT

Lipids from inactive and active temporal glands of the African elephant, *Loxodonta africana*, were isolated and fractionated. The inactive gland had a much higher total lipid content per gram of fresh tissue than the active gland. Lipids from the inactive gland consisted predominantly of neutral lipids while the active gland contained large quantities of phospholipids. Neutral lipids from the active gland contained much more hydrocarbons, cholesterol, and alkoxy glycerides than neutral lipids from an inactive gland. The alkoxy glyceride fraction did not contain any alkenyl glycerides. The hydrocarbons consisted of a mixture containing predominantly straight chain even numbered saturated and unsaturated hydrocarbons from C_{18}-C_{30}. Fatty acids from various fractions were investigated by gas liquid chromatography. Those from the active gland were characterized by a higher percentage of unsaturated acids. The change from inactive to the active state involves mainly a reduction in palmitic and an increase in oleic acid content.

INTRODUCTION

The temporal gland of the African elephant, *Loxodonta africana*, which has been described by Sikes (1) as an apocrine cutaneous gland is situated on each side of the head in the temporal depression. In active state, the gland produces a strongly smelling serous secretion which is not of lipid nature (S.H.W. Cmelik, unpublished results). The activity of the gland has been originally associated with the sexual activity of the elephant (2) but this has been disputed later on (3). Sometimes young, usually immature animals show a copious secretion of the gland (O. Bristow, personal communication). Except for some histological studies (4,5) carried out on the temporal gland of the African and Indian elephants, no other work has been done on the chemical composition of the gland and its secretion. It has been, decided, therefore, to investigate in the first instance the neutral lipids from both active and inactive glands in order to establish to what extent the change in activity would affect the lipid composition.

MATERIAL AND METHODS

Reagents

All solvents were of the highest purity. Chloroform and methanol were distilled before use. Unisil was obtained from Clarkson Chemical Co. (Williamsport, PA). Precoated Silica Gel G plates and activated aluminum oxide II-III were obtained from Merck A.G. Darmstadt, Germany. Fatty acid methyl esters and hydrocarbon standards were supplied by Applied Science Laboratories (State College, PA) and NuChek Prep. (Elysian, MN). Long chain alcohols were obtained from Fluka A.G. (Buchs, Switzerland).

Animal Material

African elephants whose temporal glands were used in this work were shot during the annual population reduction exercise in the

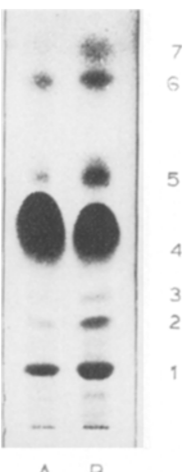

FIG. 1. Thin layer chromatogram of neutral lipids from an inactive (A) and an active (B) temporal gland of the African elephant. The plate was developed twice in petroleum-ether-ether-acetic acid (95:5:1) and charred with 80% sulfuric acid, w/v. Legend of the spots: 1, cholesterol; 2, free fatty acids; 3, long chain alcohols; 4, triglycerides; 5, alkoxy glycerides; 6, cholesteryl esters; 7, hydrocarbons.

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TABLE I

Chemical Composition of Total Lipids from the Temporal Gland of the African Elephant

Preparation	% Weight of total lipids							
	Hydrocarbons	Cholesteryl esters	Free cholesterol	Alkoxy glycerides	Triglycerides	Free fatty acids	Phospholipids	Unidentified
Inactive gland	<0.1	0.5	2.3	<0.2	76.2	0.1	19.8	1.5
Active gland	1.4	1.2	5.7	2.6	22.8	0.5	62.8	3.0

TABLE II

Field Data and Lipid Yield for the Temporal Gland of the African Elephant

	Temporal gland	
	Inactive	Active
Sex of animal	♂	♂
Age of animal (years)	10	18
Weight of glands (g)	150	670
mg Lipids/g of fresh tissue	53.5	20.7

Wankie National Park in Rhodesia. The glands were excised together with the surrounding fatty tissue, packed in ice, and delivered to the laboratory with the minimum delay.

Histology of the Gland

Histological preparations were made from both inactive and active glands and stained with haematoxylin-eosin. The presence of lipids was shown by staining with oil red O.

Extraction and Separation of Lipids

Since the gland was embedded in a layer of fatty tissue, all the surrounding tissue had to be carefully removed before extraction. The glandular tissue was then homogenized in a Waring blender with chloroform-methanol (2:1) in a ratio of 1:15 (w/v). The suspension was filtered through glass wool, and the extraction of the residue was repeated once more. The joined extracts were evaporated to dryness in a rotary evaporator, the residue taken into chloroform, and the insoluble residue spun off in a centrifuge. The total chloroform-soluble lipids were separated into neutral and phospholipids on a Unisil column (6). Total cholesterol in neutral lipids was determined by the use of a modified Liebermann-Burchard reagent (7). For the quantitative determination of esterified cholesterol, the free cholesterol was previously precipitated with digitonin.

Isolation of Various Lipid Fractions

Neutral lipids (Figure 1) were fractionated on Unisil columns (8) and on preparative Silica Gel G plates which were developed twice in petroleum ether-ether-acetic acid (95:5:1). The plates were given only a very light sprinkling with 4,5-dibromofluorescein which was sufficient to make bands visible under UV light. Individual bands were marked, scraped from plates, and extracted with ether. Values given in Table I were compiled from colorimetric determinations, and the yields from preparative column and thin layer chromatography (TLC).

Because of the contamination with triglycerides, alkoxy glycerides had to be rerun on

TABLE III

Percent Fatty Acid Composition of Various Neutral Lipid Fractions from Inactive and Active Temporal Glands of the African Elephant

	Cholesteryl esters		Alkoxy glycerides	Free fatty acids		Triglycerides	
Carbon number	Inactive	Active	Active	Inactive	Active	Inactive	Active
12:0	---	---	---	---	0.9	---	0.4
14:0	10.4	9.3	9.8	1.1	8.8	6.4	6.5
14:1	---	---	1.9	---	---	---	1.2
14:2	2.1	2.3	---	0.6	0.9	0.8	---
16:0	41.7	16.3	20.6	29.5	15.9	25.6	29.3
16:1	6.2	5.8	5.9	4.5	3.1	6.8	8.9
16:2	4.2	3.5	2.9	2.3	2.6	1.7	1.6
17:0	2.1	2.3	1.6	1.1	2.6	2.6	1.6
18:0	10.4	7.0	2.9	10.2	8.0	4.7	4.1
18:1	10.4	35.0	36.3	33.0	41.6	47.0	41.5
18:2ω6	6.2	7.0	4.9	3.4	4.9	1.3	3.2
19:0	---	---	---	1.1	---	---	---
18:3ω3	---	2.3	2.9	2.3	0.9	---	---
20:1ω9	---	3.5	---	1.1	3.5	3.0	1.6
20:2	6.2	3.5	5.9	---	2.6	---	---
20:3ω6	---	---	---	5.1	0.9	---	---
20:4ω6	---	---	2.0	2.3	2.6	---	---
20:5ω3	---	2.3	2.5	---	---	---	---
22:2ω9	---	---	---	2.3	---	---	---
Σ Unsat. acids	35.6	65.1	65.1	57.0	63.8	60.7	58.1

300-μ plates in the same solvent system. The purified alkoxy glycerides were hydrolyzed with 10% KOH in methanol, and the fatty acids were analyzed by gas liquid chromatography (GLC) (9). An unhydrolyzed portion of the alkoxy glyceride fraction was examined by two dimensional TLC according to Schmid and Mangold (10) for the presence of alkenyl diglycerides.

The fraction containing hydrocarbons and cholesteryl esters was hydrolyzed with 10% KOH in methanol, and the fatty acids were analyzed by GLC. The hydrocarbons in the nonsaponifiable part were separated from cholesterol on an aluminum oxide column (11) and analyzed by GLC.

Samples of the surrounding fatty tissue were extracted and processed in the same way. Examination of the neutral lipids by TLC showed only the presence of triglycerides.

Identification of the Products of Hydrolysis

Cholesterol obtained by column chromatography was identified by mixed melting point with pure cholesterol. Fatty acids were converted into methyl esters (12) and analyzed by GLC on a 20% (diethyleneglycerolsuccinate) column. Individual fatty acids were identified as described previously (13). Hydrocarbons were analyzed on a 2.5% SE 30 column at 220 C, and the retention times were compared with those from standard hydrocarbons.

RESULTS AND DISCUSSION

Histology of the Gland

The histological examination has revealed a distinct difference between the active and inactive gland. While the active glnad contained large secretory cells, cuboidal or columnar, and had a large, deep staining, well defined nucleus, the inactive one had only small cuboidal cells with a small nucleus.

The results of the histological examination have been confirmed by personal observation of the authors. During the removal of the inactive gland from the dead animal, not even traces of a secretion were observed, while the excision of the active gland was followed by a copious secretion of a viscous fluid with a typical "elephant odor."

Distribution of Lipid Fractions

The total lipid content of the inactive gland was more than twice as high as in the active one (Table II) indicating that large quantities of lipids must have been stored within the cells. This could also be seen from the greatly increased amount of glycerides and the reduced phospholipids in the inactive gland (Table I).

Another notable feature of the active gland was the appearance of larger quantities of alkoxy lipids. Fatty acid yields from the alkaline hydrolysis showed that they must have been present in the form of diglycerides. No

TABLE IV

Fatty Acid Composition of the Triglycerides from the Adipose Tissue of the African Elephant

Carbon number	Percent of total	
	Temporal gland	Perinephric fat (17)
14:0	3.6	5.0
14:2	0.7	—
15:0	—	0.8
16:0	31.9	44.5-46.7
16:1	8.0	2.8-5.0
16:2	1.4	—
17:0	2.2	1.5
17:1	—	1.0
18:0	3.6	5.5-6.4
18:1	47.1	33.0
18:2	1.4	0.4-0.9
18:3	—	1.8

alkenyl ethers were detected after the two dimensional chromatography and hydrolysis with HCl. No alkoxy glycerides have been found in the fatty tissues surrounding the temporal gland, but they seem to be present in the neutral lipids from the mitochondria of the adrenal gland (14) of the elephant.

Fatty Acid Composition

Fatty acids esterified to cholesterol (Table III) in the temporal gland differ from cholesteryl ester fatty acids from other elephant tissues (14) and blood plasma (15,16) by a very low content of linoleic and higher polyunsaturated fatty acids. The difference between the two glands in mainly reflected in a higher content of palmitic and lower content of oleic acids in the inactive and a reversed ratio of these acids in the active gland. Such differences exist also in the free fatty acid fraction by not in the triglycerides. Fatty acids from alkoxy glycerides of the active gland were similar in composition to other lipid fractions from the active gland. It should be noted that all fractions were characterized by a fairly high content of myristic acid.

GLC of the fatty acids from the fatty tissue surrounding the temporal gland has shown that their spectrum differs very little from the fatty acid spectrum of the triglycerides in perinephric fat (17) (Table IV). Although these fatty acids differ in composition from fatty acids in ruminant depot fat (18), particularly by a higher content of palmitic acid and a very much lower content of stearic acid, the almost complete absence of polyunsaturated fatty acids indicates that extensive hydrogenation must be taking place in the digestive tract of the elephant (19).

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Occurrence of 5,9,19-Octacosatrienoic, 5,9-Hexacosadienoic and 17-Hexacosenoic Acids in the Marine Sponge *Xestospongia halichondroides*

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ABSTRACT

The marine sponge *Xestospongia halichondroides* contains 13% 28:3Δ5,9,19, 27% 26:2Δ5,9, and 21% 26:1Δ17 in its total fatty acids. The *cis*-5,*cis*-9,*cis*-19-octacosatrienoic acid structure is unknown in other living organisms.

INTRODUCTION

Recent work in our laboratory (1,2) has identified a new family of C_{24}, C_{25}, C_{26} and C_{27} polyunsaturated fatty acids with isolated (nonmethylene interrupted) double bonds in the marine sponge *Microciona prolifera.* All contained Δ5,9 unsaturation. Specific acids identified were 24:2Δ5,9, 25:2Δ5,9, 26:2Δ5,9, 26:3Δ5,9,17, 26:3Δ5,9,19, 27:3Δ5,9,19, and 27:3Δ5,9,20.

The high level (48%) of C_{24}-C_{28} chain lengths in *Microciona* fatty acids and similar findings in two other sponges by Bergmann and Swift (3) led us to examine 20 sponge genera from the class Demospongiae for the fatty acid chain lengths present (4). All the sponges studied contained unusually high levels (34-79%) of characteristic C_{24}-C_{30} "demospongic acids," many of which had gas liquid chromatography (GLC) retention times indicating new and novel structures. One of these sponges, *Xestospongia halichondroides,* contained 39% C_{26} and 13% C_{28} chain lengths in its total fatty acids. We report here a full characterization of the three major C_{26}-C_{28} acids of *Xestospongia.*

EXPERIMENTAL PROCEDURES

Materials

Xestospongia halichondroides sponge samples collected in the Gulf of Mexico in May 1976 were purchased from the Gulf Specimen Company, Panacea, Florida. The live sponges were packed in seawater and shipped by air to our laboratory. Upon arrival, the sponges were washed in seawater, carefully cleaned of all visible algae and debris, and cut into small cubes. Immediate extraction with chloroform-methanol 2:1 (5) yielded 3.5% (dry wt) total lipids.

Methyl esters of the total *Xestospongia* lipids were prepared by KOH-catalyzed methanolysis (6) and purified by preparative thin layer chromatography (TLC). This purification was carried out on 1.0 mm thick silicic acid (Silica Gel G60, E. Merck, Darmstadt, Germany) impregnated with rhodamine 6G and developed in petroleum ether-diethyl ether 97.5:2.5. The methyl esters (located under UV light by comparison with known standards) were separated into two distinct bands; each was scraped off the plates, and the esters were eluted from the silicic acid using diethyl ether.

The following standards were purchased from reliable sources and used in the identification of fatty acids and of the products of reductive ozonolysis by gas liquid chromatography: methyl esters of 14:0, 16:0, 18:0, 18:1Δ9, 20:0, 22:0, 24:0, and 30:0; a mixture of C_{10} to C_{20} α-olefinic hydrocarbons; 1,5-hexadiene; and 1,11-dodecadiene. Methyl esters of *Limnanthes douglasii* seed fat [containing 20:1Δ5 (7)], linseed oil, and *Tropaeolum speciosum* seed fat [containing 22:1Δ13, 24:1Δ15, 26:1Δ17 (8)] were prepared by KOH-catalyzed methanolysis (6).

Methods

GLC analyses of methyl esters were run isothermally on 1.80 m x 2.4 mm ID columns packed with 10% EGSS-X or 10% Silar-10C on 100/120 mesh Gas Chrom Q (Applied Science Laboratories, State College, PA) at 185-200 C using a flame ionization detector. Peaks were identified using cochromatography, by comparison of retention times with known standards, and by graphic correlation of equivalent chain length (ECL) values using semilog plots (9-11). All ECL values given in this report were measured on an EGSS-X column at 200 C.

Methyl esters were separated according to the degree of unsaturation by preparative TLC on 1.0 mm thick $AgNO_3$-impregnated silicic acid (23% w/w) developed in petroleum ether-diethyl ether 80:20. Bands were located under UV after spraying with 2′,7′-dichloro-

fluorescein and were recovered by extraction with diethyl ether. Further purification of the individual bands by Ag^+-TLC used 97:3, 80:20, or 60:40 petroleum ether-diethyl ether solvent systems.

Infrared analyses were carried out in CCl_4 solution using a Perkin-Elmer 700 spectrophotometer. Ultraviolet spectra were run in hexane solution on a Beckman DB spectrophotometer. The 60 MHz PMR spectra were obtained with a Varian T-60 instrument using 10-25 mg samples dissolved in CCl_4 with $Si(CH_3)_4$ as a reference marker. Mass spectrometric analyses were performed on a Hitachi/ Perkin-Elmer RMU-7 mass spectrometer operated at 70 eV with direct insertion of the sample probe at 120 C.

Reductive ozonolyses of 10-50 μg methyl ester samples were carried out in CH_2Cl_2 at -70 C or purified petroleum ether at -10 C using the method of Stein and Nicolaides (12) with a Supelco micro-ozonizer. Aldehydic products were analyzed by GLC as described above except that isothermal column temperatures of 90-190 C were used, according to the chain lengths of the aldehydic products. Peaks were identified by cochromatography with known standards and by semilog retention time plots for homologous series. To eliminate extraneous GLC peaks, the petroleum ether was purified before use (stirred 12 hr with conc. H_2SO_4, washed repeatedly with water, dried over Na_2SO_4 and then alumina, distilled).

Methyl esters were hydrogenated in methanol solution using a PtO_2 catalyst (13).

RESULTS

Purification of Methyl Esters

Xestospongia halichondroides fatty acid methyl esters were separated by TLC into two distinct bands. GLC analysis of the original esters and their fully hydrogenated derivatives revealed that the lower band contained mostly polyunsaturated C_{16}-C_{22} esters while the C_{24}-C_{28} and more saturated C_{14}-C_{20} chains were present in the upper band. The three major C_{26}-C_{28} peaks having ECL values of 26.70, 27.00, and 29.52 on an EGSS-X column at 200 C were selected for detailed analysis. These peaks constituted 21, 27, and 13% by weight, respectively, of the total methyl esters from the sponge.

Ag^+-TLC of the upper band separated monoene, diene, and triene fractions corresponding to the three major C_{26}-C_{28} GLC peaks. Each of these fractions was repurified by Ag^+-TLC in varying petroleum ether-diethyl ether solvent systems. The products exhibited only one spot by Ag^+-TLC, but GLC analysis before and after hydrogenation revealed minor homologues one or two carbons shorter or longer than the major component.

Identification of 28:3

The methyl ester having an ECL value of 29.52 was recovered in the triene band from Ag^+-TLC. It was 80% pure by GLC with a 27.60 ECL peak (14%) and a 28.54 ECL peak (4%) as the major impurities. Both the ECL values and the Ag^+-TLC migration of these impurities, as well as their subsequent behavior in hydrogenation/GLC, mass spectrometry, PMR, and ozonolysis, pointed to their being structural analogues of the 28:3 component. Their influence on each analytical procedure was carefully considered; but as far as we could determine, they did not interfere in any way with the positive identification of the 80% major component.

Hydrogenation of the unknown methyl ester produced an n-28:0 peak as characterized by GLC. Thus, an n-28:3 was indicated. Spectrographic evidence also supported and further defined the methyl octacosatrienoate structure. Mass spectrometric analysis gave a molecular ion at m/e 432. The ester showed no absorption bands in the 220-300 nm region, indicating the absence of conjugated double bonds (14). Its infrared spectrum exhibited the usual 2970-2820 cm^{-1} hydrocarbon and 1712 cm^{-1} ester bands and had no prominent absorption at 980-960 cm^{-1} indicating *cis* rather than *trans* unsaturation (15). The absorption bands in the 1450-1121 cm^{-1} region were distinctively different from methyl oleate but exactly matched the published spectrum of methyl *cis*-5,*cis*-9,*cis*-12-octadecatrienoate (16). The PMR spectrum corresponded to that of an unsaturated methyl ester. Integration of the CH_3O- and -HC=CH- signals gave a 3.0:5.9 ratio indicating a trienoic compound. However, there was no =C-CH_2-C= signal at 2.7 δ, showing the absence of any 1,4-diene structures (17).

Reductive ozonolysis of the ester produced four major GLC peaks, as expected for a 28:3. These peaks were identified as a C_5 aldehyde-ester, a C_4 dialdehyde, a C_{10} dialdehyde, and a C_9 aldehyde. Either a 28:3Δ5,9,19 or a 28:3Δ5,15,19 structure could have produced these fragments. To identify the position of the middle double bond, the 28:3 ester was partially hydrogenated with hydrazine (18), a reaction that is not accompanied by double bond isomerization. The 28:1 reaction products were isolated by Ag^+-TLC and subjected to reductive ozonolysis. Two of the main fragments identified by GLC analysis were a C_9

aldehyde-ester and a C_{19} aldehyde.

We conclude that the unknown compound was the methyl ester of *cis*-5,*cis*-9,*cis*-19-octacosatrienoic acid.

Identification of 26:2

The methyl ester of 27.00 ECL was identified in the same manner as the 28:3. The ester was recovered in the diene band from Ag^+-TLC. It was 80% pure by GLC with two principal impurities (14% ECL 27.93 and 4% ECL 26.05) which appeared to be structural analogues and did not interfere with positive identification of the major component.

Hydrogenation of the ECL 27.00 unknown produced an n-26:0 peak on GLC, indicating a 26:2 fatty acid. Mass spectrometry gave a molecular ion at m/e 406, and the ester had no UV absorption bands between 220 and 300 nm. Its infrared spectrum was identical with that of the 28:3Δ5,9,19 discussed above and showed no indications of *trans* unsaturation at 980-960 cm^{-1}. PMR of the 26:2 yielded a normal methyl ester spectrum with a 3.0:3.9 ratio of the CH_3O- and -HC=CH- signals, confirming the dienoic structure. Again there was no $=C\text{-}CH_2\text{-}C=$ signal at 2.7 δ, indicating isolated (nonmethylene interrupted) double bonds.

Ozonolysis of the 26:2 produced three major GLC peaks: a C_5 aldehyde ester, a C_4 dialdehyde, and a C_{17} aldehyde. Hence, the unknown compound was the methyl ester of *cis*-5,*cis*-9-hexacosadienoic acid.

Identification of 26:1

The ECL 26.70 methyl ester was identified by the same methods used for the 28:3 and 26:2. It was recovered in the monoene band from Ag^+-TLC and was 94% pure by GLC. Hydrogenation produced an n-26:0 GLC peak, indicating a 26:1 structure. Mass spectrometry gave a molecular ion at m/e 408 with the usual M^+-32 base peak expected for monoene methyl esters (19). The ultraviolet and infrared spectra of the ester were identical with those of methyl oleate. PMR of the 26:1 produced a normal fatty acid methyl ester spectrum with a 3.0:1.9 ratio of the CH_3O- and -HC=CH- signals, as one would expect of a monoene. Ozonolysis yielded two products, a C_{17} aldehyde-ester and a C_9 aldehyde. Thus, the unknown compound was the methyl ester of *cis*-17-hexacosenoic acid.

DISCUSSION

The three *Xestospongia* fatty acids identified here are typical of the C_{24}-C_{30} demospongic acids that are characteristic of sponges in the class Demospongiae (4). The 28:3Δ5,9,19 structure has not, to our knowledge, been previously reported in living organisms. The 26:2Δ5,9 acid is also found in the spong *Microciona prolifera* (1,2) but is unknown in other types of organisms. The 26:1Δ17 structure is uncommon, but it has been found in both higher plants (8,20) and fish (21).

Our results suggest that 26:3 and 27:3 analogues of 28:3Δ5,9,19 and that 25:2 and 27:2 analogues of 26:2Δ5,9 are also present in *Xestospongia.* As mentioned above, minor GLC peaks with appropriate homologous ECL values were found in the 28:3 and 26:2 fractions isolated, and the positions of their double bonds were such that they did not interfere with spectrometric and ozonolysis analyses. However, definitive characterization of these minor components was not attempted, and their identification remains tentative.

Morales and Litchfield (22) have shown that the sponge *Microciona prolifera* possesses a very active fatty acid chain elongation system that produces C_{24}-C_{28} acids from normal chain length precursors. Following elongation, Δ5,9 desaturation may occur. It seems likely that similar biosynthetic pathways occur in *Xestospongia.* The new 28:3Δ5,9,19 structure could be produced from oleic acid

$$18{:}1\Delta9 \rightarrow \rightarrow 26{:}1\Delta17 \rightarrow 28{:}1\Delta19 \rightarrow 28{:}2\Delta9,19 \rightarrow 28{:}3\Delta5,9,19$$

in the same way that 26:3Δ5,9,19 originated from palmitoleic acid in *Microciona* (22). The 26:2Δ5,9 in *Xestospongia* is probably produced by the same pathway as in *Microciona:*

$$16{:}0 \rightarrow \rightarrow 26{:}0 \rightarrow 26{:}1\Delta9 \rightarrow 26{:}2\Delta5,9$$

The 26:1Δ17 in *Xestospongia* is no doubt a direct elongation product from oleic acid.

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Studies on Biosynthesis of Waxes by Developing Jojoba Seed Tissue

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ABSTRACT

Slices of developing jojoba cotyledons incorporated a variety of precursors into wax, free alcohols, and polar lipids. ^{14}C-Decanoic and ^{14}C-lauric acids were elongated and desaturated, whereas ^{14}C-myristic and ^{14}C-longer chain fatty acids, although incorporated into wax, were insignificantly modified. Exogenously added ^{14}C-acetate contributed mainly to chain elongation of endogenous oleic acid, whereas ^{14}C from added glucose was uniformly distributed throughout the acyl chain of the fatty acids. These data suggest the existence of metabolically separate pools of acetate and/or sites for de novo synthesis and elongation of acyl chains.

INTRODUCTION

The jojoba shrub [*Simmondsia chinensis* (Link)], which is native to arid regions of southwestern North America, produces seeds which store lipid in the form of simple wax esters of long chain (predominantly 20-22 carbon) fatty acids and alcohols (1). The wax which makes up 40-60% of the dry weight of the seed has properties similar to sperm whale wax. The ban since 1970 by the United States on importation of sperm whale oil has accelerated interest in agronomic development of the jojoba as a source of high quality lubricating oil.

The synthesis by developing jojoba seeds of fatty acids having chain lengths of 20 and 22 carbons rather than the more usual 16 and 18 carbons and their storage as wax esters to the exclusion of triacylglycerols prompted us to investigate these rather unusual aspects of lipid synthesis.

These investigations were restricted to a period of ca. 1 month (June 1977) at which time developing jojoba seeds were available and suitable for biochemical work. Since wax biosynthesis was essentially completed by the end of June, the seeds were no longer useful after this time for our biosynthetic studies. Further work will be carried out June 1978.

MATERIALS AND METHODS

Developing jojoba seeds of undetermined age (i.e., days after fertilization) were generous gifts from Dr. D.M. Yermanos, University of California, Riverside, and Ms. Kay Klier, Rancho Santa Anna Botanical Gardens, Claremont, California. Most experiments were carried out with seeds harvested on June 17 or June 29, 1977, at which time the wax constituted ca. 10% of the fresh weight of the cotyledons.

[1-^{14}C] Radioactive fatty acids with the following specific activities (Ci/mol) were purchased from Amersham Searle (Arlington Heights, IL): decanoic, 21; lauric, 32; myristic, 45; palmitic, 58; stearic, 58; and oleic, 54. [1-^{14}C] Acetate (58 Ci/mol) and D-[U-^{14}C] glucose (9 Ci/mol) were obtained from New England Nuclear (Boston, MA). [2-^{14}C] malonate (37 Ci/mol) was purchased from ICN (Albany, CA). [1-^{14}C] Oleyl alcohol was prepared by the reduction of methyl oleate (5 μCi, 54 Ci/mol) with excess lithium aluminum hydride in dry diethyl ether.

Tissue Slice Incubation

The shell and seed coat were removed from developing jojoba seeds, and the cotyledonous tissue was cut into cubes of 2-4 mm size. Each incubation mixture contained 0.24-0.35 g of tissue in 0.5 ml of buffer. The tissue was incubated aerobically with gentle shaking at 26 C, and the reaction was terminated by the addition of 0.5 ml isopropanol and by heating for 2-5 min at 80-100 C; The incubation buffer consisted of 0.1 M $NaPO_4$, pH 7.0 containing 50 mM glucose and sucrose. Some incubations were performed at pH 5.0 using morpholinoethanesulfonic acid as the buffer or at pH 7.0 with 20 μmoles of $KHCO_3$. Neither of these alterations influenced the relative proportion of products derived from ^{14}C-acetate although at pH 5.0 the overall incorporation of ^{14}C-acetate into lipid was 30-50% higher than at pH 7.0. Fatty acids were added to the incubation buffer as their potassium salt dissolved in 90% ethanol. Oleyl alcohol was added in 95% ethanol. Incubations with fatty acids or alcohols as the radioactive precursor also included 0.1 mM sodium acetate. The incorporation of the fatty acids into lipids was enhanced by adding unlabeled carrier fatty acid. For example, the percentage

of oleic acid incorporated into lipids was increased from 7% to 25% when the fatty acid concentration was increased from 15 μM to 140 μM.

Extraction and Lipid Analysis

The incubations were stopped by adding 0.5 ml isopropanol and heating for 3-5 min at 80 C. The tissue slices were then crushed with a Teflon pestle in 3-4 ml of chloroform-methanol-acetic acid, 50:50:1 (by vol). The slurry was heated at 80 C for 10-20 min in a capped tube. After cooling, the tube stood for 8 hr and then 3 ml of chloroform and 3 ml of water were added, mixed, and the lower layer drawn off. Further extractions with chloroform-methanol, 2:1 (by vol), and chloroform were carried out until essentially no additional radioactivity was removed.

Portions of the lipid extracts were separated by thin layer chromatography (TLC) on Silica Gel G plates developed first halfway, then fully, in petroleum ether-diethyl ether-acetic acid, 80:20:1 (by vol), the R_f values for wax esters and free alcohols being 0.9 and 0.4, respectively. The ^{14}C material remaining at the origin (referred to subsequently as polar lipid fraction) was recovered by repeated extraction of the silica with chloroform-methanol, 1:1 (by vol), and was rechromatographed by TLC on Silica Gel G plates developed with chloroform-methanol-water, 65:25:4 (by vol), or with chloroform-methanol-7 M aqueous ammonium hydroxide, 90:10:1 (by vol).

For assessment of the incorporation of radioactivity into various lipid classes, the silica gel from the plates was scraped into liquid scintillation vials and counted in 10 ml of a toluene-triton based scintillant which gave 100 ± 5% recovery of radioactivity applied to the TLC plates. For further analysis of individual lipid classes, the material was eluted three times from silica gel with appropriate solvents.

For the assessment of recoveries and quantitation of mass, methyl esters of undecanoic, pentadecanoic, and heptadecanoic acids and oleyl alcohol were added to the lipid extracts at various stages as internal standards.

Ethanolysis of wax ester fractions was carried out by the procedure of Duncan et al. (2). The ethyl esters and alcohols gave quantitative recovery of ^{14}C activity and were analyzed at 220 C on a Varian Aerograph model 920 gas chromatograph equipped with a thermal conductivity detector. The chromatograph was equipped with a 1.8 m x 6.4 mm OD stainless steel column packed with 14% SE 30 on Chromosorb W, AW, 60/80 mesh (Analabs, North Haven, CT). Similar columns packed with 10% SP-1000, 10% SP-2330, 10% EGSS-X, and 10% DEGS on Chromosorb W supports (Supelco, Bellefonte, PA) were also used during this work.

The effluent from the gas chromatograph was monitored for radioactivity with a Nuclear Chicago Biospan No. 4998 gas flow proportional counter (referred to as radio-GLC). Areas of the mass and radioactive peaks were integrated electronically. The response of the thermal conductivity detector to long chain fatty alcohols was observed to be 0.74 times the response to an equal weight of long chain fatty acid ethyl ester.

The fatty acid composition of the polar lipid fractions was similarly analyzed except that transesterification was accomplished with 10% BCl_3 in methanol (Applied Science Laboratories, State College, PA) and GLC analysis was on 10% EGSS-X (Supelco).

The ^{14}C-labeled free alcohols obtained from the total lipid extract were identified on the basis of their chromatographic behavior. They co-chromatographed with stearyl alcohol in petroleum-diethyl ether-acetic acid, 80:20:1 (by vol), and petroleum-diethyl ether, 1:1 (by vol), TLC solvent systems. The ^{14}C-free alcohols recovered from TLC eluted with standard alken-1-ols on a SE 30 GLC column, and when derivatized to trimethylsilyl ethers by co-injection with N,O-bis-(trimethylsilyl)-trifluoroacetamide plus 1% trimethylchlorosilane (Pierce Chem. Co., Rockford, IL), they eluted with the standard compounds on SE 30 and DEGS columns.

In order to minimize losses during concentration of the volatile short chain fatty acid esters, no heat was applied during evaporation of the solvents under N_2. Nevertheless, recovery of the methyl 11:0 internal standard relative to oleoyl alcohol revealed that ca. 25-50% of this short chain standard was lost, and, therefore, the incorporation of the short chain radioactive fatty acids from the incubations may also be expected to be underestimated.

Degradation of ^{14}C Fatty Acids and Alcohols

The individual fatty esters and alcohols from ethanolysis of the wax esters were isolated by preparative GLC (SE 30) for degradative studies.

Reductive ozonolysis of the ethyl esters and alcohols was carried out on a microscale in hexane according to the method of Stein and Nicolaides (3). The ratio of radioactivity of the aldehyde to aldehyde-ethyl ester or aldehyde-alcohol fragments was determined by radio-

GLC (SP-1000, 180 C).

For α-oxidation, the ethyl esters were first saponified, and the individual monoenoic acids and alcohols were reduced to the corresponding saturated compounds by catalytic hydrogenation. An aliquot was esterified with ethereal diazomethane and checked for complete reduction by GLC (DEGS or SP-2330). Chemical α-oxidation of the saturated acids and alcohols was accomplished essentially by the method of Harris et al. (4). The procedure worked as satisfactorily for long chain alcohols as it did for acids. After extraction, the mixture of shorter chain length saturated acids was esterified with diazomethane and analyzed by radio-GLC (SP-1000, 180 C).

An estimation of the percent ^{14}C in the carboxyl carbon of some of the saturated acids derived from the monoenoic ethyl esters was obtained by the Schmidt decarboxylation. The method of Aronsson and Güther was used (5).

RESULTS

Incorporation of ^{14}C-Substrates

The time course of ^{14}C-acetate incorporation into lipids by tissue slices of developing jojoba seeds is shown in Figure 1. In these experiments, a maximum of ca. 35% of the ^{14}C-acetate added to the incubation was incorporated into lipids. After extraction of lipids, most of the remaining radioactivity from the incubation was soluble in the aqueous phase and was volatile under acidic conditions, suggesting it was unincorporated ^{14}C-acetate.

The ^{14}C-acetate which was incorporated into lipid was found in three components of the lipid fraction as shown in Figure 1. Increasing the concentration of ^{14}C-acetate to 10 mM or reducing the pH of the incubation to 5 did not alter the distribution of products as summarized in Figure 1. Slices of the seed coat or leaf tissue did not incorporate ^{14}C-acetate into wax (nor could we detect simple wax esters in these tissues), thus wax is synthesized primarily in the cotyledon rather than being transported to the cotyledon from the leaves or seed coats.

TLC of the extract from the immature jojoba cotyledons used for incubations indicated the wax esters as the major mass component (>90%), with less than 1% of free acids or alcohols. As shown in Figure 1,[1-^{14}C]acetate was incorporated primarily into three fractions. Although wax esters represented the major mass, the most rapidly labeled product was a polar material, ^{14}C-simmondsyl acetate. It had an R_f value of 0.60 in chloroform-methanol-water (90:10:0.5) TLC solvent system, and on

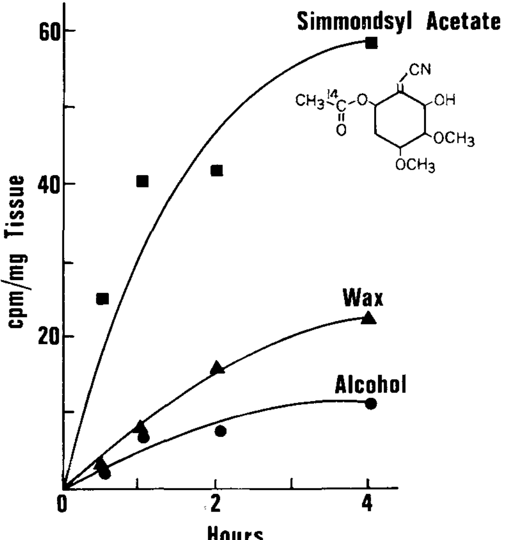

FIG. 1. Incorporation of ^{14}C-acetate into lipids by slices of developing jojoba seed cotyledons as a function of time. Simmondsyl acetate, wax, and alcohol constituted greater than 95% of the ^{14}C-acetate incorporated into the lipid extract. Details of the experimental procedures are described in the Materials and Methods section.

a 1.5% SE-30 (150 C) GC column, it had an equivalent chain length of 15.6. Simmondsyl acetate is related to the structure of simmondsin (6) (which constitutes ca. 2% of the dry weight of mature jojoba seeds) by replacement of glucose with acetate. Its isolution and characterization have been completed, and the results will be submitted to *Phytochemistry* for publication (M.R. Pollard, J.B. Ohlrogge, and P.K. Stumpf, unpublished data). Simmondsyl acetate is apparently formed as a result of our incubation conditions, since we could not detect this compound in cotyledons which had been extracted without incubation. That simmondsyl acetate probably does not serve as an acetyl donor in the elongation of oleic acid is suggested by the absence of the usual precursor-product curve in Figure 1. No incorporation of exogenous [1-^{14}C]acetate into acyl components of phospholipids was detected. Another unusual feature observed in Figure 1 is the occurrence of free ^{14}C-alcohols and the total absence of free ^{14}C-fatty acids. This would suggest that the acyl thioesters are rapidly reduced to alcohols which then serve as substrates for their esterification with acyl thioesters to form waxes. Equally striking is the complete absence of triacylglycerols, even as transitory intermediates.

A typical gas chromatographic analysis of

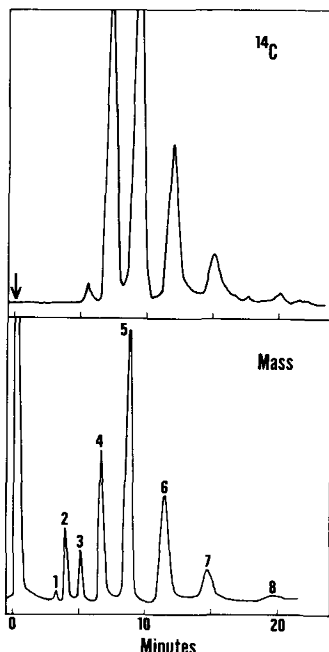

FIG. 2. Gas liquid radiochromatographic pattern (on 14% SE-30) of [^{14}C] fatty acids (ethyl esters) and of ^{14}C-alcohols formed by incorporation of ^{14}C-acetate into slices of developing jojoba cotyledons. Peaks 1 to 8 in the lower tracing of mass represent ethyl palmitate, oleoyl alcohol (added as an internal standard), ethyl oleate, eicosenol, ethyl eicosenoate, docosenol, ethyl docosenoate, and tetracosenol, respectively.

the ethanolysis products of the wax ester fraction from seeds incubated with [1-^{14}C] acetate is shown in Figure 2. The fatty acid and alcohol composition seen in Figure 2 is similar to that observed by Yermanos (1) and Miwa (7) for developing and mature jojoba seeds. Although routine GLC analysis was done on a SE 30 column, which will separate fatty ethyl esters and alcohols according to chain length but not degree of unsaturation, some representative samples were also analyzed on high polarity columns (DEGS, SP-2330). These GLC traces showed an absence of C_{18-24} saturated and polyenoic entities, both in terms of mass and ^{14}C activity. Thus, wax biosynthesis in developing jojoba seed is committed to the formation of C_{20} and C_{22} monoenoic acids and the corresponding alcohols with the exclusion of di- and triunsaturated fatty acid formation. As previously observed by Spencer et al. (8), reductive ozonolysis, discussed later, showed that the monoenoic double bond position is always $\omega 9$. Analysis of the composition of the polar lipids of the developing cotyledon revealed more than 90% of the acyl chains as 16:0, 18:1, or 18:2, with less than 10% contributed by 20:1 and 22:1. Thus, either by compartmentation or enzyme specificity, the C_{20} and C_{22} acids are largely excluded from esterification into cotyledon polar lipids. The fatty acid profile of the total lipid extract of jojoba leaf tissue appeared typical for higher plant leaves: i.e., 25% 16:0, 18% 18:1, 7% 18:2, and 46% 18:3. with less than 1% of C_{20} and C_{22} acids. These results are similar to those observed for castor bean, rapeseed, and safflower seeds where acyl chains uniquely characteristic of the stored lipid are generally not found in the polar lipids or leaf lipids (9).

Table I summarizes a detailed study on the incorporation of a number of ^{14}C substrates into ^{14}C waxes. Both [1-^{14}C] acetate and [2-^{14}C] malonate were incorporated essentially into the same products. [U-^{14}C] glucose was incorporated, in addition, into palmitic and oleic acids. ^{14}C-label supplied by ^{14}C-decanoic and ^{14}C-lauric acids (Table I) was recovered in the wax fraction as longer chain fatty acids. In contrast, ^{14}C-myristic, ^{14}C-palmitic, and ^{14}C-stearic acids showed little if any elongation and no detectable desaturation. We suggest that these differences derive from the capacity of the tissue to funnel shorter chain precursors onto the "ACP track" (10) which makes possible the specific desaturation of stearoyl-ACP to oleoyl-ACP, whereas exogenous longer chain fatty acids (C_{14}, C_{16}, C_{18}) may only be activated to CoA thioesters. Interestingly, [1-^{14}C] oleyl alcohol was not oxidized to the corresponding acid nor was it significantly incorporated into wax. It is conceivable, however, that this substrate was poorly transported into the cotyledonous tissue.

Though only 4% of the added [1-^{14}C] oleic acid was incorporated into wax, a significant amount was readily elongated to eicosenoic acid and also reduced to eicosenol. The observation that exogenous free ^{14}C-oleate was elongated to 20:1 and 22:1 would support the suggestion that the elongation reactions employed CoA thioesters rather than ACP thioesters since there is no evidence that an oleate:ACP ligase occurs in plants and the evidence for an oleoyl-CoA:ACP transacylase is fragmentory. Studies on other plant systems that produce erucic acid are underway in this laboratory in order to test this proposed substrate specificity for elongation.

In experiments with less mature developing seeds incubated with ^{14}C-acetate or ^{14}C-malonate, we consistently observed that the amount of radioactivity in the fatty acid portion of the wax ester was greater than that found in the alcohol moiety. This difference

was observed both by TLC and GLC analysis of the ethanolysis products. As the time of incubation increased, the ratio of ^{14}C-acid to ^{14}C-alcohol in the wax diminished (Table II). However, if the total sum of ^{14}C-alcohols (free, and derived from ethanolysis of the wax) is compared with that for the ^{14}C acids found in the wax (negligible amounts of ^{14}C acids are found free or esterified to other lipids), a ratio of approximate unity, fairly constant with time, was observed. Specific activity measurements on the free alcohols (mainly eicosenol and docosenol) gave the amount of free alcohol per gram of tissue as ca. 0.75 mg. Of this quantity, it is not known how much represents alcohol available for wax ester biosynthesis or how much may be present in the bulk of the wax and, therefore, inaccessible. The data shown in Figure 1 and Table II are consistent with a metabolic free alcohol pool. Thus, the ^{14}C-alcohol synthesized from [1-^{14}C] acetate, presumably via the corresponding acid, is diluted by mixing with unlabeled alcohol, and, therefore, wax biosynthesis occurs with acid and alcohol moieties having different specific activities. With increased incubation time, the specific activity of the free alcohol pool increases, so that the proportion of ^{14}C acid and ^{14}C alcohol in the wax became more equal.

A contrast was observed between the patterns of incorporation of ^{14}C-acetate (or ^{14}C-malonate) and ^{14}C-glucose. [1-^{14}C]-acetate, as shown in Figure 1, was incorporated primarily into simmondsyl acetate (~60%) with minimal (<5%) incorporation into acyl polar lipids. However, there was essentially no label incorporated from [U-^{14}C] glucose into simmondsyl acetate or simmondsin. The major (40%) ^{14}C-component from the glucose incubations was an acyl polar lipid with an R_f value of 0.44 (phosphatidyl choline and ethanolamine had R_f values of 0.36 and 0.57, respectively) on Silica Gel G TLC in chloroform-methanol-water, 65:25:4 (by vol). This polar lipid was not identified but had the following unusual distribution of ^{14}C-labeled acyl groups: 9% 16:0, 34% 18:1, 17% 20:1, and 40% 22:1.

Degradation Studies

Degradation studies of ^{14}C-20:1 and ^{14}C-22:1 acids and their corresponding alcohols from the wax fraction formed from [1-^{14}C] acetate revealed that the ^{14}C-label occurred principally in the carboxyl carbon of ^{14}C-20:1 and in the carboxyl and the C(3) carbon of ^{14}C-22:1 (Table III). The conclusion consistent with these degradation data would be that either ^{14}C-acetyl-CoA or ^{14}C-malonyl-

TABLE I

Precursor Incorporation into Wax

			% Incorporated		% Radioactivity in wax									
					Acids							Alcohols		
Precursor	[mM]	Specific activity μCi/μmole	All lipids	Wax	10:0	12:0	14:0	16:0	18:1	20:1	22:1	18:1	20:1	22:1
Acetate[a]	0.1	58	35	14	---	---	---	---	2	45	10	---	24	19
Malonate[b]	0.1	37	11	3	---	---	---	---	---	45	8	---	27	20
Glucose[c]	7.2	9	10	4	---	---	---	3	15	37	5	---	28	12
10:0[a,d]	0.14	16	75	27	43	16	10	3	11	4	---	---	4	9
12:0[a,d]	0.14	25	80	22	---	61	28	2	2	4	---	---	3	---
14:0[a,d]	0.14	21	65	30	---	---	94	6	---	---	---	---	---	---
16:0[a,d]	0.14	30	45	15	---	---	---	100	---	---	---	---	---	---
18:0[a,d]	0.14	30	< 3	< 1	---	---	---	---	---	---	---	---	---	---
18:1[a,d]	0.14	32	25	4	---	---	---	---	43	30	11	5	18	3
18:1-OH[a]	0.14	32	5	< 1	---	---	---	---	---	---	---	100	---	---

[a][1-^{14}C] substrate.
[b][2-^{14}C] substrate.
[c][U-^{14}C] substrate.
[d]All these incubations also included 0.1 mM sodium acetate.

TABLE II

Incorporation of [^{14}C] Acetate into Wax Components in Developing Jojoba Cotyledons as a Function of Time[a]

Incubation time	Ratio: ^{14}C-acid/^{14}C-alcohol
30 min	4.9
1 hr	2.8
2 hr	2.0
Pulse chase[b]-7 hr	1.3

[a]Details of the experimental procedure are described in the Materials and Methods section.
[b]Cotyledon slices were incubated for 30 min with 0.1 mM [1-^{14}C]acetate, and then 10 mM nonradioactive acetate was added and the incubation continued until 7 hr.

CoA is added onto a pre-existing cold endogenous oleoyl-CoA pool for elongation to the C_{20} and C_{22} products. Thus, the unusual aspect of these results is that exogenously added ^{14}C-acetate or ^{14}C-malonate appeared to be largely excluded from de novo synthesis of the 18:1 precursor. In sharp contrast, when [U-^{14}C]glucose was incubated with tissue slices, the ^{14}C-label was now found uniformly distributed in the hydrocarbon chains of the C_{20} and C_{22} products (Table III). Thus, the acetyl and/or malonyl thioesters derived from glucose were being utilized for de novo synthesis as well as elongation of the newly formed oleoyl thioester. We interpret this difference in radiolabeling as evidence that the de novo synthesis and the elongation of acyl chains in the jojoba cell utilize separate pools of acetate. These results imply that de novo synthesis and elongation may take place in separate sites of the cell.

Lipid Synthesis by Germinating Cotyledons

It was of interest to examine the capacity of *germinated* cotyledon tissue to synthesize ^{14}C-fatty acids from ^{14}C-substrates. Slices of germinated cotyledons (6 days after imbibition) were incubated with [1-^{14}C]acetate, and 34% of the radioactivity was recovered in the ex-

TABLE III

Degradation Studies on the ^{14}C-Labeled Fatty Acids and Alcohols from the Wax Esters of Developing Jojoba Cotyledons

A: 0.1 mM [1-^{14}C]acetate as substrate	Distribution of ^{14}C-activity							
	Ozonolysis[a] (%)		α-Oxidation[b]					
Compound degraded	C9A	AE/AA	C_n	C_{n-1}	C_{n-2}	C_{n-3}	C_{n-4}	
18:1 acid	33%	66% (C_9)	1	0.8	0.9	0.7	0.9	
20:1 acid	5%	95% (C_{11})	1	0	0	0	0	
22:1 acid	<5%	>95% (C_{13})	1	0.4	0.6	0	0	
20:1 alcohol	<5%	>95% (C_{11})	1	0	0	0	0	
22:1 alcohol	<5%	>95% (C_{13})	1	0.7	0.6	0.1	0	
B: 10 mM [1-^{14}C]acetate as substrate								
	Ozonolysis[a]		Schmidt decarboxylation[c]					
20:1 acid	15%	85% (C_{11})	91% $^{14}CO_2$					
22:1 acid	10%	90% (C_{13})	---					
C: 0.1 mM [2-^{14}C]malonate as substrate								
	Ozonolysis[a]		Schmidt decarboxylation[c]					
18:1 acid	37%	63% (C_9)	---					
20:1 acid	13%	87% (C_{11})	---					
22:1 acid	5%	95% (C_{13})	---					
D: [U-^{14}C] D-β-glucose as substrate								
	Ozonolysis[a]		Schmidt decarboxylation[c]					
18:1 acid	52%	48% (C_9)	---					
20:1 acid	46%	54% (C_{11})	8% $^{14}CO_2$					
22:1 acid	45%	55% (C_{13})	---					

[a]Distribution of radioactivity between aldehyde (A), and aldehyde-ester (AE) or aldehyde-alcohol (AA) fragments, of specified carbon chain length.
[b]Approximate specific activity, in counts/unit response of mass detector, of the shorter chain length acids from α-oxidation, relative to the specific activity of the parent acid.
[c]% of $^{14}CO_2$ trapped by Schmidt decarboxylation relative to ^{14}C activity of reactant acid, i.e., % ^{14}C in carboxyl carbon.

tracted lipids. Although the major (>90%) mass component was wax esters, on ethanolysis of the total extract only 6% of the incorporated label was present in fatty acyl groups and none was present in fatty alcohols. ^{14}C-palmitate and ^{14}C-oleate were the principal labeled acids, with ^{14}C-C_{20} and C_{22} acids absent. Simmondsyl acetate represented 89% of the radioactivity in the total lipid extract. In a similar experiment with [U-^{14}C]glucose and germinated tissue, there was less than a 0.2% incorporation of radioactivity into fatty acyl groups. Thus, the elongation of oleate to eicosenoate and docosenoate was found only in the developing cotyledon, concurrently with wax ester biosynthesis and alcohol formation.

DISCUSSION

We would suggest that in the biosynthesis of waxes, the developing jojoba seed employs primarily glucose, presumably derived from photosynthates, as the source of C_2 substrates. In the cotyledon, there may be at least two separate compartments involved in wax biosynthesis. Glucose, or its metabolites, can enter either compartment I where it is degraded to acetyl-CoA or to compartment II where it is also degraded to acetyl-CoA. It is, however, in compartment II where a de novo as well as a palmitoyl-ACP elongation and stearoyl-ACP desaturase pathway are localized. In this regard, a preliminary investigation has revealed the presence of an active stearoyl-ACP desaturase in homogenates of developing jojoba cotyledons (T.A. McKeon, and P.K. Stumpf, unpublished data). Thus, we suggest that the entire synthetic sequence from C_2 to 18:1 occurs on an ACP track as proposed by previous investigations (10). We further speculate that free oleic acid is now transported to compartment I where a specific system is present for the elongation of oleoyl-CoA to eicosenoyl-CoA and finally to erucoyl-CoA. We expect that these CoA thioesters would now be reduced to their corresponding alcohols which, in turn, serve as substrates with the long chain acyl-CoAs for wax formation as demonstrated by Kolattukudy in wax ester biosynthesis of broccoli (11).

When acetate is added to a tissue slice, it can readily enter compartment I where it is rapidly converted to acetyl-CoA. As such, it may remain in compartment I since the membranes enclosing compartment II presumably are impermeable to acyl thioesters (12). [1-^{14}C]-acetate was extensively incorporated into ^{14}C-simmondsyl acetate, whereas this compound was not detected (<5%) in incubations with [U-^{14}C]glucose. According to our compartmentation concept, this competing reaction for acetate or acetyl-CoA utilization may be localized in compartment I. In the incubation conditions employed, it could be another effective control preventing exogenous acetate from entering compartment II, though the in vivo significance of this reaction, if any, is not clear.

There is already precedence for the compartmentation of fatty acid synthetase enzymes and their ancillary enzymes in plant tissues, i.e., in chloroplasts (13,14), in castor bean cotyledon proplastid (15-17), and in avocado mesocarp proplastid (18). Furthermore, recent evidence suggests that there may be different pools of acetyl-CoA as well as different origins of acetyl-CoA in plant tissues (19). This two compartment hypothesis fully explains our experimental data presented in Table III. A direct test of this hypothesis will be carried out in June 1978 when developing jojoba seeds will again become available.

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Protective Effect of Biliary Lipids on Rat Pancreatic Lipase and Colipase

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ABSTRACT

The effect of biliary components on the inactivation of rat pancreatic lipase and colipase was studied. In vitro incubations of these proteins were performed at 37 C in the presence or absence of trypsin, under various conditions. The influences of bile lipoprotein complex, bile salts below or above the critical micellar concentration (CMC), or albumin were investigated. The results showed that albumin and bile salts below the CMC had no protective effect on the inactivation rate of lipase or colipase. Under both denaturating conditions, bile salts above the CMC had a very slight effect, whereas the presence of the bile lipoprotein complex maintained lipase and colipase activity. The magnitude of this effect was related to the biliary phospholipid concentration. By means of gel filtration, the protective effect of bile was found to be due to associations of bile lipoprotein complex with these proteins in presence of bile salts. A correlation between the amount of colipase and the protection of lipase in the presence of biliary phospholipids was observed. Intestinal content of rats with normal and diverted bile secretion was submitted to the same in vitro incubation, and the enzyme was more stable in the segments containing biliary phospholipids. This suggests that the interaction between the bile lipoprotein complex, colipase and lipase in the presence of bile salts could have an important role in the intestinal lumen by retaining the enzyme activity.

INTRODUCTION

Several authors (1-3) have shown that pancreatic lipase can be inactivated at interfaces and that colipase or bile salts can prevent this denaturation. In vivo, the lipid-water interface could include, in addition to glycerides and bile salts, other biliary lipids, i.e., phospholipids, as observed during lipolysis in the intestine of rats (4). Lipase can associate with the bile lipoprotein complex, mainly composed of phospholipids, in the presence of colipase and bile salts (5). This led to the study of the lipase and colipase inactivation under thermal and trypsin treatment in the presence or absence of biliary lipids.

MATERIALS AND METHODS

Rat bile and rat pancreatic juice were collected separately as previously described (4). Bile was stored at 4 C, and pancreatic juice was frozen until use. Phospholipids were assayed as follows: phospholipids were separated by thin layer chromatography (TLC) and then each class was measured (6). 3α-OH bile salts were analyzed using an enzymatic automatic method (7).

Rat pancreatic lipase and colipase were separated by gel filtration of pancreatic juice on Sephadex G-100 (5). The colipase preparation (specific activity: 530 units/mg protein) was free of lipase activity and lipase (specific activity: 700 units/mg protein) exhibited a 10% direct activity in the absence of excess added cofactor. The presence of contaminant amounts of trypsin and chymotrypsin was examined in the lipase and colipase preparations. No significant activity could be detected on the specific substrates of these proteolytic enzymes [N-benzoyl L-arginin ethyl ester and N-acetyl L-tyrosine ethyl ester, respectively (8)].

Lipase activity was determined potentiometrically at 25 C at pH 9.0 according to the procedure of Desnuelle et al. (9) in the presence of 2.5 mM taurocholate and 2.5 mM deoxycholate. Excess amounts of colipase (prepared from bovine pancreas by L. Sarda) were added to the assay system to determine the total amount of lipase activity. The activating effect of the cofactor toward colipase-free lipase was used to estimate the colipase activity under the same conditions (10).

In vitro inactivation of pancreatic lipase and colipase was studied at 37 C in the presence or absence of trypsin. Lipase and/or colipase were used in diluted solutions, i.e., 80 units/10 ml final concentration in 100 mM Tris, 20 mM $CaCl_2$ buffer at pH 8.0.

Several experiments were performed by adding to the incubation media bovine serum albumin (Merck Co., Darmstadt, Germany) at 0.04 mg/ml final concentration, Na taurocholate (Calbiochem, La Jolla, CA, A grade) at

5 mM final concentration or bile aliquots containing a known amount of phospholipid, diluted in 5 mM Na taurocholate. For details, see tables and figure legends. Trypsin (Worthington Biochemical Co., Freehold, NJ, lyophilized trypsin, 182 units/mg) was added to the incubation media at 0.1 mg/ml final concentration. All incubation mixtures (10 ml) were prepared in ice and allowed to stand for 15 min. The mixtures were then incubated at 37 C under gentle stirring for various time periods up to 90 min.

By another method, two mixtures were prepared as follows: (a) 300 lipase units were added to 1 ml of rat bile containing 3 mg of phospholipids, and (b) 455 colipase units added to 1 ml of rat bile. After stirring for 1 hr at 4 C, the mixtures were separated by gel filtration on Bio-gel A5M agarose columns (80 x 1.5 cm) equilibrated and eluted with 0.1 M NaCl containing 6 mM Na taurocholate. This was carried out at 4 C and at a constant flow rate of 10 ml/hr. As previously reported (5), lipase was eluted in two peaks: one together with the biliary phospholipids (2.0 void volumes, 23% of the eluted lipase activity) and the other with the elution volume obtained for gel filtration of lipase alone (2.30 void volumes). Colipase was eluted in part with the biliary phospholipids (2.0 void volumes, 78% of the eluted colipase activity), and the remainder emerged with 2.5 void volumes as that obtained for colipase alone. These four kinds of fractions were submitted to the inactivation procedure as described above.

In vivo experiments were performed on three rats with a normal bile secretion and three other ones with a total bile diversion. In all cases, pancreatic secretion was maintained. The study started 24 hr after the surgical intervention. The six rats received saline glucose solution (4 ml, 0.5% NaCl-5% glucose) by stomach intubation. Three intraperitoneal injections of pancreozymin-secretin (5 units/kg - 8 units/kg) were done 15 min, 30 min, and 45 min, respectively, after the intubation. The six rats were sacrificed 30 min after the last injection. The contents of three equal intestinal segments were diluted in the incubation buffer and used for studying the lipase inactivation.

RESULTS

A physiological ratio of pancreatic lipase and biliary phospholipids was maintained similar to that found in rat intestinal content (4), i.e., 175 lipase units/mg phospholipid. Biliary phospholipids were not purified, since it is known (11) that these are involved in the natural lipoprotein complex which also includes a polypeptide component (4% of phospholipid weight) and cholesterol (8% of phospholipid weight). The phospholipid composition of rat bile was established (12) as follows: phosphatidyl choline, 90%; lysophosphatidyl choline, 2.7%; spingomyelin, 5.4%; and cephalin, 1.8%.

Inactivation of Lipase and Colipase at 37 C

The effect of incubation at 37 C on dilute solution of pancreatic lipase is shown on Table I. Lipase activity rapidly decreased to 3% of its initial value after 90 min. The same results were obtained in the presence of albumin. No difference was observed in a dilute solution of Na taurocholate (0.23 mM) below its critical micellar concentration [CMC: 4.5 mM (13)]. However, this bile salt showed a small protective effect on lipase when present at a concentration above its CMC, i.e., 5 mM. The lipase behavior was clearly different in the presence of rat bile for after 90 min at 37 C it retained 63% of its initial activity.

All colipase activity was preserved after 90 min at 37 C under the several sets of conditions used. This was in good agreement with the known heat stability of the pancreatic cofactor (14).

Inactivation of Lipase and Colipase by Trypsin at 37 C

The presence of large quantities of trypsin in pancreatic juice secretion and, of course, in intestinal lumen during digestion prompted us to study the effect of the proteolytic enzyme at 37 C (see Table I). The enzyme lost its activity after 60 min. Na taurocholate, in dilute (0.23 mM) or concentrated (5 mM) solution, had no effect on the enzyme inactivation rate. Results were unchanged by the presence of albumin but were very different with solutions containing rat bile. After 90 min, lipase kept 41% of its initial activity.

Table I shows that colipase was rapidly inactivated by trypsin with or without Na taurocholate above its CMC. Bile exerted a stronger positive effect on colipase than on lipase. The cofactor retained 90% of its initial activity after 90 min incubation. One might postulate the existence of an inhibitor of trypsin activity in the normal bile secretion. Under standard conditions, the activity of trypsin on its specific substrate N-benzoyl L-arginin ethyl ester was unchanged in wide ranges of bile concentrations. Therefore, this

hypothesis can be ruled out.

Effect of Bile Lipoprotein Complex on the Inactivation of Lipase and Colipase

The above data suggest that some biliary components other than bile salts or albumin are responsible for the protective effect of bile on lipase and colipase inactivation. The influence of concentration of biliary phospholipids (lipoprotein complex) in the presence of 5 mM Na taurocholate on the lipase and colipase stabilities are presented in Figure 1. Increasing the concentration of biliary phospholipids in the media markedly increased the stability of the enzyme and its cofactor, in the presence or absence of trypsin. Colipase was protected to a larger extent (88%) than was lipase (50%) under trypsin action in presence of 0.10 mg/ml biliary phospholipids (0.13 mM). A maximum protective effect of lipase and colipase activities was obtained with the same biliary phospholipid concentration (0.05 mg/ml: 0.065 mM).

In an earlier study, mixing rat bile with colipase or lipase led to association between the bile lipoprotein complex and these proteins (5). In the present study, such experiments were performed (see Materials and Methods) to explain the mode by which the bile lipoprotein complex can protect lipase and colipase against inactivation. Inactivation data obtained for lipase and colipase present in eluted fractions after gel filtration of mixture with bile are summarized in Table II. Associations with the bile lipoprotein complex greatly protected lipase against inactivation and completely maintained colipase activity. In the absence of association with the bile lipoprotein complex, lipase or colipase in Na taurocholate solution lost almost all its activity under conditions causing inactivation.

Effect of Colipase on the Inactivation of Lipase

Lipase inactivation by trypsin at 37 C was studied in the presence of increasing amounts of colipase under various conditions (Fig. 2). Added colipase had no positive effect in the presence or absence of Na taurocholate below its CMC (0.23 mM). Above the CMC, a small protective effect could be observed, but this was independent of added colipase. Clearly different results were obtained only in the presence of the bile lipoprotein complex. A relationship between the amount of colipase and the protection of lipase activity was obtained. Under the most effective con-

TABLE I

Inactivation of Lipase and Colipase at 37 C in Absence or Presence of Trypsin[a]

Incubation media[c]	37 C			37 C Trypsin[b]			37 C Trypsin[b]		
	% Remaining lipase activity						% Remaining colipase activity		
	30 min	60 min	90 min	30 min	60 min	90 min	30 min	60 min	90 min
Buffer solution	41	20	3	19	2	0	50	27	16
Na taurocholate 5mM	45	28	14	24	3	0	55	34	18
Rat bile solution[d]	69	63	63	67	58	41	96	92	90

[a]Incubation conditions were those as described in Materials and Methods. Lipase and colipase were assayed after 30 min, 60 min, and 90 min incubation time periods.
[b]Trypsin concentration: 0.1 mg/ml.
[c]All solutions were prepared in 100 mM Tris, 20 mM Cal$_2$, pH 8.0 buffer.
[d]Concentration of rat bile lipids in the medium; phospholipids: 0.05 mM; bile salts: 5mM.

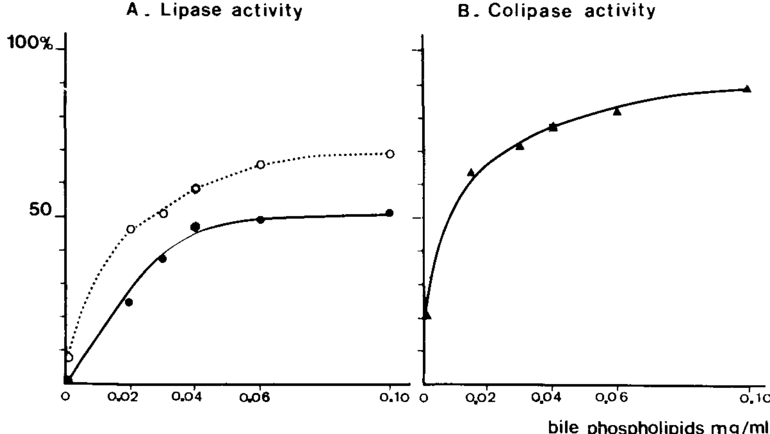

FIG. 1. Inactivation of lipase and colipase in presence of increasing amounts of biliary phospholipids. All biliary phospholipid dilutions were made in 5 mM taurocholate. Incubation conditions are those described in Materials and Methods. Activities are expressed in percentage of remaining activities after 90 min incubation time. ○---○, lipase inactivation at 37 C; ●——●, lipase inactivation with trypsin (0.1 mg/ml) at 37 C; ▲——▲, colipase inactivation with trypsin (0.1 mg/ml) at 37 C.

TABLE II

Inactivation of Pancreatic Lipase and Colipase Separately Eluted with or without Biliary Phospholipids (PL) in Na Taurocholate Buffer[a]

	% Remaining activities after 90 min inactivation	
	37 C	37 C Trypsin[b]
Lipase + biliary PL[c]	90%	81%
Lipase in buffer[d]	7%	0%
Colipase + biliary PL[c]	100%	97%
Colipase in buffer[d]	99%	13%

[a]Gel filtration procedure and incubation conditions are those as described in Materials and Methods.
[b]Trypsin concentration: 0.1 mg/ml.
[c]Biliary phospholipids concentration (PL): 0.13 mM. Taurocholate concentration: 5 mM.
[d]Taurocholate concentration: 5 mM.

ditions, in presence of 0.04 mg/ml biliary phospholipids (0.05 mM) and a colipase/lipase molar ratio above the unity, 63% of the enzyme activity was maintained.

Inactivation of Lipase Present in Intestinal Contents

The physiological implication of these in vitro experiments was investigated by a study of the lipase stability in the intestinal content of normal and bile-diverted rats (Table III). In bile-diverted rats, biliary phospholipids of the lipoprotein complex could not be quantitatively determined in the intestinal contents as previously reported (4). In normal rats, the amount of biliary phospholipids decreased from the upper segment to the lower one. The final concentrations obtained after dilution in the incubation media were very close to those used before in vitro (see

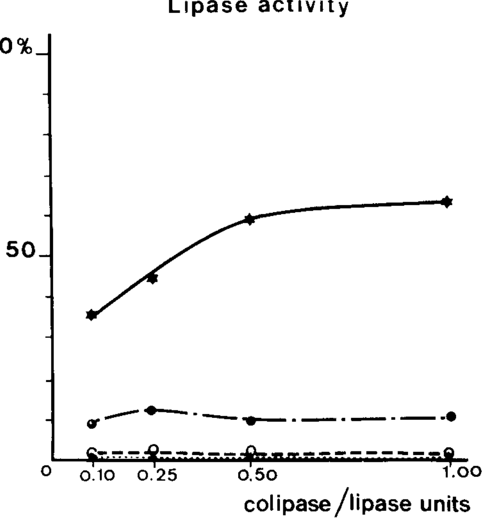

FIG. 2. Effect of increasing amounts of colipase on the inactivation of lipase by trypsin at 37 C. Incubation conditions are those described in Materials and Methods. Activities are expressed in percentage of remaining activities after 90 min incubation time. Trypsin concentration: 0.1 mg/ml. ---------, buffer; ★——★, buffer containing rat bile (phospholipids concentration: 0.05 mM; bile salts concentration: 5mM; ○ — — ○, buffer containing 0.23 mM Na taurocholate; ● - - - ●, buffer containing 5 mM Na taurocholate.

TABLE III

Inactivation of Lipase Present in the Intestinal Contents of Normal and Bile Diverted Rats[a]

Intestinal contents	Normal rats		Bile diverted rats	
	PL mg/ml	% Remaining lipase activity	PL mg/ml	% Remaining lipase activity
Upper part	0.050	56%	trace	19%
Middle part	0.012	21%	---	11%
Lower part	trace	6%	---	0

[a]Diluted intestinal contents were submited to 90 min incubation time in presence of trypsin (0.1 mg/ml) at 37 C. Incubation media contained 70 lipase units/10 ml and the indicated biliary phospholipids (PL) concentrations. Na taurocholate concentration: 5 mM.

Table III). In all cases, direct lipase activity was 75%-90% of that measured in presence of excess added cofactor. In case of bile-diverted rats, pancreatic lipase lost almost all its activity in the upper and middle segment and all its activity in the lower segment under conditions of inactivation. In normal rats, the enzyme was more stable. From the upper segment to the lower segment, enzyme stability decreased and this may be related to a decreasing bile lipoprotein complex concentration.

DISCUSSION

Rat pancreatic lipase was found to be a rather unstable enzyme which rapidly loses its activity at 37 C in dilute solution. This behavior is very similar to that reported by Kimura et al. (15) concerning human pancreatic lipase. According to Brockerhoff (1), it seems unlikely that the magnitude of this inactivation process could be the result of an additional denaturation at the air-water interface. While the latter author showed a protective effect of bile salts on the interfacial inactivation of lipase, taurocholate below or above the CMC does not enhance the enzyme stability in solution. Trypsin, normally present in pancreatic juice, causes a drastic decrease in rat lipase activity in addition to the effect of thermal inactivation as previously observed by Vahouny et al. (16) for lipase activity of total rat pancreatic juice. Rat pancreatic colipase activity is also greatly affected by the action of trypsin as well as pancreatic lipase cofactor from human source (17).

Therefore, under these conditions, pancreatic lipase and colipase have a low stability which does not agree with the important physiological role of these proteins. Albumnin is the main protein provided by bile (18) and is known to have some stabilizing properties. In the present studies, no protective effect was observed.

Bile salts are the most abundant lipid class in bile (19) and play several roles in the lipolysis process (20). Moreover, taurocholate used in this work is recognized as the main species in rat biliary secretion (21). Although bile salts are known to bind to many proteins (22) and specifically lipase (23) or colipase (24), taurocholate exerts no significant effect on the stability of the proteins tested, in agreement with others (16). The well known ternary association of lipase, colipase, and bile salt micelles in solution (25) exerts no significant protection.

Bile lipoprotein complex has been found to be present in the rat intestinal lumen during digestion, and the main part of the phospholipids of this complex is not hydrolyzed (4). From the results presented here, it is apparent that this biliary complex greatly enhances the pancreatic enzyme and cofactor stabilities. This protective effect was observed under both thermal inactivation and trypsin treatment. Maximum protection is provided for the two proteins by the same concentration range of biliary phospholipids and with relative amounts of biliary phospholipids and lipase as that found in the rat intestinal content (4).

The stabilities of lipase and colipase in the presence of bile and under the action of trypsin at 37 C were compared. Colipase kept almost all its activity after 90 min (90%) while less than half of the lipase activity (41%) was maintained. After gel filtration of a mixture of colipase and a bile sample, 78% of colipase was found associated with the phospholipid-containing fractions and maintained full activity. This explains why almost all colipase activity remained after incubation of the cofactor in presence of a bile solution (Table I). In contrast, only 23% of the lipase mixed with a bile sample emerged from the gel column with biliary

phospholipids and kept more than 75% of its activity. The main part of lipase eluted without biliary phospholipids and lost all its activity (Table II). Van Dam Mieras et al. (26) showed the same phenomenon concerning pancreatic phospholipase A_2. When adsorbed on amphipatic lipids, this enzyme was greatly protected against trypsin hydrolysis and was inactivated in solution without lipids. Because only a small portion of lipase is bound to the bile lipoprotein complex and therefore stabilized, lipase is only protected to a limited extent in the presence of a bile solution (Table I).

If colipase is added to lipase in a mixture with bile, the main part of the enzyme is associated with biliary phospholipids after gel filtration (5). Thus, if almost all lipase may associate with lipoprotein complex only in the presence of colipase and if these protein: lipid associations are responsible for a protective effect, increasing the concentration of colipase in the presence of a bile solution should enhance both associations and thus lipase stability. This has been verified, but protection of the lipase activity is not total. This likely corresponds to a limited protective effect of bile lipoprotein complex on lipase activity, because in vivo lipase protection was also found to be only partial, as also reported by Vandermeers-Piret et al. (27). This phenomenon seems evident and is related to the biliary phospholipid concentration from the upper to the lower intestinal segment (28), thus giving an explanation of the previous findings of Khayat et al. (29).

Lipase is one of the more unstable enzymes of pancreatic juice (29). An interaction promoted by colipase between the enzyme and the bile lipoprotein complex normally occurring in intestinal lumen, enhances enzyme stability. Thus, the quaternary association lipase-colipase-bile lipoprotein complex-bile salts could be the functional state exerting full enzymatic activity under the physiological conditions.

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Diet and Sterol Biohydrogenation in the Rat: Occurrence of Epicoprostanol

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ABSTRACT

The fecal sterols from rats fed several types of semipurified or commercial diets were analyzed by a combination of thin layer and gas liquid chromatography. In rats fed semipurified diets with lard, sucrose, and casein, increasing proportions of lard (0, 8, 20, 65%) enhanced the fecal coprostanol/coprostanol + cholesterol ratio (from 0.50 to 0.85). This ratio was reduced by replacing lard with triolein or a mixture of calcium oleate and linoleate (1:1) and did not change when trierucin was substituted. No coprostanol formation was observed in rats fed a diet with tripalmitin or tristearin. The addition of sodium hyodeoxycholate (0.5%) or cholestyramine (2%) to the basal diet was without effect on the coprostanol/coprostanol + cholesterol ratio in the feces. The addition of sodium taurocholate (0.2, 0.75, and 4%) strongly reduced coprostanol formation, while a chronic bile duct ligation led to an enhancement. Cholesterol feeding (0.05, 0.2, and 0.5% in the diet) slightly increased (from 51 to 66%) coprostanol formation. Trace amounts of epicoprostanol were generally found in the feces. However, in some cases a very high proportion (up to 60%) of this sterol was observed. Possible relationships between the presence of epicoprostanol and the nature of the diet are discussed.

INTRODUCTION

In rats and in other species, Δ5-3β hydroxysteroids are converted into saturated compounds by microbial 5β-H reduction of the 5-6 double bond during the passage of these compounds through the caecum and colon (1). A pure strain of bacteria (*Eubacterium* 21 408) which promotes such a biohydrogenation has been isolated (2). This anaerobic Gram positive bacterium requires a high concentration of Δ5-3β hydroxysteroids for growth (for example, 1.5 to 2 mg/ml of cholesterol). Lathosterol[1], dehydrocholesterol, and stigmasterol are not reduced, but *Eubacterium* 21 408 can transform a sterol with a 3 oxogroup to the corresponding 5β3β hydroxysterol (for example, Δ4 cholestenone to coprostanol). Other bacterial strains can also hydrogenate cholesterol to coprostanol (3). Whereas the mechanism of this microbiological conversion has led to the formulation of several hypotheses (4), data concerning the conditions promoting biohydrogenation of cholesterol are scarce (5-8). Moreover, the reason for the occurrence of epicoprostanol in fecal sterols, described in man and the dog (9,10), is obscure. With regard to these problems and in connection with our studies of cholesterol metabolism under various experimental conditions in the rat (11-15), the fecal sterols were also analyzed in detail. The results of this work are presented here.

EXPERIMENTAL PROCEDURES

Animals and Diets

Wistar rats were grown in our own animal house (16). After weaning, the animals were fed a *semipurified basal diet* (T) (Table I) which contained casein (23%), sucrose (53%), lard (8%), calcium oleate and linoleate (1:1) (2%), skim milk (4%), salt mixture (5%), yeast (2.3%), agar-agar (2.3%), and vitamins (0.2%) (17). After they were 3 months old, the rats were fed their experimental diet for 4 months. Feces were collected during the last month of the experiment and were pooled weekly. Fecal sterol composition has previously been studied after changing the proportions of lard, casein, or sucrose in a semipurified diet (11), the nature of dietary lipids (Lutton and Chevallier, unpublished data), the enterohepatic circulation of bile acids (13), the thyroid status (14), and the fiber or salt mixture content of the diet (15). In all these experimental conditions, the weight of rats at time of sacrifice was between 380 g and 500 g.

Fecal Sterols Analysis

The techniques used have been described previously (17). Feces were ground in a "Servall" blender and homogenized in a mini-

[1]Systemic name: cholesterol: cholest-5-en-3β-ol; epicholesterol: cholest-5-en-3α-ol; lathosterol: 5α-cholest-7-en-3-ol; dehydrocholesterol: 5α-cholesta-5,7-dien-3β-ol; stigmasterol: 24α-ethylcholesta-5,22-dien-3β-ol; Δ4-cholestenone: cholest-4-en-3-one; coprostanol: 5β-cholestan-3β-ol; epicoprostanol: 5β-cholestan-3α-ol; β-sitosterol: 24β-ethylcholest-5-en-3β-ol; β-coprositostanol: 24β-ethylcholestan-3β-ol.

mal volume of water (≤ 200 ml). After addition of 4 volumes of ethanol, the mixture was filtered and extracted for 48 hr in a Soxhlet apparatus. After saponification (ethanol, 2N KOH, for 3 hr), the sterols were extracted with petroleum ether (against ethanol-water, 1:1, v/v, 1N KOH). Fecal sterols were analyzed either in the unsaponifiable fraction or in the digitonide complex by gas liquid chromatography (GLC) either alone or in combination with thin layer chromatography (TLC).

Gas Liquid Chromatography

Sterol separations were performed with a Varian Aerograph 1400 equipped with a flame ionization detector. A stainless-steel column (3 mm ID, 150 mm long) was packed with 2% QF1 on Gas Chrom Z (100-120 mesh). Temperatures of the oven, injector, and detector were 200, 240, and 240 C, respectively; the flow rate of carrier gas (nitrogen) was 20 ml/min, and the pressure was 3.5 bars. Sterols were dissolved in hexane containing cholestane as internal standard (0.1 $\mu g/\mu l$) for injection. Chromatograms generally showed eight to ten peaks. Their identification has been reported previously (18). Two methods were combined for the quantitation of sterols: (a) peak areas were measured by triangulation to calculate the relative proportions of each sterol in a mixture (19); (b) an internal standard method using the ratio between the height of the major peak (coprostanol or cholesterol) and that of the internal standard (cholestane) (20). The validity of these methods has been discussed (19,20). Rigorous determination of the amounts of coprostanol and cholesterol obviously require that no other compound interferes with their peaks. Under our GLC conditions, no known fecal sterol has a retention time identical to that of coprostanol (Peak 1, see reference 18). In contrast, peak 2 could be cholesterol or epicoprostanol (9).

Thin Layer Chromatography

The TLC separation of fecal sterols was carried out either on 0.5 mm and 1 mm plates of Silica Gel G (Carl Schleicher and Schüll) using hexane-ethyl acetate (8:2) to separate ketonic compounds (coprostanone and cholestenone), triterpenic alcohols, and 5β sterols from Δ5, Δ7, and 5α saturated sterols according to their polarity, or on alumina-silver nitrate plates using chloroform-hexane-acetone (60:30:3) to separate 5β and 5α sterols or Δ7 and Δ5 sterols according to their unsaturation.

With both solvent systems, each fraction contained the main sterol (C_{27}) possibly with corresponding phytosterols. On gas liquid chromatograms, plant sterols migrated as peaks 4 and 5 (24-methyl-5β-cholestan-3β-ol and campesterol), 6 and 7 (24-ethyl-5β-cholestan-3β-ol and stigmasterol), or 7 and 8 (coprositostanol and β-sitosterol).

Isolation and Identification of Epicoprostanol in Feces

An initial simple procedure to detect important amounts of epicoprostanol in feces consisted in comparing the relative GLC percentages of peaks 1 and 2 in the total unsaponifiable fraction and after digitonin precipitation, since epicoprostanol (3α-OH) does not form a complex with digitonin.

Purified epicoprostanol was obtained by TLC of the nonprecipitable digitonin sterols fraction. After filtration of precipitable sterols, the filtrate was evaporated, dissolved in water, and extracted with diethyl ether. A second digitonin precipitation was performed to remove any contaminating traces of coprostanol. The diethyl ether extract was purified by TLC on silica gel. The sterol which migrated with the same R_f as coprostanol was eluted, and its purity was checked by GLC. Its GLC retention time was identical to that of cholesterol. Finally its 5β-H saturated structure was confirmed by mass spectrometry, and coprostanone was obtained after oxidation of the 3-hydroxyl according to Curtis et al. (21).

RESULTS AND DISCUSSION

Hydrogenation of Cholesterol to Coprostanol

Influence of the proportions of lard, sucrose, and casein in a semipurified diet (Table I): When the animals were fed a lipid-free diet (group Q) or a diet containing 2% calcium oleate-linoleate (1:1) to provide a source of essential fatty acids (group R and S) (11), the mean percentages of cholesterol transformed into coprostanol in their feces were less (48-67%) than that of rats fed a diet with 8, 20, or 64% lard (76-85%). Thus, dietary lard apparently promotes the growth of bacteria which reduce cholesterol to coprostanol. For a given lard concentration, we did not observe any significant variations of the coprostanol/coprostanol + cholesterol ratio when the proportions of casein or sucrose were changed.

Influence of dietary fatty acids: The replacement of lard (20%) (group U, Table I) by a mixture of triglycerides simulating its composition (group PSa, Table II) (Lutton and Chevallier, unpublished data) did not modify coprostanol formation. While trierucin (group Er) was without any significant effect, triolein

TABLE I

Total Sterols and Percentages of Coprostanol and Cholesterol in Feces of Rats Fed Semipurified Diets Containing Various Proportions of Lard, Sucrose, and Casein.

Group	Percentages of main dietary components[a]				Number of rats	Total Sterols (mg/day)	Coprostanol	Cholesterol	$\frac{A}{A+B}$ (%)
	Lipids								
	Calcium Oleate: Linoleate (1:1)	Lard	Sucrose	Casein			A (%)	B (%)	
Q	0	0	69.3	23	4	7.0 ± 0.3[b]	41 ± 3	27 ± 1	60 ± 2[c]
R	2	0	74	10	4	14.4 ± 0.5	47 ± 2	23 ± 1	67 ± 3[c]
S	2	0	0	84	8	7.0 ± 0.4	32 ± 1	35 ± 1	48 ± 2[c]
T	2	8	53	23	4	9.3 ± 1.0	58 ± 2	11 ± 1	84 ± 4
U	2	20	54	10	8	14.1 ± 0.6	48 ± 3	15 ± 1	76 ± 4
V	2	20	0	64	6	9.3 ± 0.9	64 ± 2	10 ± 1	86 ± 2
W	2	64	10	10	9	14.1 ± 0.5	72 ± 1	12 ± 1	86 ± 1
X	2	64	0	20	10	21.1 ± 1.2	75 ± 1	13 ± 1	85 ± 1

[a]For detailed composition of diets, see ref. 11.
[b]Mean ± SEM, four determinations per rat.
[c]$P < 0.01$ vs. diet T.

TABLE II

Total Sterols and Percentages of Coprostanol and Cholesterol in Feces of Rats Fed Semipurified Diets Containing Triglycerides, Bile Acids, or Cholestyramine.

Group	Experimental conditions	Number of rats	Total sterols (mg/day)	Coprostanol A (%)	Cholesterol B (%)	$\frac{A}{A+B}$ (%)
	Lipid replacing lard in diet U[a]					
Psa	"Reconstituted lard"	4	19.0 ± 1.4[b]	59 ± 2	17 ± 3	78 ± 3
Pa	Tripalmitin	4	12.7 ± 1.6	0	70 ± 3	0
St	Tristearin	4	25.6 ± 1.0	1	84 ± 2	0
Oℓ	Triolein	5	13.0 ± 0.9	35 ± 3	38 ± 3	49 ± 4[c]
Er	Trierucin	3	20.5 ± 1.8	53 ± 4	23 ± 3	70 ± 5
LL	Calcium oleate linoleate (1:1)	4	26.6 ± 1.8	8 ± 1	51 ± 1	14 ± 1[c]
LC	Calcium oleate linoleate (1:1) +2% cholestyramine	3	35.0 ± 2.0	8 ± 1	51 ± 1	14 ± 2[c]
	Addition to diet T[a] or experimental conditions					
$TC_{0.2}$	Sodium taurocholate 0.2%	4	9.3 ± 0.9	20 ± 2	43 ± 2	32 ± 3[d]
$TC_{0.75}$	Sodium taurocholate 0.75%	4	9.3 ± 0.7	22 ± 3	42 ± 1	34 ± 3[d]
TC_4	Sodium taurocholate 4%	3	17.1 ± 1.1	15 ± 2	47 ± 2	24 ± 3[d]
$H_{0.5}$	Sodium hyodeoxycholate 0.5%	4	26.6 ± 1.8	49 ± 3	18 ± 3	73 ± 5
CY_2	Cholestyramine 2%	4	25.3 ± 1.3	55 ± 1	23 ± 1	71 ± 2
L	Bile duct ligature	4	30.6 ± 3.4	76 ± 3	9 ± 1	90 ± 3

[a]See Table I.
[b]Mean ± SEM, four determinations per rat.
[c]$P < 0.01$ vs. diet U (Table I).
[d]$P < 0.01$ vs. diet T (Table I).

(group OL), or more particularly, a mixture of calcium oleate and linoleate (1:1) (groups LL and LC), strongly decreased cholesterol biohydrogenation (Table II). Coprostanol formation was practically totally inhibited in rats fed a diet containing 20% tripalmitin (group Pa) or tristearin (group St).

These experiments show that dietary triglycerides composed of saturated fatty acids (palmitic and stearic) and oleic acid do not promote the biohydrogenation of cholesterol in the large intestine of the rat. Moreover, oleic acid seems to be more effective when it is given in the diet as free acid. Our data elaborate on Wilson's previous observations (7). According to this author, coprostanol excretion is depressed by free palmitic and oleic acids in the diet and is accelerated by linoleic acid.

The mechanism by which fatty acids do or do not stimulate growth of reducing bacteria in the caecum is unclear. Numerous Gram positive bacteria from the digestive tract are inhibited in vitro by long chain fatty acids (22). However, in contrast to observations in vivo, this effect is seen with long chain unsaturated fatty acids (C_{18}, 1, 2, or 3 double bonds) as well as the C_{12}-C_{18} saturated fatty acids. Moreover, calcium ions and cholesterol reverse the fatty acids effect in vitro (22).

Influence of bile acids: Feeding sodium taurochenodeoxycholate (0.03%) (two rats, results not reported in Table II), sodium hyodeoxycholate (0.5%), or cholestyramine (2%) in the basal semipurified diet (13) did not change the coprostanol/coprostanol + cholesterol ratio in the feces (Table II). Three weeks after a bile duct ligation (no bile acids in the intestinal contents) the mean percentage of cholesterol transformation into coprostanol was increased. In agreement with others (6,8), we found that the addition of taurocholate to a diet (0.2 and 0.75%) decreased this ratio. A more pronounced effect was seen with a diet containing 4% sodium taurocholate. This bile acid effect was very rapid. A day after the ingestion of a single dose of sodium taurocholate (80 mg), the mean fecal copostanol/coprostanol + cholesterol ratio (4 rats) decreased from 64 to 53% (Fig. 1). This ratio returned to its initial value as exogenous bile acids were eliminated into the feces. The reasons for the specificity of sodium taurocholate as an inhibitory factor for coprostanol formation are unknown.

Miscellaneous factors: The coprostanol/coprostanol + cholesterol ratio was unchanged in the feces from adult male rats 5 or 24 months old, or from virgin, pregnant, or lactating females fed the basal semipurified diet. It was also the same among thyroidectomized,

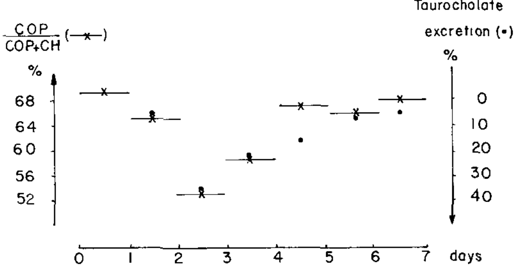

FIG. 1. Coprostanol/coprostanol + cholesterol ratio in daily pooled feces from four rats before and after ingestion of 80 mg sodium taurocholate mixed with the meal fed on day 1. Percentage of exogenous ^{14}C taurocholate excreted daily in feces. COP: coprostanol. CH: cholesterol.

hyperthyroid, or hypoiodine diet fed rats (14). The addition of fiber (cellulose or bran) or phytosterols (15) did not change this ratio. In contrast, cholesterol feeding (0.05, 0.2, and 0.5%) in a basal semipurified or in a commercial diet (Extralabo: Piétrement, Sainte Colombe, 77160 Provins, France) slightly enhanced coprostanol formation (mean ratios: from 0.51 to 0.67 and from 0.30 to 0.45, respectively). A similar observation has been reported in vitro with *Eubacterium* (2). However, Cohen et al. (8) observed that fecal biohydrogenation of cholesterol was reduced after a 1.2% cholesterol supplement to a commercial stock diet. This observation can probably be explained as follows: the size of the bile acid pool in the intestine and caecal contents was probably not greatly changed by moderate concentrations of cholesterol in the diet (≤0.5%) but was strongly increased by higher (1-2%) levels (23). Thus, the stimulating effect of exogenous cholesterol on coprostanol formation would be counteracted by a simultaneous enhancement of the sodium taurocholate level in caecum, which would inhibit this transformation.

Dietary β-sitosterol (0.15% supplement to the basal semipurified diet) was partly transformed into β-coprositostanol by bacterial flora. In agreement with results of Subbiah et al., in humans (24) we found that the transformation of cholesterol into coprostanol in the rat in vivo was higher (80%) than that of β-sitosterol to its corresponding saturated derivative (43%).

Hydrogenation of Cholesterol to Epicoprostanol

Only trace amounts of epicoprostanol (≤ 1%) were found in most samples of fecal sterols. In some rats from four other groups, however, epicoprostanol was present in a unexpected amount (up to 60%) (Table III). Three

TABLE III

Percentages of Fecal Digitonin-Precipitable Sterols and Proportions of Peaks 1 and 2 on GLC Chromatograms from the Unsaponifiable Fraction or after Digitonin Precipitation in Rats Fed Semipurified or Commercial Diets

Group and experimental conditions	Number of rats	Digitonin precipitable sterols (%)	Unsaponifiable fraction			Digitonin precipitable sterols		
			Peak 1[a] (%)	Peak 2 (%)	Peak 1 / Peaks 1+2 (%)	Peak 1 (%)	Peak 2 (%)	Peak 1 / Peaks 1+2 (%)
BR Semipurified diet + 10% bran[b]	3	79±3[d]	49±1	13±1	79±1	---	---	---
	1[c]	60	28	29	49	---	---	---
TY2 Semipurified diet hyperthyroids rats[e]	2	89±0	68±4	10±1	87±2[f]	63±7	10±2	85±3
	2[c]	47±0	14±3	59±1	20±3[g]	28±4	26±5	53±8[h]
NC Semipurified diet[i] no coprophagy	1	69	41	15	73	32	18	63
	3[c]	44±3	31±3	40±4	47±6	32±1	20±1	61±2
GP Commercial diet[j] + 0.5% cholesterol	2	84±3	52±1	28±1	65±1[k]	61±1	20±2	75±3
	4[c]	63±1	27±1	50±2	35±2[l]	48±8	24±4	66±7[m]

[a]Peak 1 = coprostanol; Peak 2 = cholesterol and epicoprostanol.
[b]see ref. 15.
[c]Rats which excreted a large quantity of epicoprostanol.
[d]Mean ± SEM.
[e]See ref. 14. Rats were fed a diet containing 2.5 mg of L thyroxine/kg diet for 2 months.
[f]See g.
[g]$P < 0.01$ vs. f, $P < 0.05$ vs. h.
[h]See g.
[i]Basal semipurified T (Table I). The rats had a tail cup to prevent coprophagy (25).
[j]Extralabo diet (15) with 0.5% cholesterol.
[k]See l.
[l]$P < 0.001$ vs. k, $P<0.01$ vs. m.
[m]See l.

TABLE IV

Comparative Mass Spectral Analysis of Authentic Coprostanol and of Our Isolated Compound (Assumed to be Epicoprostanol). Relative Percent Intensity of Main Peaks Compared to That of Molecular Ion

Compound	Conditions	m/e 388 Molecular ion	373	370	355	257
Coprostanol	T: 160 C; 70 eV	100	25	19.4	16.6	17.5
Epicoprostanol	T: 150 C; 70 eV	100	18.5	92	22.2	14.8

groups of animals ingested a semipurified type T diet (Table I). Coprophagy was not possible for rats from the NC group (Lutton, unpublished data). The rats of the TY2 group were rendered hypertyroid by the ingestion of 2.5 mg L thyroxine/kg of diet (14). The semipurified diet of BR rats was supplemented with 10% bran (15). The fourth group of rats (GP) was fed a commercial stock diet (15). The quantity of fecal epicoprostanol was aleatory in rats from the same group. One, two, or three rats out of four and four rats out of six, respectively, excreted a very large proportion of epicoprostanol (for example, 20 to 30% in four rats of the GP group, i.e., 8-15 mg/day, and almost 60% in two rats of the TY2 group, i.e., 10-14 mg/day). For these rats, we found an unusual decrease in digitonin-precipitable sterols and large variations in the proportions of peaks 1 and 2 on GLC chromatograms obtained from total sterols or after digitonin precipitation (Table III). Epicoprostanol was purified from the fecal sterols of these rats. By TLC of the nondigitonin precipitable sterol fraction, we isolated a compound with a same R_f as coprostanol which showed a GLC retention time identical to that of cholesterol. This compound was probably a 3α-hydroxysterol since it did not form a complex with digitonin. After oxidation of its 3-hydroxyl group (21), we obtained coprostanone which indicated a 5β structure. The mass spectrum of the compound presented a major difference in the relative intensity of fragment ion m/e 370, corresponding to a loss of water from the molecular ion m/e 388, compared to that of coprostanol (Table IV). These data showed that the structure of our isolated compound corresponds to 5β-cholestan-3α-ol (epicoprostanol). In the feces of rats fed the GP diet containing 0.06% β-sitosterol, we also found some epicoprositostanol, which shows that 24-ethyl on the side chain does not affect the biohydrogenation of the 5-6 double bond of the steroid nucleus.

Epicoprostanol was first found in dog feces (10) and subsequently was observed in trace amounts in human feces (9). However, this sterol was not detected in the rat. Epicoprostanol, as well as coprostanol, is due to sterol transformations by bacterial flora. After feeding antibiotics or in germfree animals, the only fecal sterols were cholesterol and some of its precursors (26). Moreover, an analysis of the digestive contents in rats of group TY2 showed that epicoprostanol formation occurs in the caecum, as seen for coprostanol (18). Although several strains of bacteria can hydrogenate cholesterol to coprostanol, the only isolated in pure culture is *Eubacterium* 21 408 (2). This bacterium probably first converts cholesterol to 4-cholesten-3-one and then stereospecifically reduces the ketone to the 5β-H-3β-hydroxy derivative (2). Thus, epicoprostanol formation cannot be explained by this mechanism, but other bacterial strains, such as *Pseudomonas testosteroni*, have a different enzymatic complement and can reversibly dehydrogenate 3β-OH and 3α-OH saturated or unsaturated steroids (27). In this case, the intermediary 5β-H 3-keto compound can yield, after reduction, two derivatives corresponding to coprostanol and epicoprostanol. Since epicoprostanol, normally present in trace amounts, sometimes appears in large proportions in rats feces, this indicates that in some cases significant growth of bacteria with these types of enzymes occurs. Precise bacterial strain determinations on the caecal contents from these rats would contribute new data concerning the strains involved in epicoprostanol formation. Our results suggest that certain conditions promote the growth of these strains. The rate of intestinal transit was accelerated for the BR and GP rats which were fed a diet very rich in fiber. Furthermore, the weight of the dry feces was 2 or 4 times higher, respectively, than that of rats fed a basal diet (15). Such an accelerated intestinal transit was also observed in hyperthyroid rats (28). The problem is more complex for rats with prevented coprophagy (NC). Their bacterial flora must be largely affected since these rats cannot consume 40% of their droppings. (29).

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A Model for Studying LCAT Reaction: In Vitro Cholesterol Esterification in Pig Ovarian Follicular Fluid[1]

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ABSTRACT

Phosphatidylcholine acyltransferase (lecithin:cholesterol acyltransferase or LCAT; EC 2.3.1.43) activity was found to be present in pig ovarian follicular fluid (POFF), in addition to pig serum (PS). The cholesterol esterification rate in both POFF and PS is linear with incubation time up to 2 hr. The mean absolute rate of POFF-cholesterol esterification was 8.1 ± 0.4 nmoles per ml per hr approximately one-fourth of that in PS. However, the fractional rate (percent of labeled cholesterol esterified per hr) of POFF-cholesterol esterification was similar to that observed in PS. There was little variation of absolute rate of cholesterol esterification in the fluid obtained from different sizes of follicles. Fatty acid or triacylglycerol did not participate in the reaction of cholesterol esterification in POFF. No appreciable change in enzymatic activity was found from storing POFF at 4 C for periods of time up to 24 hr or at -70 C up to 2 months, but activity was lost thereafter. On the other hand, PS showed a much longer period of stability (5 days at 4 C and 9 months at -70 C). A discrepancy between the fatty acid composition of cholesteryl esters formed by the LCAT reaction and the fatty acid composition at the C-2 position of phosphatidylcholine led us to propose a two-step mechanism for the LCAT reaction. It is concluded that the LCAT of POFF, as well as that of plasma, is specific for individual fatty acids rather than for the fatty acid composition of phosphatidylcholine. The fatty acid concentration of lysophosphatidylcholine decreased during prolonged incubation times (6 to 21 hr) suggesting that the increased lysophosphatidylcholine formed as a product of the LCAT reaction may be reused as substrate for the LCAT reaction or for hydrolysis by lysophosphatidylcholine hydrolase.

INTRODUCTION

Esterification of cholesterol in plasma (or serum) has been actively investigated since Sperry (1) first discovered that human plasma had the ability to esterify cholesterol. The major source of the transferred fatty acids is the phosphatidylcholine of the plasma high density lipoproteins (HDL) (2-4). This reaction is catalyzed by a plasma phosphatidylcholine acyltransferase (lecithin:cholesterol acyltransferase or LCAT; EC 2.3.1.43). LCAT is found to be synthesized by liver (5,6) and is secreted by this organ into plasma (7,8). In rat tissue, transferase-like activity was found only in plasma (9,10). The mechanism of the LCAT reaction has, therefore, been studied only in plasma (or serum).

The fluid of ovarian follicle plays an important role in ovarian physiology (11). This fluid is secreted from the thecal blood vessels through the membrana limitans, and is modified by cells of the membrana granulosa (12). It is assumed that the proteins of the follicular fluid have been filtered through the so-called "blood-follicular barrier." Therefore, in health large molecular weight materials, e.g., low density lipoproteins (LDL), very low density lipoproteins (VLDL), and chylomicrons do not enter follicular fluid. An analysis of the lipoprotein profile of follicular fluid showed that only HDL was present (12,13).

In studying pig ovarian follicular fluid, we found that ca. 80% of its cholesterol was present in the esterified form which was similar to what had been found in pig serum. Since the LCAT reaction is thought to take place on or within HDL molecules (14), the possibility that pig ovarian follicular fluid itself, like pig serum, had the ability to esterify cholesterol was explored in the present paper. The mechanism of the LCAT reaction using this fluid has been studied.

EXPERIMENTAL PROCEDURES

Pools of pig serum (PS) and pig ovarian follicular fluid (POFF) from small (1-2 mm), medium (3-5 mm), and large (6-12 mm) follicles were collected according to the procedure reported previously (13).

The method used to assay the in vitro rate of cholesterol esterification (LCAT activity) in PS and POFF has recently been described by Yao and Dyck (14). In a typical experiment, 0.5 ml of a fresh sample of fluid was pipetted into a 15 ml centrifuge tube containing 0.05 μCi of (4-^{14}C)cholesterol (specific activity, 56 mCi/μmole, Amersham/Searle Co., and radiochemical purity, as tested by TLC, greater than 98%) previously dissolved in 50 μl (for POFF)

[1]Presented at the AOCS Meeting, New York, May 1977.

TABLE I

Cholesterol Concentration in Pig Ovarian Follicular Fluid (POFF) and Pig Serum (PS)

Sample	N	Cholesterol concentration		
		Total (mg/dl)	Free (mg/dl)	Esterified (%)
POFF				
Small, 1-2 mm	3	28.2 ± 1.7	6.7 ± 0.7	76.1 ± 2.7
Medium, 3-5 mm	9	29.6 ± 4.2	5.2 ± 1.1	82.3 ± 2.7
Large, 6-12 mm	4	20.3 ± 5.6	3.6 ± 0.5	81.7 ± 4.7
PS	5	102.4 ± 19.4	20.3 ± 3.5	80.0 ± 3.1

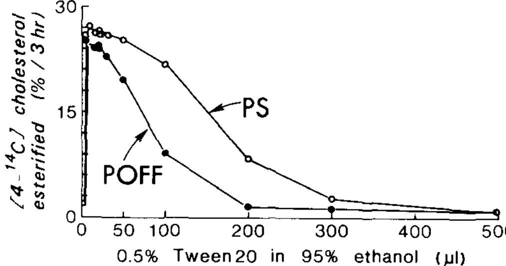

FIG. 1. Effect of Tween 20 on in vitro rate of cholesterol esterification by pig serum (PS) and by pig ovarian follicular fluid (POFF).

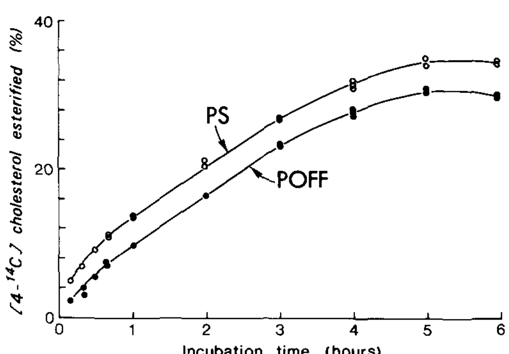

FIG. 2. Relationship of incubation time to rate of cholesterol esterification in pig serum (PS) and in pig ovarian follicular fluid (POFF).

or 100 μl (for PS) of 95% ethanol containing 0.1% of Tween 20. The ethanol was evaporated under nitrogen before the sample was added. The incubation times for PS and POFF are 5 hr and 4 hr respectively, or as indicated.

Lipids were extracted from PS and POFF according to the procedure described previously (14). The radiolabeled cholesteryl esters formed during the incubation period were isolated by thin layer chromatography (TLC) (15). The saturated, monoethenoic (Δ1), dienoic (Δ2), and polyenoic (⩾Δ4) cholesteryl esters were further separated by another TLC on Silica Gel G impregnated with $AgNO_3$, with benzene-hexane (1:1, v/v) as the developing system (16). The radioactivity of total and separated cholesteryl esters was determined after TLC by the method described elsewhere (14).

The total and free cholesterol concentration was measured by gas liquid chromatography (GLC) according to the method of MacGee et al. (17). Cholesterol esterifying acticity was expressed as either the percentage of labeled cholesterol esterified per unit time (R_a) or the number of nanomoles of cholesterol esterified per milliliter of sample per hour of incubation (R_b), with the assumption that there was isotope equilibrium between added labeled cholesterol and endogenous lipoprotein cholesterol (14).

The phosphatidylcholine and lysophosphatidylcholine fractions were separated from total phospholipids of PS and POFF by TLC (18). The position of the fatty acids in the phosphatidylcholine molecule, prior to cholesterol esterification, was determined by hydrolysis with phospholipase A (Crotalus terr. terr. solution in 50% glycerol, 146, B-grade, Calbiochem). The preparation of lysophosphatide from phosphatidylcholine was carried out in essentially the same manner as described by Parijs et al. (19). The methyl esters of fatty acids, phosphatidylcholine, and lysophosphatidylcholine were prepared by the use of boron trifluoride-methanol reagent using the method of Morrison and Smith (20) using pentadecanoic acid (15:0) as an internal standard. The fatty acid concentration was expressed as μg per ml of POFF. The cholesteryl esters were transesterified by sodium methoxide reagent (15,21). All the methyl ester derivatives were purified by TLC (15,22) before GLC analysis. The method used to measure the distribution of fatty acid composition by GLC has previously been reported (15).

RESULTS

The cholesterol concentration of POFF and of PS are shown in Table I. Approximately 80% of the cholesterol of POFF and of PS, regardless of the size of the follicles, is esterified. This result was similar to that found in PS, except that the cholesterol concentration of PS was about four times higher than that of POFF.

Figure 1 shows the effect of Tween 20 on incorporation of (4-^{14}C)cholesterol into cholesteryl esters by PS and POFF. Less than 1% of added (4-^{14}C)cholesterol was esterified by PS and by POFF during 3 hr incubation if (4-^{14}C)cholesterol was not dispersed into Tween 20 before incubation. The optimum condition for cholesterol esterification was obtained when (4-^{14}C)cholesterol was dispersed into 95% ethanol containing 0.10-0.15 μl and 0.05-0.10 μl of Tween 20 (normally provided by 0.1% Tween 20 in 95% ethanol; see experimental procedures for details) per 0.5 ml of PS and POFF, respectively. However, an excess of Tween 20 in the incubation medium could also lead to inhibition of cholesterol esterification in both PS and POFF.

TABLE II

In Vitro Rate of Cholesterol Esterification in Pig Ovarian Follicular Fluid (POFF) and Pig Serum (PS)

Sample	In vitro rate of cholesterol esterification R_a (%/hr)	R_b (nmoles/ml/hr)
POFF		
Small, 1-2 mm	4.7 ± 0.3	8.5 ± 0.3
Medium, 3-5 mm	7.2 ± 0.4	8.2 ± 0.5
Large, 6-12 mm	7.5 ± 0.3	7.7 ± 0.2
PS	6.7 ± 0.2	37.8 ± 0.6

The rate (R_a) of cholesterol esterification in PS and POFF was linear with incubation time from 45 min to 3 hr (Fig. 2). The initial nonlinear period (first 45 min) was probably due to dysequilibrium between radiolabeled precursor and endogenous substrates (14). In order to simplify the assay procedure and to decrease

TABLE III

Composition and Positional Distribution of Fatty Acids of Phosphatidylcholine in Pig Ovarian Follicular Fluid (POFF) and Pig Serum (PS)

Fatty acid	POFF Total (%)	POFF C-1 (%)	POFF C-2 (%)	POFF Reconst. (%)	PS Total (%)	PS C-1 (%)	PS C-2 (%)	PS Reconst. (%)
16:0	20.4	33.1	3.1	18.1	19.4	28.8	1.3	15.1
18:0	24.8	53.7	1.1	27.4	26.0	58.7	0.7	29.7
18:1	15.0	6.9	23.5	15.2	14.5	7.4	19.3	13.4
18:2	12.9	1.6	21.2	11.4	15.7	1.4	26.4	13.9
20:3	1.9	---	4.4	2.2	1.4	0.1	3.2	1.6
20:4	18.0	0.7	33.9	17.3	17.4	0.7	36.4	18.6
22:4	1.8	0.2	3.9	2.0	1.4	---	3.1	1.5
22:5	2.0	---	4.8	2.4	1.6	---	3.8	1.9
22:6	0.6	---	1.5	0.8	0.6	---	1.9	0.9

TABLE IV

The Fatty Acid Composition of Cholesteryl Ester Synthesized by the LCAT Reaction and of Phosphatidylcholine at the C-2 Position

Fatty acid	POFF Cholesteryl ester GLC[a] (%)	POFF Cholesteryl ester TLC(^{14}C)[b] (%)	POFF Phosphatidylcholine C-2 (%)	PS Cholesteryl ester GLC (%)	PS Cholesteryl ester TLC(^{14}C) (%)	PS Phosphatidylcholine C-2 (%)
Saturated	15.9	15.7 ± 0.4[c]	4.4	15.3	12.9 ± 0.2	2.2
Monoene	32.2	32.2 ± 0.3	24.0	31.1	29.3 ± 0.4	19.5
Diene	42.3	36.8 ± 0.3	21.6	44.2	42.3 ± 0.2	27.0
Polyene	9.6	15.3 ± 0.3	50.0	9.0	15.7 ± 0.3	51.3

[a]Distribution of fatty acids in the cholesteryl ester fraction was determined by gas liquid chromatography.
[b]Distribution of radioactivity was determined by thin layer chromatography.
[c]Mean and SD of nine determinations in which incubation time varied from 10 min to 6 hr.

TABLE V

The Change of Fatty Acid Concentrations at C-1 and C-2 Positions of POFF-Phosphatidylcholine with Different Incubation Time

	C-1			C-2		
Fatty acid	0 hr	6 hr	21 hr	0 hr	6 hr	21 hr
		(μg/ml)			(μg/ml)	
16:0	16.5	13.5	12.9	5.0	4.8	4.2
18:0	37.8	31.0	30.9	4.8	4.7	3.6
18:1	6.6	5.6	5.7	26.3	20.5	13.3
18:2	0.9	0.8	0.6	22.2	15.0	8.5
20:3	---	---	---	3.8	2.4	1.6
20:4	---	---	---	56.8	43.1	24.8
22:4	---	---	---	4.0	3.2	1.8
22:5	---	---	---	5.2	4.1	2.3
22:6	---	---	---	2.4	1.9	1.1

TABLE VI

The Distribution of Fatty Acids at C-1 and C-2 Positions of POFF-Phosphatidylcholine with Different Incubation Time

	C-1			C-2		
Fatty acid	0 hr	6 hr	21 hr	0 hr	6 hr	21 hr
		(%)			(%)	
16:0	26.7	26.5	25.8	3.8	4.8	6.9
18:0	61.2	60.9	61.7	3.7	4.7	5.9
18:1	10.7	11.0	11.4	20.2	20.6	21.7
18:2	1.5	1.6	1.2	17.0	15.1	13.9
20:3	---	---	---	2.9	2.4	2.6
20:4	---	---	---	43.5	43.2	40.5
22:4	---	---	---	3.1	3.2	2.9
22:5	---	---	---	4.0	4.1	3.8
22:6	---	---	---	1.8	1.9	1.8

the sample volume, a single 4-hr incubation, based on the criteria we have described previously (14), was selected for the assay of in vitro rate of cholesterol esterification in POFF, and 5-hr incubation for PS.

No radiolabeled cholesteryl esters were synthesized if PS or POFF was incubated in the presence of (1-^{14}C)oleate or tri(1-^{14}C)oleoyl glycerol under the same experimental condition described for incubation of (4-^{14}C)cholesterol.

The rate of cholesterol esterification in POFF from three different sizes of follicles and in PS are shown in Table II. The R_a of POFF small-size follicles was much less than that of POFF medium-size follicles and that of POFF large-size follicles. However, there was little variation of R_b among the different sizes of follicles. On the other hand, R_b of POFF is about one-fourth of that found in PS, but R_a of POFF is similar to that of PS.

No appreciable change in LCAT activity was found from storing PS at 4 C for periods of time up to 5 days or at -70 C for up to 9 months (37.12 ± 1.07 nmoles/ml/hr). On the other hand, POFF was less stable—for only 24 hr at 4 C and for only 2 months at -70 C (8.65 ± 0.18 nmoles/ml/hr).

The fatty acid composition at the C-1 and C-2 positions of phosphatidylcholine from POFF and PS are shown in Table III. In both POFF and PS, palmitic (16:0) and stearic (18:0) acids are predominately at the C-1 position. However, at the C-2 position, arachidonic (20:4) acid occurs most commonly while oleic (18:1) and linoleic (18:2) acids occur next commonly.

The percentage distribution of fatty acids of endogenous cholesteryl esters of both POFF and PS is similar to that of the radiolabeled fatty acids of cholesteryl esters formed by the LCAT reaction in vitro (Table IV). Saturated (16:0 and 18:0) monoethenoic (18:1) and dienoic (18:2) fatty acids occurred more frequently and polyenoic (20:4, 22:4 and 22:5) fatty acids less frequently in the newly synthesized (4-^{14}C)cholesteryl esters than expected,

TABLE VII

The Fatty Acid Concentrations of POFF-Lysophosphatidylcholine with Different Incubation Time

Fatty acid[a]	Incubation time			Change	
	0 hr	6 hr	21 hr	0→6 hr	6→21 hr
		(μg/ml)		(μg/ml)	
16:0	7.5	11.8	7.6	+ 4.3	− 4.2
18:0	6.9	14.3	10.0	+ 7.4	− 4.3
18:1	3.9	5.9	1.9	+ 2.0	− 4.0
18:2	1.3	1.2	0.8	− 0.1	− 0.4
20:4	1.4	1.4	1.2	---	− 0.2

[a]The minor fatty acids (14:0, 15:0, 16:1, 17:0, 18:3, 20:0, 20:3, 20:5, 24:0, 24:1, 22:5, and 22:6) were omitted from the final tabulations.

knowing that < 5% of saturated fatty acids and ~50% of polyenoic fatty acids (mainly 20:4) are at the C-2 position of phosphatidylcholine (Table III).

The change in fatty acid concentrations at the C-1 and C-2 positions of POFF-phosphatidylcholine after 6 hr and 21 hr of incubation were compared (Table V). Decreased 16:0 and 18:0 concentrations at the C-1 position were found at 6 hr, but no further decrease has occurred at 21 hr. However, the concentration of various fatty acids, including 20:4 at the C-2 position of phosphatidylcholine, fell at 6 hr and even more at 21 hr.

No significant change of percentage distribution of fatty acid composition at C-1 position of POFF-phosphatidylcholine was found after 6 hr and 21 hr incubations (Table VI). On the contrary, at C-2 position, the percentage distribution of 18:2 to total fatty acids was considerably less after 21-hr incubation than it had been before incubation (Table VI). In the same experiment, the percentage distribution of fatty acids of radiolabeled cholesteryl esters as formed by LCAT reaction remained unchanged after 6-hr and 21-hr incubations. No appreciable change of concentration of free 20:4 was found during the same incubation period.

The fatty acid concentration of POFF-lysophosphatidylcholine after 6 hr and 21 hr of incubation was also compared (Table VII). It is interesting to note that the fatty acid concentration increased during the first 6-hr incubation, then decreased by the end of 21-hr incubation.

DISCUSSION

The fluid from pig ovarian follicles, like pig serum, is able to esterify cholesterol (Fig. 2). The enzyme which catalyzes cholesterol esterification in POFF is phosphatidylcholine acyltransferase (LCAT) (Table V). Therefore, we assume that the cholesterol esterifying enzyme in POFF has the same origin as for PS.

The linearity between enzyme activity and incubation time in POFF is similar to that observed in PS (Fig. 2). However, the rate (R_b) of cholesterol esterification in POFF is much less than that of PS (Table II). Since the rate (R_b) of cholesterol esterification is correlated with the concentration of free cholesterol in human serum (15,23-25), it is likely that the lower rate of cholesterol esterification of POFF is simply due to its lesser concentration of free cholesterol (Table I).

As compared to other enzymes, the LCAT activity in serum is quite stable. The lesser stability of LCAT in POFF leads us to speculate that the presence of large molecular lipoproteins (such as LDL, VLDL, or chylomicron) in serum might be involved in maintaining this stability. Since the experiment to test whether LCAT activity in POFF might be prolonged by the addition of serum LDL and VLDL failed, we have not been able to verify our hypothesis yet.

LCAT is thought to catalyze the transference of fatty acids from the C-2 position of phosphatidylcholine to free cholesterol (2-4). To test the correctness of this proposed mechanism, we have compared the distribution of various radiolabeled cholesteryl ester fractions, as formed by the LCAT reaction, to the distribution of fatty acids at the C-2 position of phosphatidylcholine. Less than one-third of polyenoic acids (mainly 20:4) at the C-2 position of phosphatidylcholine have been transferred to free cholesterol by LCAT (Table IV). This result suggests that LCAT reaction in POFF and PS does not follow the pattern of fatty acid composition at the C-2 position of phosphatidylcholine. Our observation is in accordance with the results of Goodman (26) and of Portman and Sugano (27).

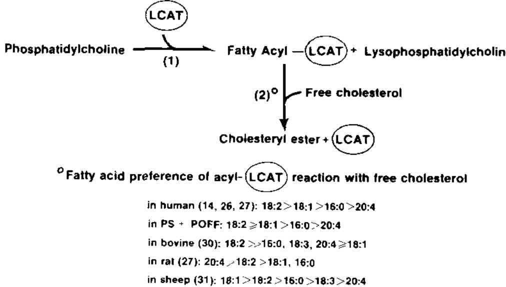

FIG. 3. A tentative mechanism for the LCAT reaction.

Further analysis of the change of fatty acid concentration during prolonged incubation times (Table V) implies that LCAT can release fatty acids from both the C-1 and C-2 positions of phosphatidylcholine, but it does so preferentially at the C-2 position. A decreased percentage distribution of 18:2 to total fatty acids at the C-2 position of phosphatidylcholine was also found after 6-hr and 21-hr incubation, suggesting that LCAT exerts a higher specificity towards 18:2 than for any other fatty acid at the C-2 position.

Glomset (28) had previously observed that about one-half the predicted amount of lysophosphatidylcholine was present in human plasma after a 24-hr incubation. Therefore, it is not surprising to find that the fatty acid concentrations of POFF-lysophosphatidylcholine continued to decrease after 6-hr incubation (Table VII). These data suggest that increased lysophosphatidylcholine from LCAT reaction may be reused as a substrate for the LCAT reaction, or for other synthesis, or for catabolism [one or more lysophosphatidylcholine hydrolases (29)].

In order to explain the difference in the percentage distribution of the amounts of fatty acids of cholesteryl esters and of phosphatidylcholine at the C-2 position, we propose a two-step mechanism for the LCAT reaction (Fig. 3). Step one involves transferring fatty acids from phosphatidylcholine to LCAT. Then the fatty acids are released from LCAT to combine with cholesterol to form cholesteryl esters. The frequency distribution of fatty acids in cholesteryl esters formed by the LCAT reaction appears to be related to a preference for certain fatty acids (in the reaction of fatty acyl-LCAT with free cholesterol) rather than to the composition of fatty acids of phosphatidylcholine. This enzyme preference varies with different animal species (15,26,27,30,31). In POFF, 18:2 is the preferred fatty acid for transferral, even though 20:4 is abundant at the C-2 position of phosphatidylcholine and can be released from phosphatidylcholine by LCAT (Table V). This enzyme preference may also explain the unique pattern of fatty acid composition of cholesteryl esters formed by the LCAT reaction which remains unchanged during the entire period of incubation from 10 min to 21 hr.

Our proposed mechanism for the LCAT reaction is also supported in part by the recent findings of Sontar et al. (32). They have demonstrated that the activation of LCAT reaction by human plasma apoproteins apoA-I and apoC-I differed, depending upon the nature of the hydrocarbon chains of phosphatidylcholine acyl donor—e.g., degree of unsaturation, chain length, etc. In other words, LCAT activity varies with the heterogenous acyl donors available for esterification in the native substrate.

The rate of decrease of arachidonic acid (20:4) which is abundant at C-2 position of POFF-phosphatidylcholine during prolonged incubation periods was similar to other unsaturated fatty acids at the C-2 position of POFF-phosphatidylcholine (Table V), although 20:4 did not completely appear at cholesteryl ester fraction via LCAT reaction (Table IV). This liberated 20:4, which was not found in the unesterified form, may possibly become a precursor for the biosynthesis of prostaglandins. As a matter of fact, Demers et al. (33) have suggested that activation of the cholesterol esterase activity was the starting point for prostaglandin synthesis facilitated by the liberation of the appropriate fatty acids.

Since the de novo synthesis of prostaglandins have already been demonstrated in the rat ovarian homogenates (34), rat corpora lutea (33), monkey ovaries (35), and rabbit Graafian follicles (36), it is likely that fluid from ovarian follicles may also have the ability to synthesize prostaglandins which, particularly, are involved in the process of ovulation (36). The possibility that prostaglandin synthesis coupled with the LCAT reaction in ovarian follicular fluid is worthy of further investigation.

In conclusion, we have demonstrated that, besides plasma (or serum), LCAT activity is present also in the fluid from ovarian follicles. The radioactivity pattern of cholesteryl esters formed by LCAT reaction in vitro does not correspond to the chemical analysis of fatty acids at the C-2 position of phosphatidylcholine. This observation and other data led us to propose a two-step mechanism for the LCAT reaction. The fatty acid composition of newly synthesized cholesteryl esters is dependent upon the specificity of LCAT for specific fatty acids rather than for the composition of fatty

acids at the C-2 position of phosphatidylcholine. On the basis of our present data and other available information, the preference of this enzyme for POFF resembles human plasma more than it does for other animal species (Fig. 3). Therefore, POFF, which contains only HDL, may provide us with a unique model for studying the LCAT reaction as affected by specific lipoproteins and of simplifying the preparation of purified LCAT.

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Effect of Dietary Cyclopropene Fatty Acids on the Octadecenoates of Individual Lipid Classes of Rat Liver and Hepatoma

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ABSTRACT

The effect of dietary cyclopropene fatty acids on the concentration of octadecenoate chain positional isomers in individual lipid classes of normal liver, host liver, and hepatoma 7288CTC has been determined. The data revealed the following: (a) Saturated and monoene fatty acid percentages of liver phosphatidylcholines and phosphatidylethanolamines were *not* affected, but the percentage of saturated fatty acids of the triglycerides and cholesteryl esters was increased while the monoene percentages decreased. (b) Oleate to vaccenate percentage ratios, previously shown to be characteristic of individual lipid classes, were completely disrupted. (c) Oleate concentrations of the two major liver phospholipids were elevated, and vaccenate levels were dramatically reduced. (d) Cyclopropene fatty acids appear to inhibit monoene elongation. (e) The elevated concentrations of oleate indicate that an alternate route of oleate biosynthesis must exist if the Δ9 desaturation is inhibited by cyclopropene fatty acids as reported previously. (f) In contrast to liver, oleate and vaccenate concentrations in hepatoma were not affected by the dietary cyclopropene fatty acids.

INTRODUCTION

Laboratory diets supplemented with cyclopropene fatty acids have been shown to cause a variety of biological effects: altered egg production and fertility (1), increased levels of saturated body fat in swine (2,3), and neonatal and postnatal mortality in rodents (4,5). Cyclopropene fatty acids act synergistically with aflatoxins in hepatoma induction (6,7) and stimulate cell division of liver paraenchymal cells (8). The mechanism of action of the cyclopropene fatty acids is not known, but it has been shown by a number of investigators (9-11) that these acids inhibit monoene fatty acid biosynthesis.

One of the most frequently reported lipid abnormalities in hepatomas is the elevation of the C_{18} monoene fraction which we have shown consists primarily of two *cis* isomers: oleic and vaccenic acids (12,13). The ratio of these two monoenes differs for each of the major lipid classes of normal liver, whereas the major lipid classes of hepatoma exhibit the same approximate ratio of oleic to vaccenic acid (13). Since cyclopropene fatty acids inhibit liver monoene biosynthesis, we wanted to determine what effect these fatty acids would have on oleic and vaccenic acid concentrations in hepatoma lipid classes. The lack of a detailed examination of the effects of dietary cyclopropene fatty acids on oleic and vaccenic acid concentration in individual phospholipid classes also prompted us to examine normal and host liver. A preliminary report of these data has appeared (14).

METHODS AND MATERIALS

Groups of three male Buffalo rats (175-225 g) were placed on a fat-free diet supplemented with either 0.5% *Sterculia foetida* oil (Diet A), or 1.0% *Sterculia foetida* oil plus 0.37% safflower oil (Diet B). The added safflower oil brought the linoleate content of diet B to 0.4%. One group of animals on diet A was implanted bilaterally with Morris minimal deviation hepatoma 7288CTC at the time the rats were placed on the diet. After 4 weeks, the normal and hepatoma bearing (host) rats were killed by decapitation. Individual livers and hepatomas were excised, weighed, lyophilized, and reweighed. Total lipids were extracted twice by the Bligh and Dyer procedure (15), fractionated into neutral lipids and phospholipids (16), and the fractions quantitated gravimetrically. Neutral lipid classes were quantitated by high temperature gas liquid chromatography (GLC) of the intact lipids (17). Phospholipids were resolved into individual classes by thin layer chromatography (TLC) as described previously (18) and quantified by the phosphorous method (19). Individual lipid classes were resolved on preparative chromatoplates and visualized by UV light after the plates were sprayed with 2′,7′-dichlorofluorescein or Rhodamin 6G. The desired lipid classes were removed from the plate and eluted quantitatively from the absorbent with chloroform-methanol-water (60:45:12). Fatty acid methyl

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esters were prepared from cholesteryl esters, triglycerides, phosphatidylcholines and phosphatidylethanolamines and quantitated by GLC as described previously (17). Octadecenoate fractions were isolated from the lipid class methyl esters by preparative GLC. The quantity of the positional isomers in the octadecenoate fraction was determined by ozonolysis, a modification of the Beroza and Bierl procedure (20), as described previously (12).

Sterculia foetida seed oil was prepared by hexane extraction of seeds. The cyclopropene fatty acid content (sterculic plus malvalic acids) was about 55%, as determined by GLC (21). The other fatty acids of *Sterculia foetida* oil consisted of palmitate (24%), oleate (10%), and linoleate (10%). The unpreserved cold pressed safflower oil was purchased locally and contained 80% linoleic acid. The fat-free diet was obtained from Nutritional Biochemicals, Cleveland, OH, formulated according to Wooley and Sebrell (22). The source and purity of lipid standards and solvents were the same as given previously (23). Other chemicals were reagent grade or better.

RESULTS

Animal and tumor growth rates were unaltered by the low levels of *Sterculia foetida* oil. Rats on diet B gained an average of 65 g during the experiment while control animals fed the fat-free diet supplemented with 0.5% safflower oil showed the same average gain (64 g).

Quantity of Lipids

The quantities of dry matter, total neutral lipids, total phospholipids, and five individual lipid classes derived from normal liver, host liver, and hepatoma of rats fed low levels of *Sterculia foetida* oil are given in Table I. Except liver triglycerides, the cyclopropene fatty acids resulted in only marginal changes in lipid concentration, which generally fell between the range of values reported previously for the respective tissues of animals fed chow and fat-free diets (23,24). Liver triglyceride concentrations were approximately half that of the fat-free levels but double the concentration of chow fed animals reported earlier (23). The reduced levels of dry matter, phosphatidylcholine, phosphatidylethanolamine, and triglycerides in the hepatoma, relative to liver, were also similar to those noted previously (23,24).

Fatty Acids of Lipid Classes

Percentages of major fatty acids in the four major lipid classes derived from normal liver,

TABLE I

Concentration of Neutral Lipids and Phospholipids in Normal Liver, Host Liver, and Hepatoma from Rats Maintained on a Fat-Free Diet Supplemented with Low Levels of *Sterculia foetida* Oil

Hepatic tissue and dietary treatment[a]	Quantity (mg/g wet wt)[b]							
	Dry matter	Total phospholipids	PC[c]	PE	Total neutral lipids	CH	TG	CE
Normal liver, diet A	288.8 ± 6.2	27.8 ± 2.9	12.1 ± 1.3	6.2 ± 0.8	25.4 ± 4.5	2.1 ± 0.5	21.3 ± 4.1	0.7 ± 0.2
Normal liver, diet B	300.7 ± 4.2	35.7 ± 0.7	15.3 ± 0.1	7.0 ± 0.1	25.4 ± 9.0	2.3 ± 0.2	20.7 ± 8.7	0.8 ± 0.3
Host liver, diet A	285.4 ± 5.2	28.9 ± 4.3	11.8 ± 2.3	7.4 ± 1.8	9.2 ± 5.5	2.9 ± 0.3	5.1 ± 5.5	0.3 ± 0.1
Hepatoma, diet A	179.3 ± 5.7	10.9 ± 0.7	4.0 ± 0.2	2.6 ± 0.1	11.0 ± 1.5	3.0 ± 0.3	4.4 ± 1.4	2.9 ± 0.3

[a]Rats were removed from stock diet and fed one of the following diets for 4 weeks: Diet A, fat-free + 0.5% *Sterculia foetida* oil; Diet B, fat-free diet + 1.0% *Sterculia foetida* oil + 0.37% safflower oil.

[b]Values represent the mean of three animals ± standard deviation.

[c]Abbreviations are: PC, phosphatidylcholine; PE, phosphatidylethanolamine; CH, cholesterol; TG, triglycerides; CE, cholesteryl ester.

host liver, and hepatoma of animals fed low levels of *Sterculia foetida* oil are given in Table II. Doubling the concentration of *Sterculia foetida* oil and increasing the level of linoleic acid in diet B to 0.4% had very little effect on the fatty acid composition. The major differences between normal liver and host liver were restricted to the increased percentage of 22:6 in phosphoglycerides and decreased percentages of 18:0 and 18:1, offset by an increase in 16:0, in cholesteryl esters, and increased triglyceride 18:2 percentages of host liver. These changes in the fatty acid composition of host liver lipid classes brought about by the hepatoma agree with those reported previously (23,24). The percentage of monoenes was reduced and saturated fatty acids increased in liver triglycerides and cholesteryl esters of animals fed the *Sterculia foetida* oil diets relative to values reported for chow and fat-free fed animals (23,24). Liver phospholipids from these animals did not exhibit significant change in monoene percentages, and stearate percentages were lower than values from animals fed chow and fat-free diets (23,24).

The fatty acid composition of hepatoma triglycerides and cholesteryl esters differed dramatically from liver, but was virtually unaffected by the *Sterculia foetida* oil diets. Palmitate and 18:1 percentages of hepatoma phosphatidylcholines and phosphatidylethanolamines were unaffected by the cyclopropene fatty acid diets, but 18:0, 18:2, and 20:4 percentages were increased relative to values reported earlier for this hepatoma obtained from animals fed chow and fat-free diets (23,24). The decreased percentages of 16:0 in hepatoma triglycerides, cholesteryl esters, and phosphatidylethanolamines, the increased percentages of C-20 and C-22 fatty acids in the neutral lipids, the decreased 22:6 percentages in phospholipids, and the higher percentages of 18:1 in phospholipids of hepatoma relative to liver agreed with earlier reports (23,24).

Isomeric Octadecenoates of Lipid Classes

The percentage distributions and concentrations of the two major *cis* octadecenoate isomers in individual lipid classes derived from normal liver, host liver, and hepatoma of rats maintained on fat-free diet supplemented with low levels of *Sterculia foetida* oil are given in Table III. The percentages and concentrations have not been corrected for five other isomers present in the octadecenoate fractions from animals fed the diets supplemented with *Sterculia foetida* oil. The sum of the percentages of these fatty acids, which have not yet been characterized and identified, represented from 10 to 25% of the octadecenoate fractions, depending on the lipid class. Values are also given in Table III for the respective tissues and classes previously obtained from animals fed only a fat-free diet (13). Despite a wide range of concentrations, the percentages of the two octadecenoate isomers in normal liver lipids of animals fed the *Sterculia foetida* diets were similar to host liver percentages from animals fed the same diet. Generally, the percentages of the Δ9 (oleate) and Δ11 (vaccenate) isomers were similar in all four lipid classes of normal and host liver when the animals were fed the diets containing cyclopropene fatty acids. The shift to the higher percentages of oleate, produced by the *Sterculia foetida* oil diets, was similar to what was observed for host liver of animals fed the fat-free diet, but not for normal livers of animals fed the fat-free diet. Each lipid class of normal liver from rats fed the fat-free diet contained different percentages of oleate and vaccenate, which we have previously shown to be affected very little by diet (13). Except for 18:1 concentrations of triglycerides, oleate and vaccenate percentages and concentrations in the hepatomas were not affected by the *Sterculia foetida* diets.

The fatty acid composition of normal liver sphingomyelin from animals fed the 1.0% *Sterculia foetida* oil diet was 16:0, 23%; 18:0, 32%; 18:1, 1.5%; 20:0, 3.2%; 22:0, 11.6%; 22:1, 3.0%; 24:0, 16.5%; and 24:1, 5.8%. The percentages of palmitate and stearate are much higher and the percentage of 24:0 and 24:1 are much lower than values reported previously for liver sphingomyelin obtained from animals maintained on chow and fat-free diets (23).

DISCUSSION

Effect of Cyclopropene Fatty Acids on Liver Tissue Fatty Acids

A comparison of the fatty acid compositions of the liver from animals fed the *Sterculia foetida* oil diets (Table II) with previously reported compositions from animals fed chow and fat-free diets (23,24) indicates that the cyclopropene fatty acids do not affect the percentage of saturated and monounsaturated fatty acids of the phospholipids, but affect only the neutral lipid percentages. The increased percentage of saturated fatty acids produced by cyclopropene fatty acids has been reported previously by a number of investigators (25-28) from analyses of total lipids, but apparently the lack of effect on the two major phospholipid classes has not been noted. Pullarkat et al. (28) have reported that the total liver phospholipids

TABLE II

Composition of the Major Fatty Acids Derived from the Four Major Lipid Classes of Normal Liver, Host Liver, and Hepatoma from Rats Maintained on a Fat-Free Diet Supplemented with Low Levels of *Sterculia foetida* Oil

Hepatic tissue and dietary treatment[a]	Fatty acid percentages[b]						
	16:0	16:1	18:0	18:1	18:2	20:4	22:6
Triglycerides							
Normal liver, diet A	50.5 ± 1.1	3.4 ± 0.2	16.9 ± 1.0	25.5 ± 1.9	1.2 ± 0.3	T[c]	
Normal liver, diet B	48.4 ± 1.2	3.8 ± 0.5	21.9 ± 0.9	23.1 ± 1.7	1.1 ± 0.3		
Host liver, diet A	37.9 ± 4.4	1.1 ± 0.2	16.4 ± 7.8	22.9 ± 6.8	11.8 ± 8.4	4.2 ± 3.0	3.7 ± 2.5
Hepatoma, diet A[d]	15.8 ± 1.4	1.3 ± 0.1	16.0 ± 0.7	21.9 ± 2.9	12.3 ± 0.2	12.9 ± 1.8	4.7 ± 0.7
Cholesteryl esters							
Normal liver, diet A	63.4 ± 1.8	4.3 ± 0.1	14.9 ± 0.9	10.1 ± 1.1	2.2 ± 0.2	3.1 ± 0.7	
Normal liver, diet B	55.4 ± 8.9	4.6 ± 1.7	21.0 ± 3.7	13.0 ± 2.4	3.8 ± 0.3	1.3 ± 0.2	
Host liver, diet A	73.7 ± 5.7	1.0 ± 0.4	8.9 ± 0.7	6.2 ± 1.9	4.3 ± 3.0	3.7 ± 2.1	0.9 ± 0.4
Hepatoma, diet A[e]	7.4 ± 0.3	1.0 ± 0.1	8.0 ± 0.8	18.1 ± 1.8	7.5 ± 0.7	19.7 ± 1.4	6.9 ± 1.2
Phosphatidylcholines							
Normal liver, diet A	26.6 ± 0.8	2.4 ± 0.2	27.6 ± 0.6	17.0 ± 0.7	11.3 ± 1.4	8.8 ± 1.0	3.5 ± 0.2
Normal liver, diet B	26.8 ± 1.1	3.3 ± 0.3	24.2 ± 0.3	16.3 ± 1.3	15.5 ± 0.8	5.9 ± 0.2	3.4 ± 0.4
Host liver, diet A	27.1 ± 3.0	T	25.0 ± 1.4	11.7 ± 4.3	13.6 ± 5.1	11.0 ± 2.9	9.0 ± 2.6
Hepatoma, diet A	25.4 ± 1.1	1.6 ± 0.4	17.8 ± 1.1	23.9 ± 1.9	13.9 ± 1.6	10.2 ± 0.9	2.1 ± 0.2
Phosphatidylethanolamines							
Normal liver, diet A	22.7 ± 1.2	T	28.1 ± 1.7	5.5 ± 0.2	5.1 ± 0.6	23.4 ± 2.6	15.1 ± 2.4
Normal liver, diet B	23.3 ± 0.5	0.8 ± 0.1	28.2 ± 0.8	6.9 ± 0.9	6.6 ± 0.1	19.8 ± 0.8	11.3 ± 1.5
Host liver, diet A	21.9 ± 3.2	T	25.5 ± 2.5	3.6 ± 1.8	6.4 ± 1.7	19.6 ± 1.8	19.5 ± 2.3
Hepatoma, diet A	5.8 ± 0.5	T	33.4 ± 0.6	20.7 ± 1.2	10.7 ± 1.2	18.1 ± 1.3	3.8 ± 0.4

[a]Rats were removed from stock diet and fed one of the following diets for 4 weeks: Diet A, fat-free diet + 0.5% *Sterculia foetida* oil; Diet B, fat-free diet + 1.0% *Sterulia foetida* oil + 0.37% safflower oil.

[b]Values represent the mean of three animals ± standard deviation. The difference between the sum of the percentage in any row and 100% represents the sum of other fatty acids not given in the table.

[c]Detectable quantities of less than 0.5%.

[d]The following fatty acids were also present in the percentages indicated: 14:0, 0.8%; 20:0, 0.8%; 20:1, 2.4%; 20:2, 0.9%; 20:3, 0.9%; 22:3 + 24:0, 1.7%; 24:1 + 22:4, 5.5%; and 22:5, 1.6%.

[e]The following fatty acids were also present in the percentages indicated: 14:0, 1.2%; 20:0, 1.2%; 20:1, 4.2%; 20:2, 1.6%; 20:3, 2.6%; 22:3 + 24:0, 1.7%; 24:1 + 22:4, 10.3%; and 22:5, 5.4%.

TABLE III

Effect of Cyclopropene Fatty Acids on the Percentages and Concentrations of Octadecenoate Isomers in Individual Lipid Classes Derived from Normal Liver, Host Liver, and Hepatomas

Hepatic tissue and dietary treatment[a]	Isomeric octadecenoate percentages and concentrations (mg/g wet wt)[b]							
	Phosphatidylethanolamines		Phosphatidylcholines		Triglycerides		Cholesteryl esters	
	Δ9	Δ11	Δ9	Δ11	Δ9	Δ11	Δ9	Δ11
Normal liver								
Diet A[c]	82(0.19)	18(0.04)	88(1.23)	12(0.16)	91(4.19)	9(0.40)	82(0.01)	18(<0.01)
Diet B[c]	74(0.24)	26(0.08)	92(1.53)	8(0.14)	94(5.62)	6(0.36)	86(0.03)	14(<0.01)
Fat-free[d]	42(0.11)	58(0.15)	54(0.82)	46(0.70)	82(12.98)	18(2.84)	73(0.08)	27(0.03)
Host liver								
Diet A	74(0.14)	26(0.04)	83(0.77)	17(0.14)	88(1.09)	12(0.11)	81(<0.01)	19(<0.01)
Fat-free[d]	55(0.06)	45(0.05)	74(0.48)	26(0.16)	88(6.56)	12(0.89)	88(0.14)	12(0.02)
Hepatoma								
Diet A	74(0.32)	26(0.10)	71(0.47)	29(0.19)	78(0.71)	22(0.20)	72(0.17)	28(0.06)
Fat-free[d]	64(0.28)	36(0.16)	66(0.47)	34(0.24)	71(1.28)	29(0.52)	70(0.11)	30(0.05)

[a]Diet A, fat-free diet + 0.5% *Sterculia foetida* oil; Diet B, fat-free + 1.0% *Sterculia foetida* oil + 0.37% safflower oil.

[b]The values given for diet A represent the mean of duplicate determinations on each of three animals, whereas the values given for diet B and the fat-free diet represent the means of duplicate determination on a composite sample from three animals. Concentrations were calculated from Tables I and II and are given in parentheses.

[c]Percentages and concentrations have not been corrected for five other components present in the octadecenoate fractions which have not been characterized and identified. The sum of these unidentified compounds ranged from 10 to 25%, depending on the lipid class, and were present only in the lipids from animals fed diets A and B.

[d]The values were taken from previously published data [Lipids 10:746 (1975)] and are given for comparison.

of young rats fed methyl sterculate contained the same percentage of palmitate and oleate as control animals, but stearate percentages were elevated. Since our results indicate that phosphatidylcholine and phosphatidylethanolamine stearate percentages were not increased, some other phosphoglyceride classes may be affected by cyclopropene fatty acids. These results now make it clear why Nixon et al. (27) found that adipose tissue (primarily triglycerides) was affected more by *Sterculia foetida* oil diets than were rat liver microsomes which contain primarily phospholipids.

Effect of Cyclopropene Fatty Acids on Octadecenoate Isomers

With the exception of Pullarkat et al. (28), who examined octadecenoate isomers of total liver phospholipids from rats fed methyl sterculate, previous investigators had not examined the octadecenoate isomers of individual lipid classes. Therefore, it was assumed that the decrease in the octadecenoate fraction of total lipids from animals fed cyclopropene fatty acids was due to a decrease in oleate. Analyses of the octadecenoate isomers in the present study indicate that dietary cyclopropene fatty acids resulted in a decrease in vaccenic acid. Actually, oleate concentrations increased in liver phospholipids relative to animals fed the fat-free diet (Table III). Liver triglycerides do not show a decrease in oleate concentrations when the comparison is made with animals fed a chow diet (13), which does not cause an accumulation of liver triglycerides as the fat-free diet does (24). Since liver oleate concentrations are not reduced by dietary cyclopropene fatty acids, an alternate pathway for oleate biosynthesis must exist because it has been demonstrated quite clearly by a number of investigators that the desaturation of stearic acid to give rise to oleic acid is inhibited by cyclopropene fatty acids (9-11,29,30). An alternate route of oleate biosynthesis in rat liver was first proposed by Reiser and Raju (9) and later in chick liver by Donaldson (31). Additional work on the alternate route has indicated that lauric acid may be involved (32). Our mass data not only supports the existence of an alternate route of oleate biosynthesis, but also indicate that this pathway is capable of maintaining cellular oleate concentrations when the stearoyl-CoA desaturase system is inhibited by cyclopropene fatty acids.

The decreased concentrations of vaccenic acid in all the liver lipid classes from animals fed the *Sterculia foetida* oil diets appear to result from the inhibition of monoene elongation. Holloway and Wakil (33) demonstrated that vaccenic acid in liver arises from the elongation of palmitoleic acid. The decreased vaccenic acid does not appear to be due to a lack of substrate (palmitoleic acid) for elongation. Normal liver triglycerides and cholesteryl esters from rats fed the cyclopropene fatty acids contained 3.4% and 4.3% palmitoleic acid, respectively. These percentages are approximately the same as 5.0% found in liver of animals fed a chow diet, but much lower than the 14.2% found in liver triglycerides of animals fed a fat-free diet (24). The higher percentage of palmitoleate, however, did not change the oleate to vaccenate ratio which we have shown to be unaffected by extremes in diet (13). The phospholipids contained as much palmitoleic acid as the phospholipids from animals fed only a fat-free diet (24). It has also been shown that palmitate and stearate desaturation is inhibited to the same approximate degree by cyclopropene fatty acids (11,30). Further supportive evidence indicating that cyclopropene fatty acids inhibit monoene elongation is the dramatic reduction of the levels of C-22, C-23, and C-24 monoenes in sphingomyelins from the animals on diets A and B, an observation made previously by Pullarkat et al. (28). The cyclopropene fatty acids may also inhibit, to some degree, the elongation of saturated fatty acids and the desaturation and elongation of polyunsaturated fatty acids. The percentage of 24:0 in liver sphingomyelin from animals fed the *Sterculia foetida* oil was approximately half that in chow or fat-free fed animals (23), and stearate percentages were doubled. Pullarkat et al. (28) have reported similar changes in 24:0 and 18:0 percentages in liver sphingomyelin from young rats fed methyl sterculate. Liver phosphatidylcholine from animals fed diets A and B contained slightly lower percentages of 20:4 and higher percentages of 18:2 than reported previously for animals fed fat-free or chow diets. Total liver phospholipids from animals fed methyl sterculate (28) and total liver lipids from animals fed for long time periods (26) also exhibited a rise in 18:2 percentages and a decrease in 20:4 percentages. This apparent lowering of 20:4 percentages may be the result of secondary effects and not due to direct inhibition by the cyclopropene fatty acids. Jeffcoat and Pollard (30) have found recently that cyclopropene fatty acids have no effect on Δ6 desaturase in vitro.

The cyclopropene fatty acids markedly disrupted oleate and vaccenate percentages (Table III) previously shown to be characteristic of individual lipid classes from normal liver and virtually unaffected by extremes in diet (13). The dramatic increase in the percentage of

oleate, particularly in the phospholipids, resulted in all lipid classes of liver having the same approximate oleate to vaccenate percentage ratios, similar to hepatoma.

Effect of Cyclopropene Fatty Acid on Hepatoma Lipids

The *Sterculia foetida* oil diets consumed by the host animals had little or no effect on the quantity of lipid classes or the concentration of octadecenoate isomers in the hepatoma relative to values of hepatomas from host animals fed fat-free or chow diet (13,23,24). These observations agree with our previous studies that showed extremes in diet had little effect on the fatty acid composition in lipid classes of the hepatoma (16,23,24). It has also been shown that the fatty acid synthesizing enzymes of tumors are also unaffected by diet (34-36). The reason why the hepatoma is not affected by the cyclopropene fatty acids is not really apparent; however, there are a number of possibilities. The cyclopropene fatty acids may have been removed by host tissues, they may not have entered the hepatoma cell, and enzymes normally affected in liver may be missing or may have already been suppressed in the hepatoma. We have previously shown that *Sterculia foetida* oil had no effect on the fatty acid composition of hepatoma cells grown in culture (17) and have demonstrated that hepatoma cells grown in culture can elongate palmitoleate and desaturate stearate (37). Other Morris hepatomas have also been shown to contain stearoyl-CoA desturase activity (36,38). These data indicate that the cyclopropene fatty acids may not be able to enter the hepatoma cell, but additional work is needed to determine if this speculation is correct.

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Initial Cholesterol Uptake by Everted Sacs of Rat Small Intestine: Kinetic and Thermodynamic Aspects

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ABSTRACT

The kinetics of initial cholesterol uptake by everted rat proximal and distal small intestinal sacs were evaluated in vitro. The mucosal incubation solution consisted of 0.05 mM cholesterol solubilized in 4.8 mM sodium taurocholate micellar solution at pH 7.4. Experiments were performed at temperatures from 26 to 38 C. The rate of cholesterol uptake followed a linear relationship when plotted against time indicating an apparent zero-order kinetics mechanism for initial uptake. An Arrhenius plot of the results of uptake versus temperature remained linear over the entire range of temperatures studied. The large free energy of activation (20 kcal/mole) suggests that an energy barrier for cholesterol uptake exists at the enterocyte luminal cell membrane and may be an important limiting step in cholesterol uptake. It is proposed that a transient association between cholesterol and a component of the enterocyte luminal cell membrane is formed during initial uptake of cholesterol. The transient association may be an activated complex formed with proteins present at or within the luminal enterocyte cell membrane.

INTRODUCTION

The two sources of cholesterol in mammals are endogenous synthesis and intestinal absorption. Since cholesterol is a precursor for hormones and bile salts and may be an important factor in the genesis of atherosclerosis, some aspects of its absorptive processes have been studied. Cholesterol is thought to be transported into the lymphatic circulation by a simple diffusion process (1) which depends on bile salts for its absorption (2-4). Cholesterol absorption in the presence of triglycerides is directly proportional to the chain length of the triglyceride fatty acid (5,6) and is modified by the simultaneous absorption of triglycerides' hydrolysis products (7).

The purpose of the present study is to determine the kinetic and thermodynamic parameters of cholesterol's initial intestinal uptake. Analysis of these parameters should provide insight into possible interactions that may occur between cholesterol and components of the enterocyte cell membrane during absorption.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Spartan, Haslett, MI) weighing 150-200 g had free access to water and Purina Rat Chow (Ralston Purina Co., St. Louis, MO). The rats were not fasted prior to experimentation. At least five rats were used for each study.

Materials

[$^{3}H_{1\alpha,2\alpha(n)}$] cholesterol (Amersham/Searle Corp., Arlington Heights, IL) with specific activity of 43 Ci/mmole was used as a tracer compound. The radiochemical purity of the compound was ascertained by thin layer chromatography (TLC) on silica gel developed in cyclohexane-ethylacetate (6:4) and was found to be greater than 98%. Nonradioactive cholesterol (Sigma Chemical Co., St. Louis, MO) had less than 1% impurities. [Carboxylic ^{14}C]-inulin (Amersham/Searle Corp.), with specific activity of 7.7 mCi/mmole, was used as a nonabsorbable marker (8). Purified grade sodium taurocholate (K & K Laboratories, Plain View, NY) was found by TLC (9) to have 2-5% impurities consisting primarily of cholic acid. Cholic acid (Sigma Chemical Co.), oleic acid (Fisher Scientific Co., Fairlawn, NY), USP grade, and glycerol monooleate (Calbiochem

TABLE I

Steady State Volumes of Adherent Fluid Compartments Determined at Various Temperatures

Temperature (C)	Volume of adherent fluid compartment[a] (µl/100 mg dry wt tissue, $\bar{X}$ ± SE) Proximal jejunum	Distal ileum
26	20.00 ± 1.22	21.00 ± 1.68[b]
28	19.87 ± 2.05	19.26 ± 1.26[b]
30	20.52 ± 1.84	19.89 ± 1.20[b]
34	21.25 ± 1.63	22.81 ± 0.57[b]
38	21.73 ± 0.66	22.02 ± 0.67[b]

[a]The figures represent mean ± standard error for five different rat experiments.

[b]$P > 0.05$; statistical comparison was made at a given temperature of distal ileum versus proximal jejunum using the Student's t-test.

TABLE II

Influence of Cholic Acid on Uptake of 0.05 mM Cholesterol

Segment	Time (min)	Cholesterol uptake, nmol/100 mg dry sac wt ($\bar{X}$ ± SE, n = 5)	
		Control[a]	Cholic acid[b]
Proximal jejunum	2	2.85 ± 0.44	2.96 ± 0.08
	4	5.25 ± 0.38	5.62 ± 0.34
	6	7.93 ± 0.45	7.68 ± 0.36
	8	9.65 ± 0.35	10.84 ± 0.40
	10	11.80 ± 0.45	12.60 ± 0.51
Distal ileum	2	2.21 ± 0.29	2.67 ± 0.04
	4	3.92 ± 0.37	4.68 ± 0.31
	6	5.64 ± 0.47	6.98 ± 0.17
	8	7.00 ± 0.47	7.32 ± 0.28
	10	8.36 ± 0.66	9.33 ± 0.35

[a]The standard micellar incubation contained 4.8 mM sodium taurocholate of 99% purity.

[b]0.24 mM cholic acid was added to the standard micellar incubation solution which contained 4.8 mM sodium taurocholate.

Co. Los Angeles, CA) were more than 98% pure. The micellar solution was prepared by ultrasound irradiation for 5 min at 60 watts of power with a sonicator (Artek Corp. Farmingdale, NY). The final micellar incubation solution contained 0.05 mM cholesterol, 4.8 mM sodium taurocholate, 0.6 mM oleic acid, 0.3 mM glycerol monooleate, 11.1 mM glucose, 130 mM sodium chloride, 0.45 mM citric acid, 12.38 mM disodium hydrogen phosphate, and tracer amounts of ^3H cholesterol and ^{14}C inulin. The solution's pH was 7.4. The osmolarity of the final solution varied between 295 and 310 mosmoles/liter (10).

Preparation of Everted Sacs

The rat was sacrificed by stunning and cervical dislocation. The small intestine was exposed by midline incision and rinsed in situ with chilled saline (NaCl, 154 mM). The entire small intestine was then removed and everted. A 10-cm segment distal to the ligament of Treitz was designated as the "proximal jejunum," and a 10-cm segment of the small bowel immediately proximal to the ileocecal valve was designated as the "distal ileum." Each segment was subdivided with sutures (silk 4-0 sutupak, Ethicon, Inc., Somerville, NJ) into five 1.5-cm sacs identified with tags indicating their exact origin. Peyer's patches were avoided. No fluid was placed in the serosal compartment of the sacs since insoluble amphiphiles similar to cholesterol are not usually transported into the serosal compartment in-vitro (11-13).

Incubation Method

The everted sacs were immediately immersed in 50 ml of the micellar incubation solution which was contained in a plexiglass incubation chamber with internal dimensions of 2 x 6 x 30 cm. The chamber was positioned in a metabolic water bath (Precision Scientific Co., Chicago, IL) and was agitated at 80 oscillations/min. The micellar incubation solution was aerated with oxygen and equilibrated to the desired temperature. Preincubation samples were withdrawn in triplicate and were used for calculation of cholesterol's specific activity. Proximal and distal sacs were removed from the chamber every 2 min for a total period of 10 min. The sacs were immediately immersed for 15 sec. in 200 ml of 1 mM sodium taurocholate solution which was stirred by a magnetic stirrer at a constant rate. The rinse was designed to remove some of the incubation solution that had remained adherent to the sac walls (12). The ratio between inulin and cholesterol in the rinse solution was similar to their ratio in the bulk incubation medium. Each sac was then dried at 50 C under 20 inches of mercury vacuum for 24 hr prior to weighing.

Radioactivity Determinations

The radioactivity of the absorbed cholesterol and the adsorbed inulin in each sac was assayed by total combustion of the tissue with a sample oxidizer (Tri-Carb Model 306, Packard Instrument Co., Downers Grove, IL). The ^3H cholesterol and ^{14}C inulin were thus converted to tritiated water and $^{14}CO_2$ gas, respectively. Monophase®-40 (Packard Instrument Co.) was used as a scintillator for tritiated water. Carbasorb® and permafluor®V (Packard Instrument Co.) were used for $^{14}CO_2$ determination. A liquid scintillation counter (Beckman LS 250, Fullerton, CA) with an automatic quench

TABLE III

Kinetic Data Obtained In Vitro for Cholesterol Uptake in Rat Small Intestine at 0.05 mM Mucosal Solution

Segment	Time (min)	Cholesterol uptake, nm/100 mg·dry sac wt ($\bar{X}$ ± SE, n = 5)				
		26 C	28 C	30 C	34 C	38 C
Proximal jejunum	2	1.83 ± 0.18	1.51 ± 0.24	2.23 ± 0.28	1.91 ± 0.28	2.85 ± 0.44
	4	3.56 ± 0.31	2.83 ± 0.14	3.88 ± 0.26	3.66 ± 0.30	5.25 ± 0.38
	6	5.26 ± 0.35	5.08 ± 0.70	5.96 ± 0.24	6.03 ± 0.59	7.93 ± 0.45
	8	6.97 ± 0.51	7.20 ± 0.70	8.14 ± 0.67	8.38 ± 0.69	9.65 ± 0.35
	10	8.68 ± 0.43	9.34 ± 0.63	9.99 ± 0.83	10.68 ± 0.19	11.80 ± 0.45
Distal ileum	2	1.94 ± 0.10	2.37 ± 0.22	2.91 ± 0.23	3.02 ± 0.07	2.21 ± 0.29
	4	3.57 ± 0.24	4.18 ± 0.39	4.51 ± 0.25	4.43 ± 0.38	3.92 ± 0.37
	6	5.45 ± 0.27	5.55 ± 0.42	5.63 ± 0.46	5.99 ± 0.33	5.64 ± 0.47
	8	6.67 ± 0.37	7.22 ± 0.42	6.86 ± 0.24	7.20 ± 0.35	7.00 ± 0.47
	10	6.91 ± 0.39	6.85 ± 0.41	7.79 ± 0.68	7.86 ± 0.82	8.63 ± 0.66

calibration at ambient temperature was used to determine all radioactivity measurements which were carried to a counting error of ± 1%.

RESULTS

Determination of Adherent Cholesterol in the Mucosal Fluid Compartment

Following the sodium taurocholate rinse, some incubation fluid remained adherent to the sac walls and, thus, added radioactive counts to those emitted by the absorbed cholesterol. To eliminate this overestimation, ^{14}C-inulin was used as a nonabsorbable marker (8) and was assumed to be homogeneously distributed between the bulk aqueous phase and the adherent fluid compartment. The specific volume of the adherent layer was determined for each segment at each time interval, and the amount of cholesterol present in that volume was then calculated and subtracted from the apparent total absorption value for cholesterol. The mean ± SE steady state volume of proximal and distal adherent fluid compartments at various temperatures were recorded (Table I). All experimental results reported in this paper were corrected by subtraction of the adsorbed amount of cholesterol from the total apparent amount of cholesterol uptake.

Influence of Cholic Acid on Cholesterol Uptake

The sodium taurocholate used in these experiments contained between 2 and 5% cholic acid. The possible influence of cholic acid on the uptake of cholesterol was investigated by adding cholic acid to the standard micellar incubation solution at a 0.24 mM final concentration. This concentration represents 5% of the final sodium taurocholate concentration. The experiments were conducted at 38 C. The addition of cholic acid to the incubation solution did not change the rate of cholesterol uptake (Table II).

Kinetic and Thermodynamic Parameters

Transport of cholesterol through intestinal membrane can be schematically depicted as follows:

$$C_1 \underset{k_b}{\overset{k_f}{\rightleftharpoons}} C_m \qquad \text{Scheme I}$$

if C_1 and C_m are the concentrations of cholesterol present at a given time in the mucosal fluid and in the enterocyte membrane respectively; and if k_f and k_b are rate constants of influx and efflux of cholesterol, respectively. If the forward and backward reactions are zero-order processes, according to the rate law, the following differential equation can be written for Scheme I.

$$dC_m/dt = k_f - k_b \qquad \text{Equation 1}$$

If $k_f - k_b = K$, and if K is an apparent zero-order rate constant of cholesterol uptake, Equation 1 can be expressed as

$$dC_m/dt = K \qquad \text{Equation 2}$$

By integrating Equation 2, Equation 3 can be obtained:

$$C_m = Kt \qquad \text{Equation 3}$$

The transfer process, noted in Scheme I, can be elaborated further by applying the activated complex theory (14) to the diffusion process and is depicted below in Scheme II. M represents the mucosal membrane component.

$$C_1 + M \rightleftharpoons (C_1 - M) \rightharpoonup C_m \qquad \text{Scheme II}$$

This scheme provides for the inclusion of an intermediate state of diffusion which may occur before the diffusion of the solute to a new equilibrium position and which is characterized by transient association of the reacting

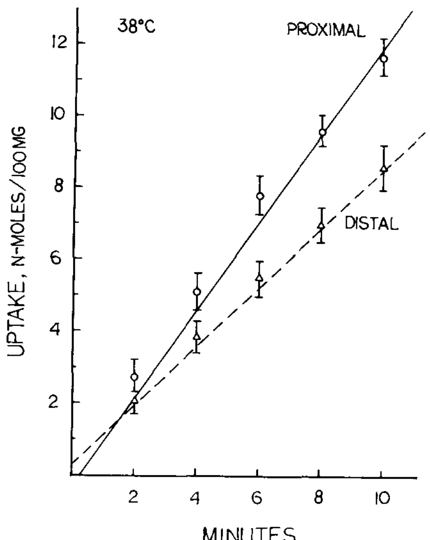

FIG. 1. Cholesterol uptake over a 10-min period at 0.05 mM concentration and 38 C incubation temperature. Each plotted point represents mean ± standard error for five different sacs from five different rat experiments. Regression lines were plotted by the least squares method. The composition of mucosal fluid, and the methods of uptake determination are described in the text.

molecules, C_1 and M, as an "activated complex."

The thermodynamic parameters of activation for mucosal membrane transfer of cholesterol can be determined as follows (Derivations are available upon request from the authors.):

$$\Delta H^* = Ea - RT \quad \text{Equation 4}$$
$$\Delta F^* = -2.303\ RT \log K^* \quad \text{Equation 5}$$
$$\Delta S^* = (\Delta H^* - \Delta F^*)/T \quad \text{Equation 6}$$

Where ΔH^* is the enthalpy of activation, Ea is the activation energy, R is the gas constant, T is the absolute temperature, ΔF^* is the free energy of activation, and K^*, which is the equilibrium constant of the activated complex, is equivalent to (Nh/RT)K where N and h are Avogadro's number and Planck's constant, respectively.

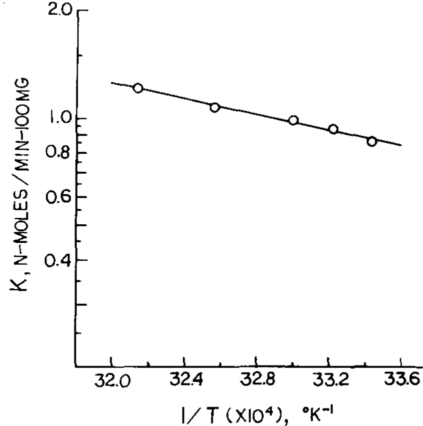

FIG. 2. Arrhenius plot of the cholesterol uptake from proximal jejunum, 0.05 mM concentrations at 26, 28, 30, 34, and 38 C incubation temperatures. The plot, which is based on log K = log A - Ea/2.303RT, was constructed on a semilog paper.

Uptake Kinetics

In order to determine the rate constant of cholesterol uptake at various temperatures, cholesterol uptake was measured versus time (Table III). A typical plot of an experimental series is shown (Fig. 1). The apparent zero-order constant of cholesterol uptake was calculated from the slope of cholesterol uptake versus time at each temperature (Table IV). The activation energy (Ea) was obtained from the slope of the Arrhenius plot of log K versus 1/T (Fig. 2). The thermodynamic parameters of activation ΔH^*, ΔF^*, and ΔS^* are listed in Table V.

DISCUSSION

A linear relationship between the amount of cholesterol uptake and time exists in the proximal jejunum and the distal ileum at incubation temperatures between 26 and 38 C (Table III). The linearity which fits best with Equation 3 indicates that cholesterol uptake by the small bowel is a zero-order process; that is,

TABLE IV

Rate Constants of Cholesterol Uptake at Various Temperatures

Uptake rate constants (nanomoles/100 mg/min)	Temperature				
	26 C	28 C	30 C	34 C	38 C
Proximal jejunum	0.8646[a]	0.9431[a]	0.9966[a]	1.0740[a]	1.2135[a]
Distal ileum	0.7231	0.7167	0.7417	0.7629	0.8063

[a]$P < 0.05$; comparison was made at a given temperature of proximal jejunum versus distal ileum.

TABLE V

Thermodynamic Parameters of Activation for the Cholesterol Uptake by Proximal Jejunum at Various Temperatures

	Temperature				
	26 C	28 C	30 C	34 C	38 C
ΔF^*, Cal/mole[a]	20,026	20,112	20,216	20,445	20,644
ΔH^*, Cal/mole[a]	4,316	4,312	4,308	4,300	3,292
ΔS^*, Cal/mole/K[a]	-52.5	-52.5	-52.5	-52.5	-52.5
Ea, Cal/mole[a]	4,910	4,910	4,910	4,910	4,910

[a]Calculations as explained in text.

the uptake rate of cholesterol is constant over the period of time studied.

The rate constant of cholesterol uptake by the proximal jejunum increased with the increase in temperature ($p < 0.05$) while that of the distal ileum was increased, but did not reach significance (Table IV). It was also observed that cholesterol uptake by the proximal jejunum was consistently greater ($p < 0.05$) than that by the distal ileum (Table III). A similar pattern of higher absorption rate by the proximal small bowel has been noted for vitamin K-1 (11), vitamin K-2 (12), vitamin D-3 (15), and retinol (13). Wilson and Dietschy (16) have calculated that the mucosal surface area including the microvillus surface of the rat's jejunum is almost twice that of the rat's ileum. The prominent difference in surface area is the most likely factor contributing to the differences in rate of cholesterol uptake observed in the present series of studies.

The linearity observed in the Arrhenius plot (Fig. 2) indicates that the mechanism of cholesterol uptake was the same over the entire range temperatures (26 to 38 C). The activation energy for cholesterol uptake of 4,910 cal/mole (Table IV) does not differ significantly from that observed by Schultz and Strecker (3). However, the large free energy of activation (ca. 20 kcal/mole) suggests that the energy barrier for cholesterol uptake across the mucosal cell membrane is higher than expected (Table IV). This value is considerably higher than the ΔF^* values for ordinary bulk diffusion, which generally fall within the range of 2-3 kcal/mole (14), indicating that the energy barrier is a major rate limiting step in cholesterol uptake.

The brush border of the intestinal mucosa consists of closely packed microvilli covered with mucopolysaccharide protein surface coat (17,18) which is believed to form an integral part of the "greater membrane" (19,20). The surface coat of the membrane is able to bind certain substances (21,22). Therefore, a barrier for cholesterol uptake could be the protein components of the cell membrane and the mucopolysaccharide surface coat. The high degree of specificity in absorption of steroids in vivo has been ascribed to lipid binding proteins at or within the enterocyte luminal cell membrane (23). Considering the above, we suggest that an activated complex could be formed by a transient association between the cholesterol molecules and proteins at the interface of the mucosal fluid and the membrane as depicted below.

$$C_1 \rightleftharpoons C_2$$
$$C_2 + M \rightleftharpoons (C_2 - M) \rightarrow C_3$$

where C_1 is the cholesterol molecules in the micelles within the luminal fluid, C_2 is the cholesterol monomers present in the immediate vicinity of the interface between the mucosal fluid and the mucosal cell membrane, $(C_2 - M)$ is the activated complex, and C_3 is the cholesterol released out of the membrane into the enterocyte's interior.

It is known that "icebergs" are promoted around the nonpolar portions of molecules in the aqueous media (24). An "iceberg" is a particular organized water structure enforced by the introduction of the hydrophobic groups into the bulk water phase. Water molecules are also absorbed to the surface of the cell membrane due to hydrogen bonding and ion-diploe interaction. Therefore, when the partially desolvated cholesterol molecules escape from the interface and collide against the surface of the cell membrane, a transient association is expected to be formed between cholesterol and the membrane proteins through hydrogen bonding of the hydroxyl polar group and the polar groups of the protein and/or mucopolysaccharides of the cell membrane as well as through hydrophobic interactions between the nonpolar groups of the cholesterol molecules and the nonpolar groups of the membrane components. In addition, there could be ion-diploe interaction between the charged sites of the membrane and the polar group of the cho-

lesterol molecule. Therefore, we propose that when the activated complex is formed between the cholesterol molecules and the components of the cell membrane through hydrogen bonding, Van der Waals interaction, and hydrophobic bonding, some water molecules associated with these interacting species are released, providing positive enthalpy of activation and large entropy values as shown in Table IV. These values of ΔH^* and ΔS^* observed for cholesterol uptake could be the net effect of all molecular interactions in the formation of the activated complex between cholesterol molecules and enterocyte cell membrane.

Analysis of the absorption of lipids out of a micellar solution is difficult because of the complexity of the system. Therefore, the proposed "activated complex" theory for cholesterol absorption should be viewed as a reasonable hypothesis which could explain the present experimental observations. However, it should be emphasized that the temperature dependency of the uptake of cholesterol by the enterocytes and the unexpectedly high free energy of activation (20 kcal/mole) could also be due in part to many of the steps which take place during transfer of cholesterol from the micelles to the organelles within the enterocyte. These steps include partitioning of cholesterol between the micelles and its monomers in the aqueous phase, permeation of cholesterol into the lipid cell membrane, diffusion through the cell membrane, and finally transfer from the cell membrane through the aqueous cytosol to the intracellular organelles (25,26). Further investigation into this complex chain of events will be necessary in order to clarify the relative contribution, if any, of each of these steps to the overall high free energy of activation which appears to be needed for the uptake of cholesterol by the enterocytes.

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ADDENDUM

Derivation of Equations to Determine Thermodynamic Parameters of Activation

The relationship between the apparent rate constant (K) for diffusion of a solute and the absolute temperature (T) is described by the Arrhenius equation (Equation 1),

$$K = A\text{Exp}(-Ea/RT) \qquad \text{Eq. 1}$$

where A is an Arrhenius constant, also known as a frequency factor, Ea is the activation energy, and R is a gas constant.

Upon taking the logarithm of both sides of Equation 1, Equation 2

$$\ln K = \ln A - Ea/RT \qquad \text{Eq. 2}$$

is derived which can be expressed as Equation 3.

$$\log K = \log A - Ea/2.303\ RT \qquad \text{Eq. 3}$$

According to Equation 3, a plot of log K versus 1/T would yield a straight line from the slope of which Ea can be calculated.

lesterol molecule. Therefore, we propose that when the activated complex is formed between the cholesterol molecules and the components of the cell membrane through hydrogen bonding, Van der Waals interaction, and hydrophobic bonding, some water molecules associated with these interacting species are released, providing positive enthalpy of activation and large entropy values as shown in Table IV. These values of ΔH^* and ΔS^* observed for cholesterol uptake could be the net effect of all molecular interactions in the formation of the activated complex between cholesterol molecules and enterocyte cell membrane.

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ADDENDUM

Derivation of Equations to Determine Thermodynamic Parameters of Activation

The relationship between the apparent rate constant (K) for diffusion of a solute and the absolute temperature (T) is described by the Arrhenius equation (Equation 1),

$$K = A\mathrm{Exp}(-Ea/RT) \qquad \text{Eq. 1}$$

where A is an Arrhenius constant, also known as a frequency factor, Ea is the activation energy, and R is a gas constant.

Upon taking the logarithm of both sides of Equation 1, Equation 2

$$\ln K = \ln A - Ea/RT \qquad \text{Eq. 2}$$

is derived which can be expressed as Equation 3.

$$\log K = \log A - Ea/2.303\ RT \qquad \text{Eq. 3}$$

According to Equation 3, a plot of log K versus 1/T would yield a straight line from the slope of which Ea can be calculated.

Based on the absolute reaction rate theory, Glasstone et al. (1) developed the relationship between the apparent rate constant and temperature by using the following sequence of reaction between A and B,

$$A + B \rightleftharpoons (A - B)^* \rightarrow C \qquad \text{Scheme A}$$

where $(A - B)^*$ is an activated complex of reactant molecules A and B formed in the transition state prior to the formation of product C. Laidler (2) used a universal frequency factor, γ, to describe the rate of product formation as follows:

$$dC/dt = \gamma(A - B)^* \qquad \text{Eq. 4}$$

where γ, defined below as Equation 5, represents the frequency of molecular collisions that occur between the reactant molecules during the reaction.

$$\gamma = RT/Nh \qquad \text{Eq. 5}$$

In Equation 5, R is the gas constant, N is the Avogadro's number, and h is the Planck's constant.

The equilibrium constant (K^*) for the formation of the activated complex for the reaction shown in scheme A is as follows:

$$K^* = (A - B)^*/(A)(B) \qquad \text{Eq. 6}$$

Rearranging Equation 6, yields

$$(A - B)^* = K^*(A)(B) \qquad \text{Eq. 7}$$

Substituting Equations 5 and 7 in Equation 4, yields

$$dC/dt = (RT/Nh)K^*(A)(B) \qquad \text{Eq. 8}$$

where $(RT/Nh)K^*$ gives a constant, and can be equated to the apparent rate constant, K

$$K = (RT/Nh)K^* \qquad \text{Eq. 9}$$

Since the free energy of activation, ΔF^*, can be determined by Equation 10

$$\Delta F^* = -2.303\, RT \log K^* \qquad \text{Eq. 10}$$

ΔF^* can be calculated on the basis of the apparent rate constant as shown below

$$\Delta F^* = -2.303\, RT \log(Nh/RT)K \qquad \text{Eq. 11}$$

The enthalpy of activation (ΔH^*) can be obtained as follows

$$\Delta H^* = Ea - RT \qquad \text{Eq. 12}$$

where Ea is obtained from Equation 3.

Since

$$\Delta F^* = \Delta H^* - T\Delta S^* \qquad \text{Eq. 13}$$

ΔS^* can be determined by rearranging Equation 13

$$\Delta S^* = (\Delta H^* - \Delta F^*)/T \qquad \text{Eq. 14}$$

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Formation of *trans*-12,13-Epoxy-9-Hydroperoxy-*trans*-10-Octadecenoic Acid from 13-L-Hydroperoxy-*cis*-9,*trans*-11-Octadecadienoic Acid Catalyzed by Either a Soybean Extract or Cysteine-$FeCl_3$ [1]

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ABSTRACT

A soybean extract or an ethanolic solution of cysteine and ferric chloride catalyzed the conversion of 13-L-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid to numerous products among which was *trans*-12,13-epoxy-9-hydroperoxy-*trans*-10-octadecenoic acid. When this fatty acid was treated further with the cysteine-ferric chloride solution, 9-hydroxy-12,13-epoxy-10-octadecenoic and 9-oxo-12,13-epoxy-10-octadecenoic acids were formed. Thus, *trans*-12,13-epoxy-9-hydroperoxy-*trans*-10-octadecenoic acid probably is an intermediate in the formation of the latter two compounds. Additionally, the *erythro* and *threo* isomers of *trans*-12,13-epoxy-11-hydroperoxy-*cis*-9-octadecenoic acid tentatively were identified as products.

INTRODUCTION

When catalyzed by aqueous extracts or lipoxygenase solutions from soybeans, hydroperoxylinoleic acid (LOOH) degrade into numerous oxygenated fatty acids. Among the product fatty acids are oxooctadecadienoic (1-3), hydroxyoctadecadienoic, dihydroxyoctadecenoic, trihydroxyoctadecenoic, dihydroxyhydroperoxyoctadecenoic (3), hydroxyepoxyoctadecenoic (3,4), and dimers (5). Many of the product fatty acids from soybean systems are structurally similar or identical to the oxygenated fatty acids obtained by the degradation of LOOH with other catalysts, such as cysteine and ferric chloride (6), hemoglobin (7), and extracts from pea (8) and potato (9). It is noteworthy that the chemical catalyst, cysteine-ferric chloride, produced so many of the products observed in biological systems. Recently, the correlation between the cysteine-ferric chloride catalyst and soybean extracts was extended to another product, oxoepoxyoctadecenoic acid (10).

In this communication, we described the degradation of 13-L-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid (I, see Fig. 1 for numerical key) into a novel product, *trans*-12,13-epoxy-9-hydroperoxy-*trans*-10-octadecenoic acid (II), by either a soybean extract or cysteine-ferric chloride catalyst. Indirect evidence pointed to the existence of other isomeric epoxyhydroperoxyoctadecenoic acids as well. Although II amounted to a comparatively small percentage of the total product mixture, it was demonstrated that II was probably an important intermediate in the formation of 9-hydroxy-*trans*-12,13-epoxy-*trans*-10-octadecenoic acid (III) and 9-oxo-*trans*-12,13-epoxy-*trans*-10-octadecenoic acid (IV).

METHODS

The methods employed were those reported previously (10) with exceptions as noted below.

The degradation of I by cysteine-ferric chloride, followed the procedures of Gardner et al. (6). The reaction was terminated before I was converted completely to products.

Product II was partially purified by column chromatography (CC) employing Mallinckrodt SilicAR CC-4 and a hexane-ether elution (6). Further purification was achieved by thin layer chromatography (TLC) on 250 μ thick plates composed of Silica Gel G. Hexane-ether-acetic acid, 50:50:1, was employed to separate II (R_f = 0.51), and hexane-ether, 6:4, was utilized to separate IIa (R_f = 0.38). After TLC isolation, product II or IIa usually contained 20-40% of an isomeric compound tentatively identified as *trans*-12,13-epoxy-*threo*-11-hydroperoxy-*cis*-9-octadecenoic acid (or its methyl ester). One TLC separation yielded IIa in better than 90% yield which permitted reasonably good analyses by spectral methods. For analytical purposes, a ferrous thiocyanate spray (destructive) was used to detect hydroperoxides on TLC plates (1).

Nuclear magnetic resonance (NMR) spectra were recorded with a Varian Model HA-100 at 100 MHz or with a Bruker WH-90 operating at 90 MHz. The samples were dissolved in

[1]Presented in part at the 13th World Congress, International Society for Fat Research, Marseilles, France, August 30-September 4, 1976, and the AOCS Meeting, Chicago, September 1976.

$CDCl_3$ with 1% tetramethylsilane as an internal reference.

Double bonds were saturated by hydrazine (11). A 2-mg sample of unsaturated fatty ester was added to 1 ml absolute ethanol containing 10 μl 64% hydrazine. Oxygen was slowly bubbled through the solution for 72 hr at room temperature, which was sufficient to achieve partial reduction. As a result of analyses by gas chromatography-mass spectroscopy (GC-MS), the saturated esters were separated from unsaturated esters.

RESULTS

Comparison of Soybean Extract with Chemical Catalyst

Soybean, I as substrate: When I was incubated with a soybean extract (pH 6.9) for 20 min, about a third of I remained unreacted (10). Since the reaction had not gone to completion, an opportunity was afforded to isolate intermediates in the formation of end products. Product II was identified as a possible intermediate that amounted to 2.2% based on the weight of I used as substrate.

Soybean, linoleic acid as substrate: A larger yield of II was obtained when linoleic acid served as the substrate in soybean extracts. In this reaction, linoleic acid presumably was oxidized sequentially, i.e., lipoxygenase oxidation to I and 9-hydroperoxylinoleic acid, and then conversion of the above to II and the corresponding isomer, *trans*-9,10-epoxy-13-hydroperoxy-*trans*-11-octadecenoic acid. From a reaction of 30 min, the yield of these isomeric epoxyhydroperoxyoctadecenoic acids was 7% based on the weight of linoleic acid substrate.

Chemical catalyst, I as substrate: Cysteine and ferric chloride converted I into a variety of products among which was a quantity of II. Because of inadvertent losses, the yield of II was not determined precisely; however, the wt % was >2 based on the amount of I used.

Previously, when cysteine and ferric chloride were used to decompose I, II was not found (6). In the previous study, I was completely degraded and, thus, other hydroperoxides, such as II, would be degraded also. In the current study, 12% I remained after the reaction was terminated.

Structure Characterization

Product II formed from I: The characteristics of II were identical whether it was isolated from reactions catalyzed by a soybean extract or cysteine-ferric chloride.

An NMR spectrum of IIa (Table I) displayed all the expected absorptions. Double irradiation experiments defined the relative positions of the functional groups. Irradiation of the C-12 epoxide proton at 3.11 δ collapsed the C-11 olefinic absorption to a doublet (J = 16 Hz). Similarly, irradiation of the C-9 proton collapsed the C-10 olefinic absorption to a doublet (J = 16 Hz). Thus, IIa is a fatty ester with an epoxy group and a hydroperoxy group located on carbons separated by a double bond. The magnitude of the olefinic coupling constants (16 Hz) is typical of *trans* olefins. The C-12, -13 coupling (J = 2 Hz) is a characteristic of *trans* epoxides.

Except for those features commonly observed with fatty methyl esters, an infrared (IR) spectrum of IIa absorbed at the following frequencies: 3600 and 3430 cm^{-1} (medium), hydroperoxide; 970 cm^{-1} (strong), isolated

I-VI: R and R_1 = H

Ia-VIa: R = CH_3, R_1 = H

IIIb, Vb, VIb: R = CH_3, R_1 = $Si(CH_3)_3$

FIG. 1. Numerical key and structures of fatty acids derived from products obtained by decomposition of 13-hydroperoxylinoleic acid by either a cysteine-ferric chloride catalyst or soybean extract. Projections shown for V and VI represent erythro and threo isomerism rather than R or S (Cahn-Ingold-Prelog) enantiomers which are not known.

TABLE I

Nuclear Magnetic Resonance Absorptions of Methyl *trans*-12,13-Epoxy-9-Hydroperoxy-*trans*-10-Octadecenoate (IIa) and Methyl 9-Hydroxy-*trans*-12,13-Epoxy-*trans*-10-Octadecenoate (IIIa)[a]

Proton	δ (Multiplicity, J, Hz)	
	IIa	IIIa
1	3.66 (s)	3.66 (s)
2	2.30 (t)	2.30 (t)
3	1.51 (m)	1.49 (m)
4-8, 14-17	1.31 (m)	1.31 (m)
9	4.33 (m)	4.14 (m)
10	5.85 (dd, 7, 16)	5.93 (dd, 6.1, 15.6)
11	5.47 (dd, 7, 16)	5.41 (ddd, 7.5, 15.6, 1.0)
12	3.11 (dd, 2, 7)	3.09 (dd, 2.1, 7.5)
13	2.84 (m)	2.81 (m)
18	0.89 (t)	0.89 (t)
19	—[b]	Variable (broad s)

[a]Obtained by $NaBH_4$ reduction of either IIa or IVa (see Fig. 1 for numerical key).
[b]Not detected.

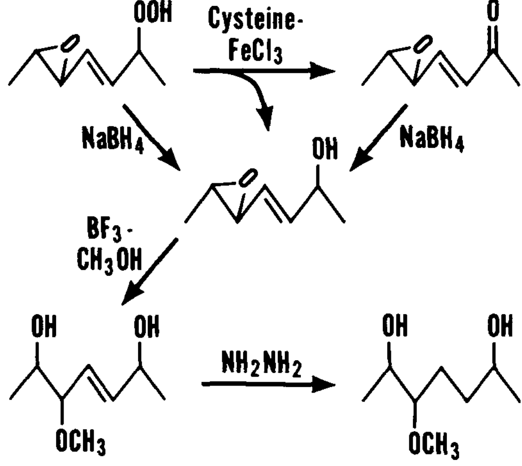

FIG. 2. Scheme showing microchemical reactions used to determine the structures of fatty acid products. Structures are abbreviated.

trans unsaturation; and 885 cm^{-1} (medium), *trans* epoxide.

Product IIa was reduced with $NaBH_4$ in order to further characterize its structure. When examined by TLC, the reduced compound exhibited more polar behavior. If the TLC plate was sprayed with the peroxide-specific ferrous thiocyanate reagent, no color developed; whereas, unreduced IIa gave a positive color reaction. Subsequently, $NaBH_4$-reduced IIa was identified as product IIIa. As outlined by Figure 2, IIIa also was synthesized via a different route, i.e., $NaBH_4$ reduction of product IVa. Product IV has been isolated and characterized previously (6).

NMR spectra of IIIa synthesized from either source (IIa or IVa) were indistinguishable. When the NMR spectrum of IIIa was compared to the spectrum of IIa, the proton at C-9 absorbed upfield significantly in IIIa (Table I). A similar shift upfield has been noted previously when NMR spectra of hydroxy fatty acids have been compared with those of hydroperoxy fatty acids (12). As expected, NMR decoupling experiments with IIIa yielded results similar to those observed with IIa.

Gas chromatography-mass spectroscopy (GC-MS) of IIIb gave a spectrum nearly identical to that reported for methyl 9-trimethylsilyloxy(OTMS)-12,13-epoxy-10-octadecenoate by Hamberg (7). Because Hamberg's spectrum, as well as the spectra obtained in this study, contained a significant 173 m/e fragment ion, it could not be ascertained whether a 13-OTMS-group was present or absent on the basis of only this experiment.

As outlined in Figure 2, chemical modification of IIIa (derived from IIa) was needed to characterize further the structure. Acid-catalyzed methanolysis of IIIa with BF_3-methanol resulted in methoxy substutution at C-12. Since C-12 is allylic, it can sustain a positive charge more readily under acidic conditions

TABLE II

Use of a Derivative[a] to Determine the Position of the Functional Groups of *trans*-12,13-Epoxy-9-Hydroperoxy-*trans*-10-Octadecenoic Acid by Mass Spectroscopy

Ion (m/e)	Possible origin	Relative intensity (%) Soybean extract	Relative intensity (%) Cysteine-ferric chloride
73	$Si(CH_3)_3$ (TMS)	41.1	60.3
103		8.2	13.1
155		16.6	13.0
173		100.0	100.0
259	b	38.1	48.4
329		1.2	2.1
381	M-(89 + 32)	3.1	6.8
397	M-(73 + 32)	1.4	3.5
402	329 + 73R[c]	9.6	20.5

[a]The derivative was synthesized according to scheme shown in Figure 2 from product isolated after decomposition of 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid by either soybean extract or ferric chloride-cysteine.

[b]Because fragmentation between sp_2 and sp_3 carbons is less favorable than sp_3-sp_3 cleavage; rearrangement probably occurs during mass spectroscopy.

[c]73R is migration of the trimethylsilyl (TMS) group at Carbon-13 to the ester carbonyl.

resulting in C-12 methoxy substitution as observed. This product, methyl 9,13-dihydroxy-12-methoxy-*trans*-10-octadecenoate was converted to its OTMS derivative for GC-MS analysis (Table II). The methoxy was located at C-12 as demonstrated by the fragment ion, 402 m/e (329 + 73). The cleavage betwen C-12 and C-13 with migration of the C-13 TMS group (73 m/e) to the ester carbonyl would occur readily as demonstrated by previous GC-MS of similar compounds (13).

The above conclusions regarding the position of the functional moieties were corroborated by hydrazine reduction as shown in Figure 2. The product, methyl 9,13-dihydroxy-12-methoxyoctadecanoate, was converted to its OTMS derivative and then subjected to GC-MS (Table III). The mass spectrum again showed the presence of a 12-methoxy group.

Homolytic Degradation of IIa

Product IIa was treated with a cysteine-ferric chloride reagent (6) for 1 hr at room temperature at a concentration of 1 mg IIa per ml reagent. Product IIa was completely converted to primarily IIIa and IVa. Quantitative data were not obtained, but it was obvious from charred TLC plates that the quantity of IIIa was greater than IVa.

Other Isomeric Epoxyhydroperoxyoctadecenoic Acids

Indirect evidence pointed to the existence of other epoxyhydroperoxyoctadecenoic isomers in addition to II. These isomers, possibly *trans*-12,13-epoxy-*erythro*-11-hydroperoxy-*cis*-9-octadecenoic acid and *trans*-12,13-epoxy-*threo*-11-hydroperoxy-*cis*-9-octadecenoic acid, were minor components of chromatographic fractions containing other fatty acids. These hydroperoxides were isolated and characterized as their $NaBH_4$ reduction products, i.e., the *erythro*- and *threo*-11-hydroxy derivatives.

Erythro isomer: The product postulated to be *trans*-12,13-epoxy-*erythro*-11-hydroperoxy-*cis*-9-octadecenoic acid was a minor component that eluted with CC fractions containing IV. When the CC fractions were applied to TLC, no evidence of a separation was obtained, except that a ferrous thiocyanate spray caused the appearance of a minor peroxide-positive spot at the lower portion of the major IV spot. After the fractions containing IV and the hydroperoxide were reduced with $NaBH_4$, two compounds separated by TLC. The mixture was methyl esterified, and the two compounds were separated and isolated by preparative TLC. By spectral methods, the major component was determined to be IIIa, which was derived from reduction and esterification of IV. The minor component was identified as methyl *erythro*-11-hydroxy-*trans*-12,13-epoxy-*cis*-9-octadecenoate (Va) by the experiments described below.

TABLE III

Mass Spectrum of a Saturated Derivative Synthesized from *trans*-12,13-Epoxy-9-Hydroperoxy-*trans*-10-Octadecenoic Acid[a]

OTMS OTMS O
OCH3
173 OCH3 259
347
331

Ion (m/e)	Possible origin	Relative intensity (%)
73	$Si(CH_3)_3$ (TMS)	58.4
103		11.5
129		15.6
141		15.8
155		18.3
173		100.0
209	331-(90 + 32)	6.0
227	?	26.9
241	331-90	29.0
259		36.1
299	331-32	7.7
315	347-32	4.4
331		2.5
383	M-(90 + 31)	2.3

[a]Derivative was synthesized according to scheme shown in Figure 2 from product isolated after decomposition of 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid by ferric chloride-cysteine.

NMR and IR spectra of Va were indistinguishable from those obtained previously for a mixture of positional isomers identified as 88% methyl 11-hydroxy-*trans*-12,13-epoxy-*cis*-9-octadecenoate and 12% methyl 11-hydroxy-*trans*-9,10-epoxy-*cis*-12-octadecenoate (6). Positional isomerism of this type has little effect on NMR and IR spectra, i.e., when the spatial relationship of the functional moieties are the same. Mercier and Agoh (14) examined similar fatty acids by NMR and reported that the *erythro* could be distinguished from the *threo* isomer by the coupling constant between the carbinol methine and the vicinal epoxide proton. For the *erythro* isomer, they found J = 3.25 Hz compared to 5 Hz for the *threo* isomer. For product Va, the coupling constant for C-11, -12 was 3.2 Hz indicating that this compound was the *erythro* isomer.

A mass spectrum of Vb was virtually identical to the spectrum published by Hamberg and Gotthammar (15) for methyl 11-OTMS-12,13-epoxy-9-octadecenoate. The dominant 285 m/e ion (100% RI), due to cleavage between C-11 and -12, was expected for an epoxide α to an OTMS group. A 199 m/e fragment ion, indicative of isomeric methyl 11-OTMS-9,10-epoxy-12-octadecenoate, was negligible (0.4% RI).

Product V was not detected when the appropriate fractions from the cysteine-ferric chloride catalyzed reaction were reduced with $NaBH_4$. No explanation is offered, except that the product could have been readily lost during workup.

Threo isomer: Another isomer, presumably *trans*-12,13-epoxy-*threo*-11-hydroperoxy-*cis*-9-octadecenoic acid, was inseparable from II by TLC and CC. Because NMR spectra of the mixture revealed minor absorptions due to the *threo* isomer, product II was the major component of the mixture. After $NaBH_4$ reduction of the mixture followed by methyl esterification, the two component mixture was separated by TLC. As expected, the major component was IIIa. The minor compound, methyl *threo*-11-hydroxy-*trans*-12,13-epoxy-*cis*-9-octadecenoate (VIa), was isolated from the TLC-spot with the highest R_f.

Product VIa was examined by NMR and IR, and the spectra were identical to the ones reported by Galliard et al. (9) who examined an identical product from potato extracts. We did not use an NMR shift reagent to better define the olefinic region of the NMR spectrum as done for VIa from potato, but we did compare our NMR spectra with copies of the original spectra of VIa from potato (Galliard, T., personal communication). The comparison proved that the compounds were identical. We measured a $J_{11,12}$ of 5.1 Hz compared to 5 Hz reported by Galliard et al. (9) and Mercier and Agoh (14) for the *threo* isomer.

As expected, mass spectra of VIb were nearly identical to those obtained for the *erythro* isomer. No feature was detected that distinguished mass spectra of VIb from those of Vb. Because the *erythro* and *threo* hydroperoxides were separated by CC prior to $NaBH_4$ reduction, this rules out the possibility that the C-11 of these two compounds bore an oxo-group rather than a hydroperoxy group.

DISCUSSION

When I was reacted with various extracts, such as from potato (9), soybeans (10,16), or peas (8), the products were analogous to those obtained with cysteine-ferric chloride (6). The analogy was reinforced by this study when we isolated II from reactions of I catalyzed by both cysteine-ferric chloride and soybean extracts. A second product, postulated to be *trans*-12,13-epoxy-*threo*-11-hydroperoxy-*cis*-9-octadecenoic acid, was also common to both. The remarkable similarity between the biological and chemical systems indicates that the basic mechanisms are similar. In the chemical

model, ferrous ion kept from being oxidized by cysteine undoubtedly is the catalyst responsible for homolytic degradation of I as follows:

$$RSH + Fe^{+++} \rightarrow Fe^{++} + RS\cdot + H^{+}$$
$$ROOH + Fe^{++} \rightarrow RO\cdot + OH^{-} + Fe^{+++}$$

In soybean extracts, ferri/ferroproteins, such as peroxidase, cytochrome, and lipoxygenase, could be responsible for homolysis of I in a manner analogous to the chemical model. After the initial homolytic cleavage of the hydroperoxy group, the pathways leading to the various products are probably similar regardless of the caralyst that initiates the primary event. Soybean lipoxygenase is one iron-containing enxyme that is known to catalyze the degradation of I to numerous products (2-5). Again, many of these products are identical to those from the cysteine-ferric chloride system. A ferri/ferro redox cycyle has been postulated to be operative in soy lipoxygenase giving rise to the observed products (17-19).

The discovery of product II provided information on one pathway by which I is degraded. The 9-hydroperoxy group of II provided direct evidence that secondary hydroperoxidation had taken place. Furthermore, it was shown by degradation of II with cysteine-ferric chloride reagent that II· could serve as a precursor to products III and IV. The role of II as an intermediate seems to be borne out by the fact that II can be isolated only from reactions that have not gone to completion. The sequence of events discussed above leads to the pathway proposed in Figure 3. This mechanism has been proposed in part by Gardner (17).

Assuming that III and IV are produced through a common intermediate, II, it is curious that the relative quantities of III and IV vary widely. The decomposition of II with the cysteine-ferric chloride catalyst resulted in formation of mostly III and minor quantities of IV. On the other hand, the decomposition of I with cysteine-ferric chloride resulted in formation of a relatively large quantity of IV and no detectable III (6). Hamberg (7) reported that the decomposition of I by hemoglobin resulted in a number of products among which was III, but he did not report the presence of IV. It is possible that IV and/or III are converted into other product(s). One possibility is solvolysis of the epoxide group, and another might be oxidation of III to IV.

Also proposed in Figure 3 is the formation of the compounds tentatively identified in this study as *trans*-12,13-epoxy-*erythro*-11-hydroperoxy-*cis*-9-octadecenoic acid and *trans*-12,13-epoxy-*threo*-11-hydroperoxy-*cis*-9-octadecenoic acid (*erythro* and *threo* isomerism not shown in

FIG. 3. Pathway proposed for formation of oxygenated epoxy fatty acids from 13-hydroperoxylinoleic acid. Structures are abbreviated.

Fig. 3). Although not directly demonstrated, it may be assumed that the 11-hydroperoxides degrade into *erythro* and *threo* 11-hydroxy (V and VI) and 11-oxo groups. Products V and/or VI have often been identified as products of the degradation of I by either biological or chemical systems (4,7,9,16); however, the corresponding 11-oxo-*trans*-12,13-epoxy-*cis*-9-octadecenoic acid has not been isolated. It is tempting to speculate that the absence of this oxoepoxyene indicates it is unstable and, thus, it could serve as a precursor to volatile aldehydes.

However, the work of Garssen et al. (4) tends to refute the addition of a second molecule of oxygen as a requirement for the formation of V and VI. These workers found that soybean lipoxygenase-1 converted I into VI with 70% retention of the two hydroperoxy oxygen atoms. They suggested that lipoxygenase-1 exerted some steric control which led to a cage reaction thereby reducing the exchange of hydroxyl function at carbon-11. In our proposed mechanism (Fig. 3), only one oxygen atom would be retained. This apparent discrepancy suggests a few possible explanations: (a) the formation of VI by lipoxygenase-1 is a unique enzymic reaction; (b) V and VI are formed via more than one pathway; (c) the precursors of V and VI may be other than the 12,13-epoxy-11-hydroperoxy-9-ene we have proposed. Since we were unable to directly isolate and characterize the precursors of V and VI, the latter explanation warrants further investigation.

Although rearrangement of the 13-hydroperoxylinoleic acid to 9-hydroperoxylinoleic acid is possible (20), we did not observe isomeric mixtures in the products. The MS of products Vb and VIb gave unambiguous proof that these structures were 11-hydroxy-12,13-epoxy-9-ene, rather than a mixture containing the latter compound and 11-hydroxy-9,10-epoxy-12-ene. Because I was prepared isomerically pure and kept at -20 C in ether (10 mg/ml) for less than 24 hr, there was little opportunity for rearrangement.

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Reaction between Peroxidized Phospholipid and Protein: I. Covalent Binding of Peroxidized Cardiolipin to Albumin

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ABSTRACT

A system is described for study of the reaction of peroxidized cardiolipin (diphosphatidylglycerol) with albumin. Covalent binding of peroxidized cardiolipin to albumin occurs in this system as evidenced partly by a decrease in lipid-extractable P and partly by lipid P being inseparable from the albumin by gel filtration in the presence of a detergent (sodium deoxycholate) under conditions known to separate the lipid moiety and apoprotein of lipoproteins. Based on analyses of the decrease in lipid-extractable P, the average number of cardiolipin molecules bound covalently per molecule of albumin is about 5 when peroxidized cardiolipin (4 moles O_2/mol cardiolipin) at about 10 times molar excess is allowed to react with albumin. However, the data of the gel filtration experiment indicate that the bound peroxidized cardiolipin molecules may not be evenly distributed on the albumin molecules. Therefore, the number of cardiolipin molecules bound per albumin molecule may actually vary over a range and be considerably higher for part of the albumin. The findings have been discussed in relation to peroxidation in vivo.

INTRODUCTION

Studies in vitro have shown that peroxidized lipids cause damage to proteins. The proteins are thereby altered with respect to their solubility (1-4), molecular weight (4-6), amino acid composition (1,3,6-8), and in the case of enzymes, their catalytic activity (6). In addition, the damaged protein exhibits characteristic fluorescence (6-8). The damage is considered to arise by cross-linking of the proteins by malonaldehyde (6), a product of lipid peroxidation, and by attack from free radicals of the lipid peroxidation (1,3,4). In the latter case, cross-linking is considered to arise by protein radicals reacting with each other, or by free radicals of the lipid peroxidation reacting with protein radicals (1,4).

Almost all investigations of the reaction between peroxidized lipid and protein in vitro have used either methyl or ethyl esters of a polyunsaturated fatty acid or the unesterified acid as model lipid. These lipids are not present as such in cellular and subcellular membranes whose lipid is almost entirely phospholipid which supposedly occurs in close contact with structural protein. Since peroxidation in vivo occurs in membrane structures, the use of the free fatty acids or their methyl or ethyl esters in model systems may give results which are not representative of what happens in vivo. This point is discussed in more detail later. In the present work, ox heart cardiolipin has been used as model lipid. This phospholipid contains an unusual high percentage (ca. 70%) of linoleic acid (9) and occurs chiefly in mitochondria (10,11). As model protein, albumin was used.

MATERIALS AND METHODS

Chemicals

Chemicals used throughout the work were of analytical grade. Albumin (human) was obtained from Schwarz/Mann, Orangeburg, NY. Silica gels for column chromatography were products of Merck, Darmstadt, W. Germany (Silica gel 60 for column chromatography, 70-230 mesh ASTM) and Mallinckrodt, St. Louis, MO (Silicic Acid, 100 mesh, analytical reagent).

Preparation of Ox Heart Cardiolipin, Na Salt Form

Total lipid was extracted according to Bligh and Dyer (12) from an ox heart obtained at the local slaughterhouse within 6 hr of slaughtering the animal. Cardiolipin was then isolated by chromatography at 2 C on a column made from 65 g activated (overnight at 100-105 C) silica gel (Merck). The silica gel was slurried into cold (2 C) chloroform, degassed by suction (water pump), and poured into a glass column with an inner diameter of 26 mm. After washing the column with 200 ml chloroform, ox heart lipid was applied as a 25 ml chloroform solution containing about 2.6 mg lipid phosphorus/ml, thus giving a load of about 1 mg lipid phosphorus per gram of silica gel. Stepwise elution with 500 ml chloroform followed by 840 ml chloroform-methanol (19:1, v/v) using a flow rate of about 2 ml/min caused

cardiolipin to be eluted with chloroform-methanol (19:1, v/v). This cardiolipin preparation was subjected to rechromatography on a column made from activated Mallinckrodt silica gel using a load of about 0.3 mg lipid phosphorus/g adsorbent. Elution was carried out with chloroform followed by chloroform-methanol (9:1, v/v). The latter eluate contained cardiolipin that was chromatographically pure when examined in the systems previously used for identification and check of purity of cardiolipin preparations (13). Finally, the cardiolipin was converted to its Na salt form as previously described (14) and stored as a chloroform solution (2 mg lipid phosphorus/ml) at -20 C in a Teflon-stoppered tube wrapped with tin foil. Under these conditions of storage, the cardiolipin was stable over the period of investigation since no change in UV adsorption (at 232 nm) or chromatographic behavior was detectable. The same batch of cardiolipin was used throughout the work.

Preparation of Cardiolipin Suspension

A cardiolipin suspension containing about 400 μg of P/ml was prepared by pipetting the calculated volume of chloroform stock solution of cardiolipin into a 15 x 100 mm glass-stoppered test tube and evaporating the chloroform in a stream of N_2. Then, a small amount of diethyl ether was added (ca. 0.1 μl/μg of P) followed by deionized water to a final concentration of 400 μg of P/ml. The addition of water was done with continuous mechanical shaking. The tube was then placed in an ultrasonic bath (Metason 5000, Struers, Copenhagen) for 10 min at room temperature after which the ether was removed by gentle blowing with N_2. Finally, sonication was repeated for another 10 min period. The lipid suspension obtained was homogenous, and its pH was 5.0-5.5.

Peroxidation of the Cardiolipin Suspension and Its Subsequent Incubation with Albumin

Peroxidation was initiated and allowed to proceed to the desired oxygen uptake in Warburg vessels (13-14 ml size, 80 oscillations per min) at 30 C in an oxygen atmosphere. For incubations longer than 1 day, it was necessary each day to flush with oxygen for a few minutes to evaporate droplets of water that condense in the tube connecting vessel and manometer. Initiation was done when not otherwise stated by the combined effects of adding $Cu(CH_3COO)_2$ (6.4 mM) to a final concentration of Cu^{++} of 5 ppm (0.79 x 10^{-4} M) and UV-irradiating the closed vessel at a distance of 28 cm for 4 hr with a mercury lamp (Osram, HQV 125 W, from which the outer fluorescent glassbulb had been removed). When the calculated amount of oxygen had been taken up, albumin (40 mg/ml H_2O; pH adjusted to 7.0-7.5 with NaOH) was added to a final concentration of 20 mg/ml after which the vessel was flushed with N_2 and incubation continued for different periods of time. When samples for analyses were to be taken, the vessel was shortly disconnected and removed from the bath. After sampling, the vessel was returned to the bath and flushed with N_2.

Lipid Extraction

Lipid extraction was done according to a modification of the Bligh and Dyer procedure (12) using an acidified extraction system as described by Kates (15). A sample containing about 100 μg of P was made up with H_2O to a volume of 980 μl in a 100 x 15 mm centrifuge tube. Then, in succession, the following additions were made with thorough mixing after each: 20 μl concentrated HCl, a mixture of 1.25 ml chloroform and 2.50 ml methanol, 1.25 ml chloroform, and finally 1.25 ml H_2O. Subsequent centrifugation for 45 min at 3000 x *g* resulted in clear upper and lower phases separated by a white layer of jelly-like consistency. This layer varied in thickness occupying for some samples a considerable part of the lower phase. In these cases, a spatula was inserted into the layer and rotated gently. Centrifugation was then repeated resulting in a great reduction in the thickness of the layer. Isolation of the lower phase which contains the extracted lipid was done by pipetting, care being taken not to include any part of the upper phase and the white layer at the interphase. To ensure quantitative transference of the lower phase, 3 ml of ideal lower phase (i.e., the lower phase of a two-phase system prepared by mixing 11.15 ml H_2O + 100 μl conc. HCl + 12.5 ml chloroform + 12.5 ml methanol) was added to the remaining upper phase plus interphase layer followed by gentle mixing with a spatula and centrifugation for 45 min. This results in a clear upper and lower phase separated this time by only a thin layer of white material. The clear lower phase was combined with that obtained in the first extraction followed by neutralization with 0.2 N methanolic ammonia and determination of the amount of phospholipid thus extracted. The tube containing upper phase +

thin layer at interphase was placed on a water bath (30-35 C), and N_2 was bubbled through its contents until no chloroform could be smelled. The resulting homogeneous solution which contains the protein and any other water-soluble compounds of the sample was analyzed for P.

Phosphorus Analysis

Phosphorus was determined by Bartlett's method (16) except for the analysis of the eluate in the gel filtration experiments where a modified micro-procedure was used (17). In this modified procedure, digestion is performed with 70% perchloric acid (instead of with 10 N H_2SO_4 and H_2O_2). In our determination of P content of aliquots of 1 ml and 2 ml eluate evaporated to dryness (corresponding to about 4 mg, respectively, 8 mg of sodium deoxycholate), it was necessary to perform the digestion slowly under controlled heating conditions to avoid dangerous explosions. The controlled heating conditions arrived at were as follows: After addition of perchloric acid and 3-5 carborundum stones to the dry samples at room temperature, heating was performed on a boiling water bath for 45 min. Then, after being cooled to room temperature and shaken shortly on a whirli-mixer, they were placed on a sand bath at 140-150 C for about 17 hr. Finally, after cooling to room temperature, the samples were placed on a sand bath at 230-240 C for 2 hr. Digestion of samples was usually complete by this procedure, but if a sample happened not to be colorless at this point, it was left at 230-240 C until colorless, indicating complete digestion. There is a small consumption of perchloric acid by this procedure amounting to some 10% and 15% for 1 ml and 2 ml eluate, respectively. Compensation for this loss was made at the end of the digestion by adding corresponding volumes of 70% perchloric acid.

Separation of Protein and Lipid by Gel Filtration in the Presence of Sodium Deoxycholate

Separation of albumin and noncovalently bound lipid by gel filtration in the presence of the detergent, sodium deoxycholate, was done according to Helenius and Simons (18).

Protein Analysis

Protein was determined according to Lowry (19).

RESULTS

Initiation of Peroxidation of Lipid Suspension

In preliminary experiments to establish conditions of peroxidation, different concentrations of Cu^{++} (0-10 ppm) were used to initiate the process. At 3 ppm, 5 ppm, and 10 ppm of Cu^{++}, induction periods were 50-60 hr, 35-45 hr, and 25-35 hr, respectively, and maximal rates of oxygen uptake were 0.09, 0.11, and 0.14 moles O_2/mol cardiolipin per hour, respectively. The long induction period would be inconvenient in the experimental work. To shorten it, lipid suspensions were intially subjected to UV irradiation. Figure 1 shows the course of peroxidation when initiation is performed by UV irradiation for 4 hr and different concentrations of Cu^{++}. Peroxidation is readily started at 3 and 5 ppm Cu^{++}, and its velocity after irradiation produced cardiolipin oxidized to any desired degree. The rates of peroxidation obtained at 1 ppm Cu^{++}, 0.5 ppm Cu^{++}, or no Cu^{++} did not differ significantly from each other and were too slow for convenient experimental work. Initiation by 5 ppm Cu^{++} combined with 4 hr of UV irradiation was chosen for preparing the peroxidized cardiolipin.

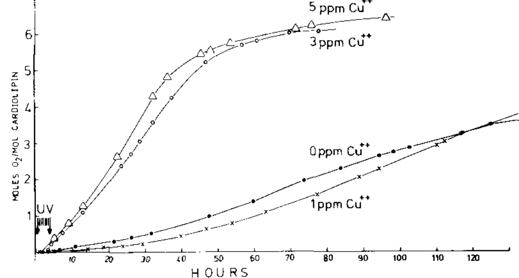

FIG. 1. Initiation of peroxidation of aqueous suspensions of sodium salt of cardiolipin at 30 C by 4 hr UV irradiation and varying concentration of Cu^{++}. Cardiolipin suspensions (0.5 ml, ca. 400 μg P/ml) containing Cu^{++} at the following concentrations: 0 ppm, 1 ppm (0.16×10^{-4} M), 3 ppm (0.47×10^{-4} M), and 5 ppm (0.79×10^{-4} M), were initially irradiated in closed Warburg vessels. The period of UV irradiation is indicated by the hatched region.

Time Study of Covalent Binding of Peroxidized Cardiolipin to Albumin

Covalent binding between albumin and peroxidized cardiolipin determined as the amount of phosphorus which resists lipid extraction was measured as a function of time (Fig. 2). It appears that binding is complete after some 12 hr. The value of 24% binding at time zero (Fig. 2) was obtained when lipid suspension and protein solution were added in succession to the complete lipid extraction system with

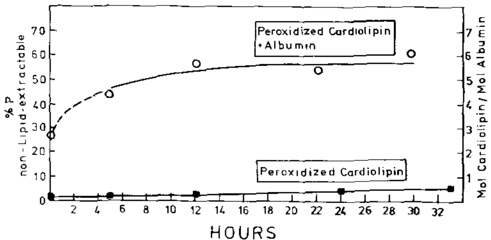

FIG. 2. Covalent binding of peroxidized cardiolipin to albumin as function of time. Peroxidized sodium salt of cardiolipin (4.2 moles O_2/mol cardiolipin) was incubated with albumin in a N_2 atmosphere. Aliquots were subjected to lipid extraction to determine the percentage of P that resists lipid extraction (left ordinate). The right ordinate gives the average number of cardiolipin molecules bound per molecule of albumin, assuming that the P which resists lipid extraction represents peroxidized cardiolipin bound covalently to the albumin (-o-). In a control experiment, peroxidized cardiolipin (4.0 moles O_2/mol cardiolipin) was incubated under the same conditions except that no albumin was present. Analysis of the percentage of P that was not lipid-extractable at different points of the incubation was similarly performed (-■-).

thorough mixing after each addition. Thus, even though contact between lipid and protein under these conditions will be very limited because the complete extraction system is heterogeneous, about half of the maximal obtainable binding occurs. This indicates that the process initially is rapid. Peroxidized cardiolipin incubated without albumin gave rise to very small amounts of nonlipid-extractable products containing P under identical conditions of incubation and extraction (Fig. 2). Nonperoxidized cardiolipin incubated with albumin was extracted completely by the present extraction procedure.

Separation of Noncovalently Bound Cardiolipin from Albumin by Gel Filtration in the Presence of a Detergent

When a mixture of peroxidized cardiolipin (4.2 moles O_2/mol cardiolipin) and albumin was incubated under N_2 for 26.5 hr and subsequently subjected to gel filtration, part of the cardiolipin is eluted together with the albumin suggesting covalent bonds between protein and lipid (Fig. 3A). In a control experiment in which albumin was incubated for the same period of time with nonperoxidized cardiolipin that had been UV irradiated and preincubated under the same conditions as the cardiolipin of Figure 3A, except that oxygen had been replaced by N_2, all of the

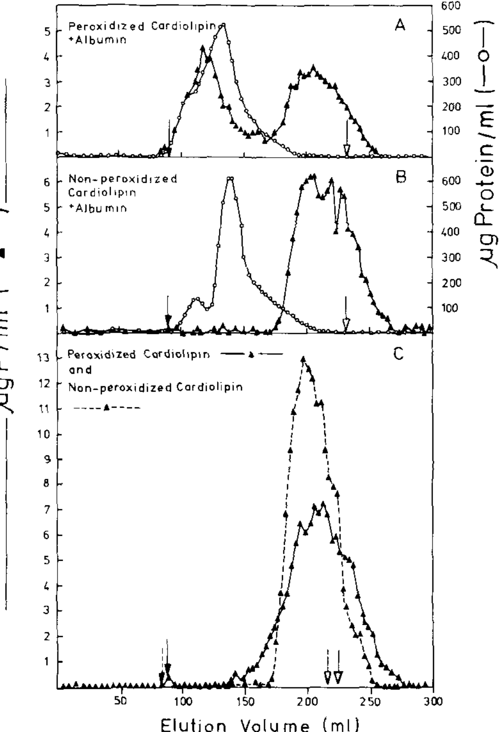

FIG. 3. Separation of albumin and noncovalently bound cardiolipin by gel filtration in the presence of a detergent. Column: Sephadex G-200 (2.5 cm ID; bed length = 45 cm). Buffer: 10 mM sodium deoxycholate-0.05 M NaCl-0.05 M sodium carbonate (pH 10). Samples: 1 ml solutions that had been incubated for 22-27 hr under N_2 at 30 C, A: 20 mg albumin + 5 mg peroxidized sodium salt of cardiolipin (4.2 moles O_2/mol cardiolipin); B: 30 mg albumin + 5 mg nonperoxidized sodium salt of cardiolipin; C: either 5 mg peroxidized sodium salt of cardiolipin (4.0 moles O_2/mol cardiolipin, solid line), or 5 mg nonperoxidized sodium salt of cardiolipin (broken line). Prior to application a sample was made into a 2 ml solution in buffer, and 288 mg sodium deoxycholate was added with stirring giving a clear solution. Pumping rate: 7.5-9 ml/hr, fractions 3-3.7 ml. Elution volumes of Blue dextran and 2-mercaptoethanol are indicated with solid and empty arrows, respectively. (0) Albumin, (▲) Phosphorus.

cardiolipin is separated from the albumin (Fig. 3B). Finally, Figure 3C shows that peroxidized cardiolipin (4.0 moles O_2/mol cardiolipin), as well as nonperoxidized cardiolipin, is eluted at a position removed from that of albumin. Figures 3A-C show that peroxidized cardiolipin reacts with albumin, whereby covalent bond(s) are formed between that protein and P-containing product(s) of cardiolipin.

DISCUSSION

That covalent binding of peroxidized cardiolipin to albumin does occur to a considerable extent under the present conditions is established by the results of the two applied methods for separating lipid and protein. Although the chemical characterization of the underlying reactions must await various analyses on the system, the data already indicate that the reactions differ significantly from those that have been observed in other systems. Figure 3A shows that the P content of the albumin after reaction varies, and the number of peroxidized cardiolipin molecules bound per molecule of albumin thus will be considerably higher than 5 for part of the albumin. There may possibly be a range of complexes varying with respect to the number of peroxidized cardiolipin molecules being bound per molecule of albumin. Also, since the cardiolipin contains more than one polyunsaturated fatty acid in its molecule, cross-linking may have occurred between albumin molecules by peroxidized cardiolipin reacting with more than one albumin molecule. Therefore, the products of reaction may also vary with respect to molecular weight. Dessai and Tappel (1) observed covalent binding in a system of peroxidizing linolenic acid and cytochrome c. Using linolenic acid -1-^{14}C, the number of peroxidized linolenic acid molecules bound covalently per molecule of cytochrome c ranged from 0.03 to 0.99 with degrees of peroxidation increasing from 0.01 to 1.0 mol O_2/mol linolenic acid and the lipid being present at about 100 times molar excess relative to the protein. Losses of those amino acids of the cytochrome c that are labile to oxidation occurred by the reaction. It was considered that damage of cytochrome c (measured as decrease in solubility) was caused only to a minor extent by the binding of peroxidized linolenic acid, and that the quantitatively important damage arose from free radical oxidation without addition of lipid. Roubal and Tappel (4), studying the polymeric products that arise in a system of peroxidizing ethyl esters of polyunsaturated fatty acids and either cytochrome c or ribonuclease, also found that only low levels of lipids were bound covalently to the protein in the damaging reactions. The products were considered to be mainly protein – protein cross-linked polymers arising by free-radical chain polymerization. The reason that much more peroxidized lipid was covalently bound to protein in the present system than in the above works (1,4) obviously must originate in differences between the experimental systems. The present system differs in several respects: it is homogeneous, its fatty acids are built into the structure of a phospholipid, protein is not present in the system during the peroxidation but added after peroxidation has taken place, albumin is used as model protein, and linoleic acid is the major fatty acid of the phospholipid used. The higher percentage of binding may arise from the system being homogeneous because this expectedly would facilitate reaction between protein and lipid. In a biphasic system, peroxidized lipid molecules may react mutually within the lipid phase. Also, the structure of a peroxidized phospholipid may be more suitable for making the kind of contact required for covalent reaction than might be free peroxidized fatty acids or their ethyl esters.

The fact that about 25% of the peroxidized cardiolipin reacts instantaneously with albumin (Fig. 2) suggests that at least that percentage of the cardiolipin has been converted to a reactive form by the peroxidation. Since the concentration of the peroxidized cardiolipin suspension (400 μg P/ml) is about 6.5 mM, the concentration of the reactive compound(s) at the moment of adding it to the albumin would be about 1.6 mM. By its order of magnitude, this concentration seems to exclude these compounds being free radicals, and the coupling of peroxidized cardiolipin to albumin probably does not arise by free radicals reacting with that protein. This is in contrast to previous suggestions that peroxidized lipid reacts with protein in the form of free radicals (1,4). However, protein and lipid were cooxidized in these studies, and the continuous production of lipid peroxy radicals would allow quantitatively important reactions with the present protein even though their steady-state concentrations are very low.

Chio and Tappel (6) obtained evidence for damage of proteins by malonaldehyde arising in the peroxidation of methyl linolenate. Whether this reaction, too, is significant in the present system is not known. Information on its extent may be obtained by fluorescence analyses for N,N′-disubstituted 1-amino-3-imino-propene which is formed in that type of lipid peroxidative damage of proteins (6,20), and also by analyses for thiobarbituric acid reacting substances in the peroxidized lipid suspension. One would not expect the reaction to be quantitatively

important because linoleic acid (the major fatty acid of cardiolipin), unlike linolenic acid and higher polyunsaturated fatty acids, does not yield detectable thiobarbituric acid-reacting substances in its early stages of peroxidation (21). The reaction may, however, occur to a limited extent because a small percentage of the fatty acids of ox heart cardiolipin (less than 10%) are higher polyunsaturated fatty acids (9) which give rise to malonaldehyde production upon peroxidation.

From a biological point of view, a most important question is whether the kind of reaction between peroxidized phospholipid and protein described here occurs in vivo. I have not found any answer to this in the literature, but then neither seems that question to have been asked. One paper, however, deals with peroxidation in microsomal membrane and states: "During peroxidation the amount of extractable phospholipid decreased" (22). Although this decrease in extractable phospholipid may have originated from enzymatic breakdown of phospholipid, it might also have been caused by covalent binding of peroxidized phosphlipid to membrane proteins. At present, the significance of covalent binding of peroxidized phospholipid to protein in vivo is unknown.

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The Effects of Certain Ecdysteroids and Inhibitory Amines and Amides on the Metabolism of 22,25-Dideoxyecdysone in Cockroach Organ Cultures[1]

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ABSTRACT

An organ culture assay system using cockroach leg regenerates and fat body has been developed in which compounds that either inhibit molting hormone metabolism or act as molting hormone antagonists can be tested. Representatives of three classes of compounds were tested in the system: ecdysteroids, azasteroids, and nonsteroidal amines and amides. Inhibitory compounds were found in all three of the classes. Certain of these inhibitors represent a new class of insect hormonal compounds with a novel mode of action – the disruption of molting hormone metabolism.

INTRODUCTION

Numerous studies have been made of the molting hormone (MH) activity or effect(s) of various ecdysteroids on cultured insect tissues (1). However, few of these studies have taken into account the possibility that the tested compound might be altered by the cultured tissue before the molting hormone effect takes place; that the tested compound might serve as a prohormone or hormone precursor. Earlier studies indicated that cockroach leg regenerate tissue from *Leucophaea maderae* (F.) was capable of converting α-ecdysone (II, Fig. 1) to 20-hydroxyecdysone (I) and that the tissue did not respond by producing cuticle until this had occurred (2,3). More recently, Marks (4) studied the molting hormone effect of the synthetic ecdysteroid 22,25-dideoxyecdysone (IV) on leg regenerates and found that the molting hormone effect was greatly enhanced by co-culturing the leg regenerates with fat body tissue. At that time, he suggested that IV was being converted to an active insect molting hormone as previously demonstrated by Kaplanis et al. (5) and King (6). Marks also proposed that the cockroach system could be used to evaluate compounds that might inhibit this conversion (4).

A number of natural and synthetic ecdysteroids (7,8) and azasteroids and nonsteroidal amines and amides (9,10) have previously been shown to inhibit molting and metamorphosis and/or steroid metabolism in insects. The present study reports the molting hormone activity of certain ecdysteroids on cockroach leg regenerates. It also reports the effect of some azasteroids and nonsteroidal amines and amides on the molting hormone activity of 22,25-dideoxyecdysone in the leg regenerate-fat body culture system.

METHODS AND MATERIALS

Late-instar nymphs of the cockroach, *Leucophaea maderae*, were isolated from the laboratory colony while still white. Twenty-four hours later the mesothoracic legs were removed at the coxotrochanteral joint. After 28 days, the coxa was removed, and the regenerating leg was dissected; at the same time, 8-10 cu mm of abdominal fat body

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[1]IUPAC equivalent names:

- I = 2β,3β,14α,20,22R,25-Hexahydroxy-5β-cholest-7-en-6-one
- II = 2β,3β,14α,22R,25-Pentahydroxy-5β-cholest-7-en-6-one
- III = 2β,3β,14α,22S,25-Pentahydroxy-5β-cholest-7-en-6-one
- IV = 2β,3β,14α-Trihydroxy-5β-cholest-7-en-6-one
- V = 14α-Hydroxy-2β,3β-bis(trimethylsiloxy)-5β-cholest-7-en-6-one
- VI = 2β,3β-Dihydroxy-5β-cholest-7-en-6-one
- VII = 2β,3β,5β,14α-Tetrahydroxy-5β-cholest-7-en-6-one
- VIII = 3β,5β,14α-Trihydroxy-5β-cholest-7-en-6-one
- IX = 3β-Hydroxy-chol-5-en-24-dimethylamine
- X = 3β-Methoxy-chol-5-en-24-dimethylamine
- XI = 3β-Cyclopentoxy-chol-5-en-24-dimethylamine
- XII = 5α-Cholan-24-dimethylamine
- XIII = 5β-Cholan-24-dimethylamine
- XIV = N,N,δ,7a-Tetramethyloctahydro-1-H-indene-1-butanamine
- XV = N,N-Dimethyl-3,7,11-trimethyldodecanamide
- XVI = N,N-Dimethyl-3,7,11-trimethyldodecanamine
- XVII = N,N-Dimethyl-11-methoxy-3,7,11-trimethyldodecanamide
- XVIII = N-Ethyldodecanamide
- XIX = N,N-Dimethyldodecanamide
- XX = N,N-Dimethyldodecanamine
- XXI = N-Ethyldodecanamine
- XXII = N,N-Dimethyl-11-methoxy-3,7,11-trimethyldodecanamine.

I R = OH
II R = H
III (22S-OH) R = H

IV R = R‴ = OH; R′ = R″ = H
V R = $(CH_3)_3SiO$; R′ = $(CH_3)_3Si$; R″ = H; R‴ = OH
VI R = OH; R′ = R″ = R‴ = H
VII R = R″ = R‴ = OH; R′ = H
VIII R = R′ = H; R″ = R‴ = OH

IX R = H
X R = CH_3
XI R = [cyclic group]

XII
XIII, 5β - H

XIV

XV

XVI

XVII

XVIII $CH_3(CH_2)_{10}CONHC_2H_5$

XIX $CH_3(CH_2)_{10}CON(CH_3)_2$

XX $CH_3(CH_2)_{11}N(CH_3)_2$

XXI $CH_3(CH_2)_{11}NHC_2H_5$

XXII

FIG. 1. Ecdysteroids, azasteroids, and nonsteroidal amines and amides.

were also removed. Two leg regenerates alone or in combination with the fat body were placed about 3 mm apart on the lower coverslip of a Rose multipurpose tissue chamber (11) and covered with dialysis strip soaked in M20S culture medium with 7% fetal calf serum (12). The chamber was then assembled and filled to a total of 2 ml with culture medium.

Since many of the test compounds were insoluble in water, a number of solvents were investigated. We selected dimethylsulfoxide (DMSO) at the level of 0.5 μl/ml of culture medium as the most successful. Ten μg of each test compound and/or the 22,25-dideoxyecdysone (IV) in 1 μl of DMSO were injected through the chamber gasket directly into the fat body with a 10-μl syringe. In the cultures without fat body, the test compound was injected under the dialysis strip, and although most precipitated immediately, the crystals remained adjacent to the periphery of the leg regenerates. It was assumed that, at the concentrations used, saturation levels of the test compounds were maintained in the vicinity of the tissues. When injected into the fat body, the test compound immediately became available to the cells.

The treated chambers were incubated at 26 C, examined at the end of 14 days with the aid of phase contrast optics, and scored for the presence of cuticle (13). Five chambers (10 leg regenerates) were scored for each treatment, and the significance of the results was determined by using 2 x 2 contingency tables based on Fischer's exact test.

The experimental compounds were tested against leg regenerates under three conditions: leg regenerates alone, leg regenerates plus fat body, and leg regenerates plus fat body and compound IV. From the results we obtained under each condition, it was

TABLE I

The Activity of Certain Ecdysteroids in Inducing Cuticle Formation in Cockroach Leg Regenerates when Tested in Cockroach Leg Regenerates Alone and in Leg Regenerates plus Fat Body and in Inhibiting the Activity of Compound IV (22,25-Dideoxyecdysone) when tested in Leg Regenerates plus Fat Body and IV

	Cuticle formation			
Ecdysteroid[a]	Leg regenerates (%)	Leg regenerates + fat body (%)	Leg regenerates + fat body + IV (%)	Inhibition (%)
I	93	---	---	---
II	82	---	---	---
III	8	0	100	0
IV	23	93	---	---
V	9	60	---	---
VI	0	0	90	3
VII	25	75	---	---
VIII	9	7	64	31[b]

[a]All ecdysteroids tested at 10 μg per chamber.

[b]Difference from IV alone significant at P>0.05. For all experiments N≥10.

possible to determine in what way the compound affected the system.

When the compound was tested against the leg regenerate alone and cuticle was formed, the experiments were stopped because the compound either possessed MH activity or was being converted to an active compound by the leg regenerate. When no cuticle was formed, the compound was tested against leg regenerates in the presence of fat body. If cuticle was formed, under these conditions, it was apparent that the leg regenerates (13) (epidermal tissue) could not convert the experimental compound to a chemical(s) with MH activity, but that the fat body could; the compound then was acting as a precursor for a compound(s) with MH activity. When no cuticle was formed in the presence of fat body, the compound was tested on leg regenerates in the presence of fat body and the ecdysteroid IV; in this case, when cuticle was formed, the compound was assumed to have no effect on the system at the concentration tested. However, when cuticle was not formed, it was apparent that the experimental compound was blocking the conversion of IV to a compound(s) that has MH activity in the tissue culture system.

RESULTS

Three groups of compounds were tested. The first consisted of synthetic ecdysteroids (I-VIII, Fig. 1) that differ structurally either in the steroid nucleus and/or the side chain. The results obtained with these compounds are summarized in Table I. With 20-hydroxyecdysone (I), 93% of the leg regenerates without fat body produced cuticle within 14 days. With α-ecdysone (II), which is readily converted to I by leg regenerates, 82% of the regenerates gave a positive response, but ecdysteroids IV and VII gave only 23 and 25% responses, respectively, which suggested a low level of conversion of these compounds by the leg regenerate. The 22-isoecdysone (III) and compounds V, VI, and VIII were essentially inactive. In the presence of fat body, V, VII, and IV produced 60, 75, and 93% activity, respectively, indicating the conversion of these ecdysteroids by the fat body to compounds with MH activity. When tested against regenerates plus fat body in the presence of compound IV, 22-isoecdysone (III) and V showed no significant inhibitory activity, but VIII produced a 31% inhibition as compared to IV alone.

The second group of compounds were 25-azasteroids (IX-XIII, Fig. 1) that disrupt steroid metabolism and molting and metamorphosis in insects (9). As expected, the azasteroids did not exhibit MH activity in either the leg regenerate system alone or in combination with fat body tissue. However, when tested against compound IV plus fat body, azasteroids IX, XIII, and X showed 100, 83, and 78% inhibition, respectively, of cuticle formation in the system (Table II). Compound XII showed a much lower level of inhibition (35%), and XI was inactive at the concentration tested.

The third group of compounds differed from the first two groups in that it consisted

TABLE II

The Effect of Certain Azasteroids and Nonsteroidal Amines and Amides in Inhibiting the Activity of Compound IV (22,25-Dideoxyecdysone) in Inducing Cuticle Deposition in Cockroach Leg Regenerates when Tested in Leg Regenerates plus Fat Body in the Presence of Compound IV

Compound[a]	Cuticle deposition (%)	Inhibition (%)
Control	93	--
Azasteroids		
IX	0	100[b]
X	20	78[b]
XI	80	14
XII	60	35[b]
XIII	16	83[b]
Nonsteroidal amines and amides		
XIV	50	46[b]
XV	70	25
XVI	50	46[b]
XVII	60	35[b]
XVIII	100	0
XIX	70	25
XX	80	14
XXI	70	25
XXII	90	3

[a]All compounds tested at 10 μg per chamber. None of the azasteroids or nonsteroidal amines and amides showed significant activity in inducing cuticle deposition when tested with either leg regenerates alone or with leg regenerates plus fat body.

[b]Difference from Control (IV alone) significant at $P>0.05$. For all experiments $N \geqslant 10$.

of nonsteroidal compounds (XIV-XXII, Fig. 1). Compound XIV contained only the C and D rings and C-18 methyl of the steroid nucleus plus the 25-azasteroid side chain, and the remaining compounds were branched and straight chain secondary and tertiary amines or amides (10). When these compounds were tested against the leg regenerates and fat body-leg regenerates, none was found to have MH activity. However, of the 12 compounds in this group, XIV, XVI, and XVII showed statistically significant ($P > 0.05$) inhibition of the conversion of IV to an active compound(s), and three other chemicals, XV, XIX, and XXI, showed marginal, but not statistically significant, inhibition. The remainder were essentially inactive at the concentration tested.

DISCUSSION

The results of our experiments show clearly that compounds with at least three different types of activity affect the molting hormone mediated processes in the organ culture test system and, presumably, in insect tissues in vivo. These are compounds that (a) possess MH activity in the system; (b) serve as precursors and are readily converted by the tissues to compounds with MH activity; and (c) block the conversion of inactive ecdysteroid precursors such as IV to compounds with MH activity in the system. A fourth type of activity may also occur when an inactive or slightly active ecdysteroid or related steroid acts as an antihormone or hormone antagonist by effectively competing for the MH hormone receptor(s). This would result in a reduction in the activity of an insect MH or some other active ecdysteroid in the system. Although it has proved difficult to distinguish between these four modes of action in vivo, this differentiation can be readily made in the organ culture system. This demonstrates the unique capability of such an in vitro system to provide information for structure-activity and mode of action studies aimed at developing new or improved types of chemicals that control or disrupt the hormone-regulated processes of insects.

The action of VIII, which showed only low levels of activity in vivo (8) and yet resulted in a reduction in the activity of IV

when tested in combination with fat body, is not readily apparent. Compound VIII may be competing with IV as a substrate for the same enzyme(s), or alternatively, VIII and/or its metabolic product(s) may be functioning as antihormones. Compound VIII has previously been reported to have anti-MH activity on *Drosophila* imaginal discs cultured in vitro in that it reduces the degree of evagination induced by minimally effective quantities of 20-hydroxyecdysone (14). However, preliminary studies with compound VIII in the leg regenerate system showed no apparent interference with the action of 20-hydroxyecdysone at the 1 μg/ml level.

The cockroach leg regenerate-fat body in vitro system effectively converts the ecdysteroids IV, V, and VII to compounds with MH-activity, most probably via side chain hydroxylation. However, the in vitro system does not appear to have the metabolic capability to effect certain modifications of the steroid nucleus: For example, compound VI, which differs from IV, and compound VIII, which differs from VII only in the absence of a 14α- and a 2β-hydroxyl group, respectively, are not converted to active compounds in the system as are compounds IV and VII. The metabolic products formed when IV is incubated with cockroach fat body have been isolated and partially characterized. The identity of these metabolites and the site(s) of inhibition of conversion by the various types of inhibitory compounds are currently being investigated.

The 25-azasteroids tested have all previously been shown to be potent inhibitors of steroid metabolism and molting and metamorphosis in insects (9). These earlier studies by Svoboda et al. (9) showed that when an azasteroid was fed to house flies, *Musca domestica* L., in combination with ecdysteroid IV, the azasteroid reversed the inhibitive effect of the ecdysteroid. Since IV severely inhibits development in house flies and X effectively reversed this effect, the investigators hypothesized that this azasteroid blocked the conversion of IV to a more inhibitive compound(s). They also found that α-ecdysone reversed the deleterious effects of certain of the azasteroids in the confused flour beetle, *Tribolium confusum* Jacquelin du Val, further evidence that MH metabolism was being affected. Our present experiments confirm this hypothesis. Unlike the ecdysteroids that may act by any or all of the four modes, all the azasteroids that showed activity in our system interfered with the conversion of IV by the fat body. Although the azasteroids IX and X showed slight cytotoxicity, compound XIII, which was highly active, was clearly not cytotoxic.

The third group of compounds were nonsteroidal in nature and, like the azasteroids, served neither as hormones nor precursors. However, three of these compounds, XIV, XVI, and XVII, showed significant inhibition of conversion of IV to an active compound in our system. Two of the three – XVI and XVII – also showed a high level of inhibitory activity against dipteran and lepidopteran insects in vivo (10). This again demonstrates a correlation between the inhibition of the conversion of IV and the in vivo efficacy of certain of the test compounds. Thus, from these and earlier studies, it is evident a new class of compounds that disrupts the hormone regulated processes of insects has been developed. These compounds exhibit a novel mode of action in that they interfere with the metabolism of the endogenous molting hormones of insects.

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The Effect of Phospholipase D on the Function of Fragmented Sarcoplasmic Reticulum

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ABSTRACT

Incubations of fresh preparations of fragmented sarcoplasmic reticulum (FSR) were carried out at pH 5.7. This pH was necessary for hydrolysis of phospholipids by phospholipase D. The pH did not influence calcium uptake or the activity of calcium-stimulated ATPase of FSR. Treatment of FSR with phospholipase D caused hydrolysis of the membrane phospholipids. The phosphatidic acid produced remained bound to the membrane. Increasing phospholipid cleavage was paralleled by loss of calcium uptake, which was complete when about two-thirds of the membrane phospholipids were hydrolyzed.

INTRODUCTION

Changes in the phospholipid content of the membranes of fragmented sarcoplasmic reticulum (FSR) have been shown to interfere with its calcium-translocating system. For example, hydrolysis of the FSR phospholipids by phospholipase C inhibits both the ATP-supported calcium accumulation and the calcium-dependent ATPase (1,2). Phospholipase A treatment of FSR abolishes only calcium uptake, whereas the calcium ATPase activity seems to be maintained by the lysophosphatidylcholines and free fatty acids, which resulted from the phospholipid hydrolysis (3). In this paper, the modification of the vesicular calcium transport system by phospholipase D is described. Since this enzyme is inactive at neutral pH (4), the stability of the membranes remaining for a definite period of time at nonneutral pH values must be known. Therefore, studies concerning this problem have been included.

METHODS

Preparation of sarcoplasmic vesicules, determination of calcium uptake in the presence and absence of oxalate, as well as the assay of ATPase activity, were performed according to Hasselbach and Makinose (5,6). Stock suspensions of sarcoplasmic vesicules were stored at 0-4 C in 0.1 M KC1 (15-20 mg/ml) and were used within 48 hr. The effect of incubations at various values of pH was studied in the following way. The stock preparation of FSR was diluted to 10 mg/ml and the pH was adjusted to the desired value within about 30 sec by the addition of 0.1 M HC1 or KOH using a Metrohm Combi Titrator. After incubation for 2 hr at room temperature, the pH was adjusted to neutrality, and 0.1 M KC1 was added to adjust the protein concentration to 5 mg/ml. This suspension now was immediately assayed for calcium uptake and enzymatic activity.

Incubation for phospholipase D hydrolysis was performed at room temperature with the pH adjusted to 5.7, as described above, using 2 mg enzyme/mg FSR protein and 10 mM $CaC1_2$. Controls were treated identically with the exception that phospholipase D was omitted. After 3, 15, 30, 60, and 120 min, an aliquot was removed and neutralized, calcium and phospholipase D were removed by three centrifugations for 30 min at 100,000 g of the FSR in 0.1 M KC1, 0.1 mM EGTA. The extent of hydrolysis was monitored by the pH stat method as described earlier (3) and by thin layer chromatography (TLC) followed by densitometry according to Fewster et al. (7). Phospholipase D (EC 3.1.4.4) with a specific activity of 0.5 U/mg was purchased from Boehringer, Mannheim.

RESULTS

Effect of 2 hr Incubation at Various pH Values on Calcium Uptake and Ca^{++}-ATPase

A typical experiment with FSR not older than 48 hr carried out by incubation of FSR for 2 hr at different pH values is shown in Figure 1. Calcium uptake in the presence of oxalate was influenced insignificantly in the range from about pH 5.5-9.5. The free calcium concentration in the assay media was low after cessation of net calcium uptake, resulting in low Ca^{++}-ATPase activity. Preincubation at pH values lower than 5.5 and higher than 9.5 inhibited calcium uptake. The calcium remaining in the medium may stimulate the Ca^{++}-ATPase. At even more extreme pH values, the Ca^{++}-ATPase seemed to be inhibited irreversibly. Calcium uptake in the absence of oxalate showed a similar behavior as in the presence of oxalate but seemed to decrease in activity more rapidly at a pH less than 5.5 or greater than 9.5.

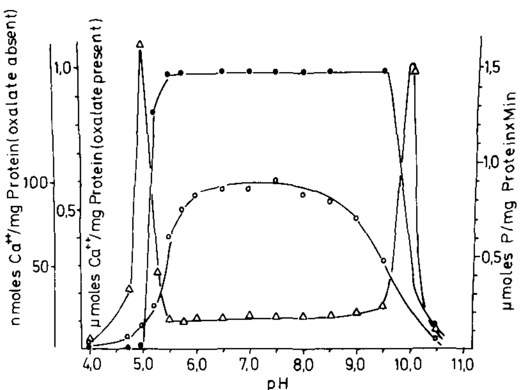

FIG. 1. Calcium uptake and Ca^{++}-ATPase of FSR preincubated for 2 hr at various pH values. The symbols represent the average of duplicate analyses and are typical of the results obtained in seven experiments. •——• Calcium uptake in the presence of oxalate (µmoles calcium/mg protein, 10 min after start of reaction). o——o Calcium uptake in the absence of oxalate (nmoles calcium/mg protein, 2 min after start of reaction). △——△ Activity of Ca^{++}-ATPase after cessation of net calcium uptake (Ca^{++}-ATPase = Ca^{++}, Mg^{++}-ATPase minus Mg^{++}-ATPase, µmoles phosphate split from ARP/mg protein per minute from minute 12 through minute 15 of incubation). Experimental conditions: ATP = $MgCl_2$ = potassium oxalate (if present) = 5 mM, histidine pH 7.0 = 20 mM, KCl = 40 mM, $CaCl_2$ = EGTA = 0.1 mM, FSR protein 0.1 mg/ml.

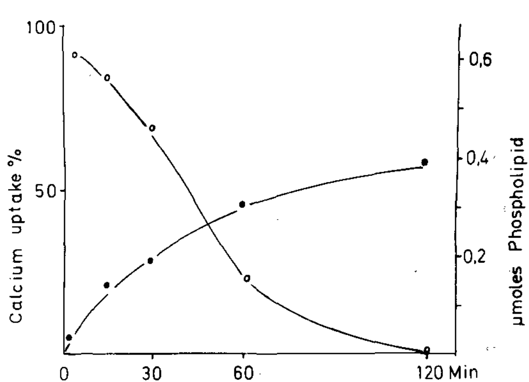

FIG. 2. Effect of phospholipase D treatment on calcium uptake of FSR. The abscissa shows the digestion time, the left ordinate the calcium uptake in presence of oxalate as percent of uptake of controls treated identically with the exception that during preincubation phospholipase D was omitted, and the right ordinate shows the hydrolysis of phospholipids/mg FSR protein in the presence of phospholipase D. Each point on the curves was done in dubplicate, and the same pattern was obtained in five other experiments. o——o Calcium uptake. •——• Phospholipid hydrolysis.

Effect of Phospholipase D on the Phospholipids and the Function of FSR

Treatment of FSR membranes resulted in the disappearance of the original phospholipids, phosphatidylethanolamines and phosphatidylcholines, and the appearance of phosphatidic acid, detectable as soon as 3 min after addition of phospholipase D. After an incubation of 2 hr, TLC revealed only traces of phosphatidylethanolamines and phosphatidylcholines, whereas the amount of sphingomyelins had not changed significantly.

The effect of phospholipase D treatment of FSR membranes on the calcium uptake can be seen in Figure 2. Depending on the degree of hydrolysis of phospholipids, the calcium uptake by FSR was diminished and was completely inhibited when about 0.4 µmoles phospholipid [i.e., about two-thirds of membrane phospholipids (3)] were hydrolyzed.

In contrast to the results on the calcium uptake, the findings concerning the Ca^{++}-ATPase revealed a biphasic behavior. Whereas at low degree of hydrolysis, a slight but consistent increase in the activity of Ca^{++}-ATPase was found; higher degrees (more than 30% of the membrane phospholipids hydrolyzed) caused an inhibition of this enzyme. When about 0.4 µmoles phospholipid (i.e., two-thirds of the membrane phospholipids) were digested, the activity was found to be still 40-60% of that found for the control.

DISCUSSION

The results concerning the preincubation experiments show that a certain pH range is tolerated by FSR without leading to an alteration of its function; this suggests that no irreversible structural damage of the membrane has occurred. This range is much broader for freshly prepared FSR than for aged preparations (not shown here) and is broader if the free calcium concentration inside of the FSR is kept low by the presence of oxalate in the uptake medium.

Beyond this range, on the acid as well as on the alkaline side, there is a rather small range in which calcium uptake is decreasing or already abolished, but calcium-stimulated ATPase is still active, i.e., Ca^{++}-ATPase continues splitting ATP with the initial rate after net calcium uptake has come to an end because little or no calcium is removed from the uptake media. For the acid side, similar results were recently obtained by Bergman et al. (8), although different experimental conditions were chosen. These authors ascribe this dissociation of Ca^{++}-ATPase and calcium uptake to an uncoupling of the energy-providing system from the calcium-

translocating system rather than to an increased leakiness of acid-treated FSR.

Our findings concerning the effect of phospholipase D on FSR function are in contrast to those of Yu et al. (9), who did not observe any effect of phospholipase D treatment on calcium uptake and only a slight stimulation of Ca^{++}-ATPase activity. An explanation for this discrepancy may be that under their experimental conditions, 2 hr at pH 7.2 (a value at which we, even after a 4 hr incubation, have seen no phosphatidic acid formed) hydrolysis of phospholipids was insufficient. Additionally, in their digestion media, these authors had no calcium present which would not only enhance the activity of phospholipase D (4) but also have a protective effect on proton inactivation of FSR (8).

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Studies on the Hydrogen Belts of Membranes: III. Glycerol Permeability of Dihydrosphingomyelin-Cholesterol Membranes

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ABSTRACT

The permeability of an N-oleoyldihydrosphingomyelin bilayer against glycerol was similar to that of a bilayer of phosphatidylcholine with identical effective hydrophobic chain length. Cholesterol at 1:1 molar ratio reduced the permeability, and also reduced the energy of activation of glycerol penetration, an effect not found for diesterphosphatidylcholine with cholesterol. The higher level of the ground state of the entropy of activation for permeability can be interpreted in terms of a hydrogen belt model which postulates lipid-lipid hydrogen bonding in membranes and explains the effect found as a disturbance of the hydrogen belt structure. Dihydrosphingomyelin can be considered to function as an "extender" in the hydrogen belt network.

INTRODUCTION

Those regions between the hydrophobic core and the polar zones of a biological membrane which we have called "hydrogen belts" (1,2) consist of hydrogen bond acceptors (CO groups of phospho- and sphingolipids) and donors (OH groups of cholesterol, sphingosine, water, possibly proteins). We have suggested that these groups may engage in lipid-lipid hydrogen bonding. In the preceding paper of this series (3), we showed that while cholesterol reduced the permeability of both diesterphosphatidylcholine and (CO-free) dietherphosphophatidylcholine bilayers, it reduced the activation entropy of permeability only in mixture with diether-, not with diester-lipid. This finding was interpreted to support our hydrogen belt model.

Sphingolipids have both a hydrogen bond accepting C=O group and a hydrogen bond donating free 3-OH group. (The NH group is a weaker donor and not, at present, considered by us.) We have, therefore, classified the sphingolipids as "extenders" in the hydrogen belt network. According to our concepts, sphingolipids added to a lipid bilayer would not disturb the existing hydrogen bond donor-acceptor balance in the belts. In a pure sphingolipid bilayer, there is already a perfect balance between donors and acceptors, and while the addition of further acceptors (CO) would lead to further hydration (1,3-5) without disturbance of the water structure at the hydrogen belt, the addition of further donors might upset this structure, in analogy to the disturbance evidenced in the dietherphosphatidylcholine-cholesterol system (3). Here we test this possibility by studying the permeation of a nonelectrolyte through bilayers (liposomes) of dihydrosphingomyelin with and without cholesterol.

We want to stress that we intentionally studied dihydrosphingomyelin rather than the more common *trans* unsaturated sphingomyelin because we wanted to eliminate any influence of the allylic double bond on the properties of the 3-OH group. We have postulated that this double bond may impart partial acceptor character on the group (2); but that is a different story.

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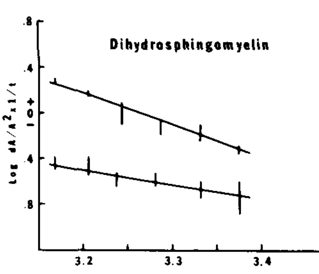
TABLE I

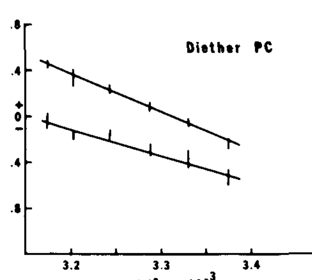
Activation Energies ΔE^* of Permeation of Glycerol through Lipid Bilayers[a]

	Without cholesterol	With cholesterol
Dihydrosphingomyelin	14.2 ± 0.9	7.2 ± 1.0
Diesterphosphatidylcholine[b]	16.6 ± 1.8	16.8 ± 1.4
Dietherphosphatidylcholine[b]	15.2 ± 1.3	10.1 ± 0.9

[a]Temperature, 27-47 C. Liposomes contained 96 mole % dihydrosphingomyelin and 4 mole % dioleylphosphate; or 48 mole % dihydrosphingomyelin, 48 mole % cholesterol, and 4 mole % dioleylphosphate.

[b]Taken from (3).





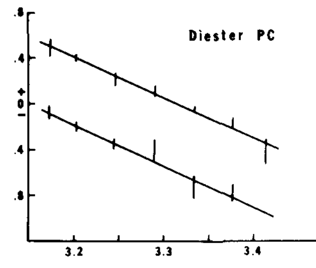


FIG. 1. Representative Arrhenius plots of relative permeabilities of glycerol through liposome bilayers. Plots for diether-PC and diester-PC taken from (3). Bars given range of data of the experiment. Upper lines: permeation of polar lipids; lower lines: polar lipid/cholesterol, 1:1.

MATERIAL AND METHODS

N-Oleoyldihydrosphingomyelin (H_2-SM) was prepared as follows. Crude beef brain lipids were separated on silicic acid (Mallinckrodt, lg/60 mg lipid) with chloroform-methanol 3:2 (discarded) and 1:4 (crude sphingomyelin fraction). From this fraction, sphingosylphosphorylcholine was prepared according to Kaller (6). This was hydrogenated (H_2Pt, in ethylacetate) and acylated with oleoyl-N-hydroxysuccinimide (7). The H_2-SM was purified by high performance liquid chromatography (Porasil®, Waters Assoc., Framingham, MA) with chloroform-methanol-water, 2:3:0:5. Base analysis (8,9) showed C_{18}-dihydrosphingosine with 21% C_{20}-dihydrosphingosine.

The syntheses of diester- and dietherphosphatidylcholine (PC) have been described (3,10). Cholesterol was recrystallized three times from ethanol. Dioleylphosphate was synthesized as described (3).

The relative permeation rates of glycerol were determined by monitoring the absorbance change at 450 nm as a function of time during isotonic swelling of liposomes (3,11). The change in absorbance, divided by the square of the initial absorbance, per second, is inversely proportional to the volume change of the liposomes and to the permeability coefficient (11). Activation energies ΔE^* were determined by Arrhenius plots of liposome swelling as a function of temperature (3).

Four mole percent dioleylphosphate was added to the lipids, and the dihydrosphingomyelin-cholesterol molar ratio was 1:1. Handshaken liposomes, 0.40 μmoles lipid in 40 μl of 0.15M KCl were injected into 1.5 ml of 0.3 M aqueous glycerol. The initial absorbance, under these conditions, was 0.65 ± 0.03 and 0.44 ± 0.03 for sphingomyelin liposomes with and without cholesterol. The corresponding values for diester- and dietherphosphatidylcholine were 0.53 ± 0.06 and 0.57 ± 0.2 without cholesterol, and 0.46 ± 0.03 and 0.44 ± 0.03 with cholesterol. The size distributions of liposomes in these preparations were, therefore, quite similar. The linear changes of absorbance between 2 and 4 sec after injection were measured. The magnitude of the permeability rates and the fact that the permeability-temperature curves were smooth between 27 C and 50 C showed that we were working above the crystalline/liquid-crystalline transition temperature, as intended; we did not determine the actual transition point. The ΔE^* values of Table I are the means of five experiments.

RESULTS AND DISCUSSION

Previous permeability studies of sphingomyelin bilayers (12,13) have used natural lipid with long chain fatty acids. Since permeation rates depend on the thickness of the bilayer, no direct comparison with the permeation rates of glycerophospholipids (which have shorter chains) could be made. We have, therefore, synthesized a compound that has the same effective hydrophobic chain length, 28, defined previously (3), as palmitoyl-oleoyl-phosphatidylcholine and the corresponding diether analog. It can be seen in Figure 1 that the permeabilities against glycerol are very similar. The activation energies ΔE^* of glycerol permeation through the lipids (without cholesterol) are also similar (Table I), though ΔE^* for dihydrosphingomyelin may be some 2 kcal lower than that for the diester-PC.

Cholesterol closes the bilayer of sphingomyelin as it does those of the phosphatidylcholines (Fig. 1). It also severely reduced the activation energy of glycerol permeation (Table I). This is similar to the reduction found for dietherphosphatidylcholine with cholesterol (3) (Table I). Diesterphosphatidylcholine shows no such decrease of ΔE^* with cholesterol (3,14) or may even show some increase (15).

Our interpretation of these findings is as

follows. The approximately fivefold difference in permeation rates between dihydrosphingomyelin without and with cholesterol at 37 C corresponds to a $\Delta\Delta G^*$, the difference between them of the free energies of activation, of around 1 kcal/mole; thus the difference of 7 kcal/mole between the ΔE^* must, according to $\Delta G^* = \Delta H^* - T\Delta S^*$, be due mainly to a difference $\Delta\Delta S^*$ in the entropy of activation. ($\Delta H^* = \Delta E^* - 0.6$ kcal/mol) (16). The difference $\Delta\Delta S^*$ between dihydrosphingomyelin without and with cholesterol is calculated as -26 cal/mole/degree. For dietherphosphatidylcholine with and without cholesterol, a difference of -16 cal/mole/degree had been found (3).

The energy and entropy of activation are a measure of the dehydration of the permeant (here, glycerol) which must cross the bilayer as an anhydrous species (3,14-16). Since only the permeant and water are involved in the transition state of dehydration, and this transition state is the same in all systems, it follows from the negative entropy change of -26 cal/mole /degree that the ground state of the entropy of dehydration must be higher by +26 cal, i.e., the structure of water near the hydrogen belts of the bilayer is more disordered. These results are in accord with our concepts of hydrogen belt structure. In both lipid bilayers, cholesterol can no longer hydrogen-bond to the polar lipids – as it can to diester-PC – because there are no CO groups available (diether-PC), or the CO available is already bound or balanced by OH groups (dihydrosphingomyelin). Thus, the cholesterol-OH proton is free to interact with the adjacent water layer and disrupt its structure (3). This leads to a higher entropy content of this layer, which is mirrored in the higher ground state of ΔS^* that we find in our experiments.

ACKNOWLEDGMENT

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Cyclopropene Fatty Acids of Selected Seed Oils from Bombacaceae, Malvaceae, and Sterculiaceae

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ABSTRACT

Fatty acid compositions of seed oils from three species of Bombacaceae, eleven from Malvaceae, and six from Sterculiaceae were determined. Each of the seed oils contains varying amounts of both malvalic and sterculic acids accompanied by one or both of the corresponding cyclopropane fatty acids. In addition, the seed oil of *Pachira aquatic* Aubl. (Bombacaceae) contains 12.8% α-hydroxy-sterculic acid.

INTRODUCTION

Cyclopropene fatty acids have been reported as constituents in the seed oils from many species of the Malvaceae, Sterculiaceae, Tilliaceae, and Bombacaceae families (1). A number of oils from these families have been investigated and found to contain both malvalic and sterculic acids, frequently accompanied by smaller proportions of one or both of the analogous cyclopropane acids (2,3). In addition, seed oils containing malvalic acid usually contain measurable amounts of epoxy acids (4).

It is evident from these earlier investigations that seed oils containing such an array of compounds are difficult to quantitatively analyze by traditional gas liquid chromatography (GLC) and hydrogen bromide (HBr) titration procedures. Recourt et al. (5) have shown that the cyclopropene acids tend to isomerize and decompose as they pass through the GLC column. In addition, GLC data show that the malvalic acid peak is masked by the linoleic acid peak (3) and that the corresponding cyclopropane acid may also be obscured by the presence of oleic acid (6). The HBr titration methods most often used to determine the cyclopropene and epoxy acids present are recognized as being subject to interference by conjugated dienols (7).

The present investigation was undertaken to determine the fatty acid composition of a series of seed oils that contains a mixture of normal fatty acids and fatty acids with epoxy, hydroxy, cyclopropene, and cyclopropane functional groups.

EXPERIMENTAL PROCEDURES

Oil was obtained from the ground seed as previously described (8). Fatty acids in the oils reacting with hydrogen bromide at 55 C were determined by the titration method of Harris et al. (9). Methyl esters for GLC were prepared by sodium methoxide catalyzed methanolysis and treated with silver nitrate by the procedure of Schneider et al. (10) to convert the cyclopropene acids to derivatives amenable to chromatography. Epoxy acids, when present, were converted to methoxy-hydroxy derivatives by treating with boron trifluoride (11). Equivalent chain lengths (ECL) of methyl esters and the reaction products were determined by GLC as previously described (6). The normal methyl esters were separated from the reaction products of the cyclopropene and hydroxy acids by thin layer chromatography (TLC) on 1.0-mm layers of Silica Gel G developed with n-hexane-ethyl ether (70:30). The normal methyl esters were separated according to degree of unsaturation on Silica Gel G (containing 20% silver nitrate) developed with benzene. Mass spectral data were obtained with a Dupont 21-491-2 mass spectrometer interfaced to a Bendix Model 2600 gas chromatograph (GC–MS). The column, 6 ft x ¼ in. packed with 5% Silar (5 cp) was held isothermally at 195 C.

Nuclear magnetic resonance (NMR) spectra were determined with a Varian HA-100 spectrometer. Samples were dissolved in deuterochloroform; tetramethylsilane was used as an internal standard. Infrared (IR) spectra were measured on liquid films with a Perkin-Elmer Model 337 spectrometer. A Beckman DK-2A spectrophotometer was used to record ultraviolet (UV) spectra. Hydroxyl groups were silylated with bis(trimethylsilyl)-trifluoroacetamide in pyridine.

RESULTS AND DISCUSSION

Preliminary Identification of Methyl Esters and Derivatives

GLC analyses of mixed methyl esters containing only normal esters and derivatized cyclopropene esters were consistent with the peaks previously reported for the esters from derivatized *Sterculia foetida* oil (10). However, GLC of those mixed esters also containing

TABLE I

Composition of Seed Oils

Sample	Oil in seed (%)	Fatty acid composition by GLC (area %)															HBr reactive acids at 55 C (%)	Spectral data	
											Cyclo-propane		Cyclo-propene						
		16:0	16:1	17:0	17:1	18:0	18:1	18:2	20:0	20:1	18	19	18	19	Epoxy[a]	Hydroxy[b]		UV	IR
Bombacaceae																			
Chorisia speciaea St. Hil.	21.7	18.7	0.5	0.2	0.2	2.8	8.4	44.7	0.7	---	0.1	0.5	12.4	10.0	0.8	---	21.0	---	---
Pachira aquatica Aubl	55.5	60.5	---	0.3	---	2.9	7.6	4.7	0.4	---	---	0.1	1.6	8.6	---	12.8	26.6	---	OH
Salmalia malabarica (DC) Schott and Endl.	23.6	30.0	0.6	0.2	0.8	5.0	16.7	25.0	---	---	tr	0.6	7.5	11.0	1.0	---	15.5	L_3[c] 1.0	---
Malvaceae																			
Althaea hirsuta L.	12.2	13.0	0.5	0.3	0.4	4.4	7.5	52.8	0.8	---	0.4	0.2	16.5	1.6	---	---	22.4	---	---
Hibiscus grandiflorus[d]	11.7	18.2	---	0.4	2.0	6.0	15.5	46.0	0.3	---	0.8	1.3	4.0	3.0	---	1.6	7.2	---	OH
Hibiscus syriacus[d]	27.7	20.0	tr	tr	0.7	2.2	10.2	42.6	0.3	---	tr	1.0	13.4	3.0	2.7	2.8	19.9	---	OH
Kitaibelia vitifolia Willd	22.6	8.0	0.3	tr	0.3	2.5	14.2	64.4	0.5	---	0.2	0.1	7.7	1.5	---	---	10.5	---	OH
Lagunaria patersonii G. Don	18.9	23.0	3.9	tr	0.4	4.7	21.5	22.3	tr	---	---	1.1	7.7	3.9	---	1.4	14.1	---	OH
avatera kashmiriana Comb.	19.1	15.0	tr	tr	0.6	3.6	10.8	50.5	0.5	0.3	0.5	tr	16.4	1.2	---	---	14.4	---	---
Malva montana Forsk	18.4	15.7	0.2	tr	0.4	3.3	10.3	55.5	---	---	tr	0.2	12.1	1.0	---	1.0	14.4	---	---
Malva parviflora L.	11.0	15.4	---	0.2	1.1	3.0	9.0	53.4	0.8	---	0.2	0.4	12.0	1.7	1.0	1.2	14.8	---	---
Malva tournefortiana L.	17.0	12.4	0.5	1.6	2.1	3.5	11.0	43.8	2.3	0.2	tr	0.3	17.8	1.5	2.0	---	23.7	---	---
Malope trifida Paxt.	17.7	18.0	0.3	0.2	1.6	3.7	6.8	44.3	0.2	---	0.2	0.3	11.5	3.1	6.5	1.2	24.9	L_2[e] 5.4	OH
Pavonia sepium St. Hil	26.6	31.0	1.0	tr	0.2	2.0	15.4	34.2	0.5	tr	tr	tr	2.3	1.3	5.1	5.7	17.6	L_2 3.6	OH
Sterculiaceae																			
Firmiana platanifolia Schott and Endl.	24.3	17.2	1.2	tr	0.2	2.4	18.2	36.0	0.7	---	tr	0.3	1.6	20.5	2.0	---	24.3	---	---
Pterygota alata Roxb.	47.8	24.4	1.6	0.2	0.3	3.3	9.9	38.5	0.6	---	0.1	0.6	12.2	2.5	2.7	1.4	19.5	---	OH
Pterospermum acerifolium (L.) Willd	21.5	17.0	tr	tr	0.3	2.9	8.6	32.3	1.0	---	---	---	32.2	3.8	---	---	39.1	---	---
Sterculia foetida L.	53.5	14.7	tr	tr	tr	1.4	4.9	4.5	1.8	0.2	tr	0.4	6.3	65.1	---	---	64.6	---	---
Tarrietia utilis[d]	29.7	29.2	0.7	0.1	0.2	2.0	20.3	18.9	0.6	tr	tr	0.2	6.8	20.2	---	---	25.7	---	---

[a]Epoxy acid is vernolic by GLC evidence.

[b]Hydroxy acid is a conjugated dienol by GLC evidence except in *P. aquatica* and *H. syriacus* which were identified as α-hydroxysterculic.

[c]L_3 conjugated triene; % in fatty acids.

[d]Authority not available.

[e]L_2 conjugated diene; % in fatty acids.

epoxy, hydroxy, or conjugated unsaturation were difficult to interpret. Esters shown to contain the epoxy function were further derivatized by converting this group to the methoxy-hydroxy derivative followed by conversion of the hydroxyl group to the trimethylsiloxy (TMS) ether (11). The resulting TMS-derivatized esters were reanalyzed by GLC. These data (Table I) indicated that the seed oils contained varying amounts of epoxy and/or hydroxy acids in addition to the cyclopropanes, cyclopropenes, and normal fatty acids. The varying amounts and wide distribution of the epoxy and hydroxy acids in these oils were as expected (1,4,12). The mixed esters derived from each oil were separated into three fractions by preparative TLC on Silica Gel G plates. GLC data indicated that fraction I was a mixture of normal long chain methyl esters and possibly the cyclopropane methyl esters. Fraction II contained the esters of the cyclopropene derivatives, and fraction III contained the hydroxy methyl esters and the methoxy-hydroxy derivatives obtained from the epoxy methyl esters. De Bruin et al. (13) reported that *Pachira aquatic* oil possibly contained an acid similar to sterculic, but which might also contain hydroxyl group(s). Therefore, the hydroxy acid component was isolated and characterized.

Characterization of the Hydroxy Acid in *P. Aquatica*

The migration pattern of the component(s) in fraction III isolated from *P. aquatica* was compared to that of a known mixture of α-hydroxy octadecanoates and methyl sterculate. The major component in the isolated material remained near the origin along with the α-hydroxy acids whereas authentic sterculate migrated nearer to the solvent front. Distinctive IR absorption bands at 1265, 1210, and 1110 cm^{-1} are indicative of α-hydroxy esters (14). Absorption bands at 1850 and 1010 cm^{-1} were also present (cyclopropene ring). A pronounced signal (singlet) in the NMR spectrum at δ4.12 suggested that a methine proton was on the carbon adjacent to the carboxyl function. Signals at δ2.3-2.4 associated with a methylene group in the alpha position were not observed. After conversion of the hydroxyl group of the major component to a TMS derivative, ECLs of 18.6 (APL column) and 20.6 (R-446 column) were observed. The ECLs were similar to those of authentic methyl sterculate (6). Mass spectral data of the isolated component were identical to those of α-hydroxysterculic acid of *Pachira insignis* as reported by Morris and Hall (15). The fragmentation patterns of the silver nitrate derivatives obtained from the isolated component were not as clearly defined. However, the appropriate molecular ions at m/e = 412 for the ketone derivative and m/e = 428 for the methoxy derivative were apparent. Also, the ion at m/e = 161 resulting from cleavage alpha to the carbon bearing the TMS group was observed.

Characterization of the Cyclopropene Acids

The silver nitrate derivatives obtained from the methyl esters of the cyclopropene acids were found in fraction II from TLC. The migration pattern and GLC elution pattern of these components were identical to the corresponding derivatives of malvalic and sterculic acids from the seed oil of *Sterculia foetida* by Recourt et al. (5). The MS fragmentation patterns are also identical to those reported by Eisele and co-workers (16).

Characterization of the Cyclopropane Acids

The cyclopropane acids were contained in fraction I from the preparative TLC along with the normal long chain fatty acids. These acids have retention characteristics similar to those of the analogous monounsaturated methyl esters (6). Therefore, it was necessary to further separate fraction I according to degree of unsaturation by $AgNO_3$-TLC prior to identification of the cyclopropane acids. The fraction collected as the saturated methyl esters from this mixture was shown by GLC to contain small amounts of dihydromalvalic acid with ECLs of 17.7, (APL column) and 18.4 (R-446 column) and/or dihydrosterculic acid with ECLs of 18.7 (APL column) and 19.4 (R-446 column). Mass spectra of these acids are similar to those of the parent monoenoic esters (17). The only apparent difference is that the cyclopropane acids show a molecular ion 14 mass units greater than the parent monoenoic ester.

Description of Seed Oils

Oil content of the seeds varied from 11% in *Malva parviflora* to 56% in *P. Aquatica.* The seed oils, except for three (Table I) showed no UV absorption indicative of conjugated unsaturation. Epoxy acids, if detected, ranged from 0.8% in *Chorisia speciasa* to 6.5% in *Malope trifida.* Cyclopropene acids ranged from 3.6% in *Pavonia sepium* to 71.4% in *S. foetida.* In general, the presence of a cyclopropene ring was established in the seed oils by HBr titration at 55 C and by the presence of absorption bands in the IR spectrum at 1010 and 1850 cm^{-1}. GLC analyses for the cyclopropene acids are probably more reliable than the HBr titration because of uncertainties in the titration method pointed out by Feuge et al. (18). Also

observed in the spectra was absorption at 3550 cm^{-1} (hydroxyl) from many of the oils. The seed oil of *P. aquatica* contained 12.8% α-hydroxysterculic acid.

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Chemical Composition of the Preen Gland Secretions from Some Ciconiiform Birds

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ABSTRACT

The preen waxes from four Ciconiiformes species are shown to be ester waxes of very different composition. Three species possess monoester waxes with branched wax acids and alcohols. In one of these waxes, homologous series of ethyl-substituted constituents are observed. In contrast to this, the uropygial gland secretion of the marabou is a triglyceride mixture.

INTRODUCTION

The comparison of the chemical composition of uropygial gland secretions has been found useful to the chemotaxonomy of birds (1) and in most cases has confirmed the natural system. The limited data on birds of the order Ciconiiformes (herons, storks, ibises, hammerhead) seem to indicate a certain heterogeneity of this group of birds. In previous papers, we have shown unbranched ester waxes and triglycerides to be present in the preen wax from *Ardea cinerea* (heron) (2), unbranched and secondary alcohols containing monoester waxes in *Nycticorax nyctocorax* (night heron) (3), and unbranched monoester waxes, 2-hydroxy fatty acids containing diester waxes as well as triglycerides in *Ciconia ciconia* (white stork) (4). Further investigation of this order of 111 different species, therefore, seems to be desirable.

This paper presents the chemical composition of the uropygial gland secretion from four more species: the buff-necked ibis (*Theristicus caudatus,* Bodd.) living in South America and three African species, the sacred ibis (*Threskiornis aethiopica,* Lath.), the marabou (*Leptoptilos crumeniferus*), and the hammerhead (*Scopus umbretta*). The results are compared with data already published.

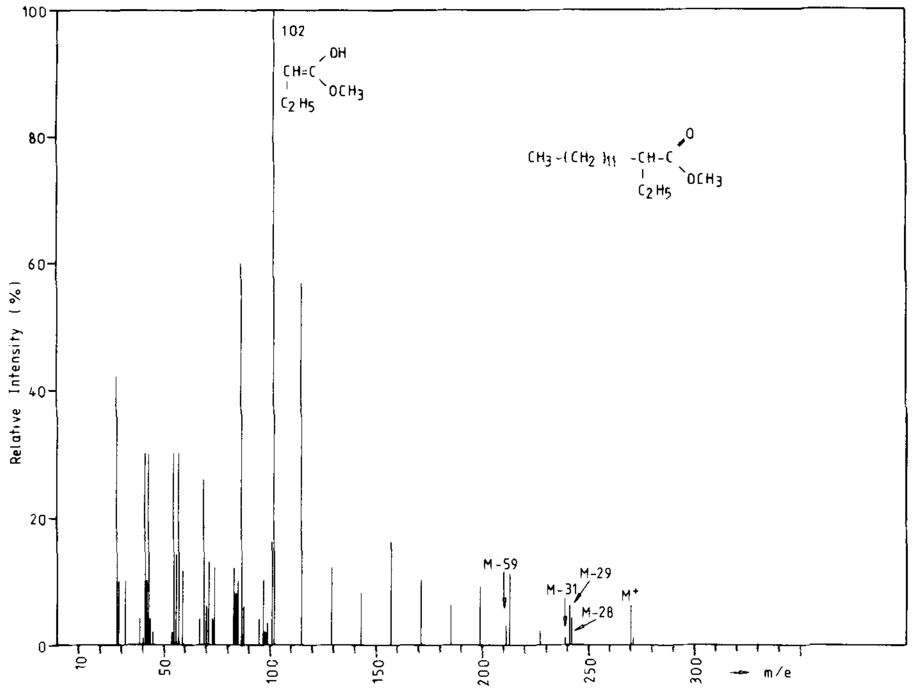

FIG. 1. Mass spectrum of methyl 2-ethyltetradecanoate.

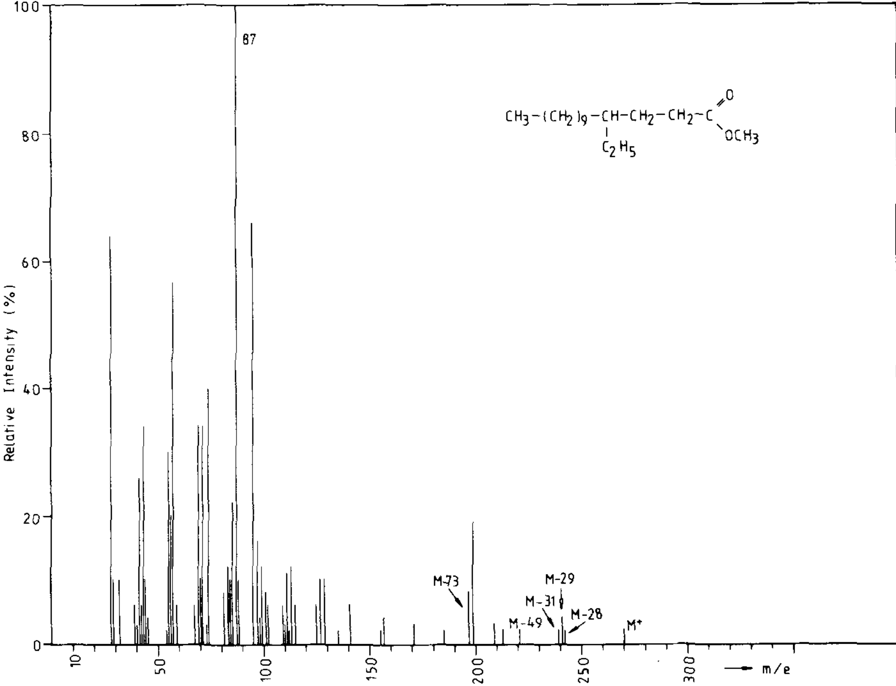

FIG. 2. Mass spectrum of methyl 4-ethyltetradecanoate.

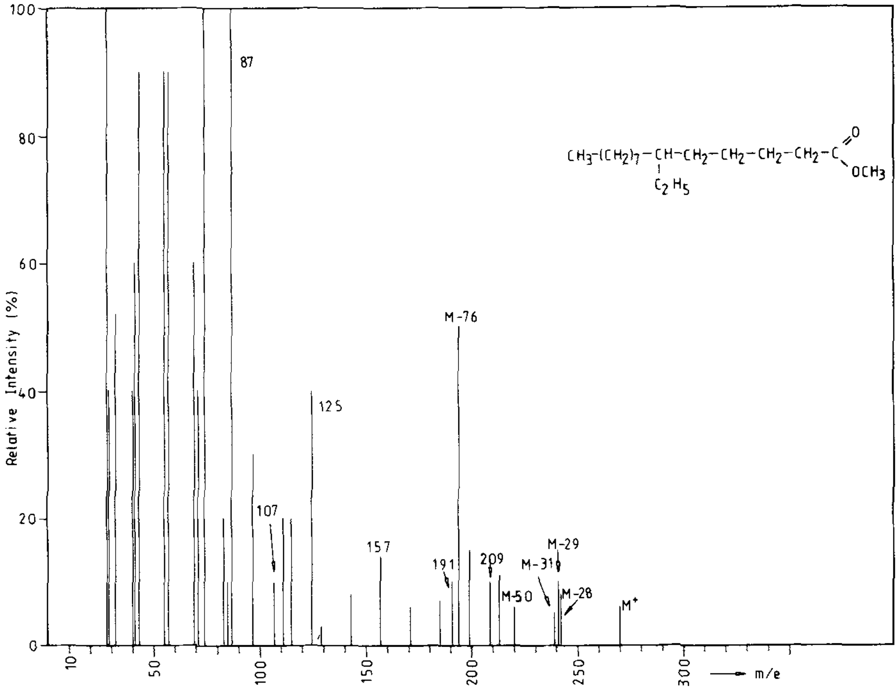

FIG. 3. Mass spectrum of methyl 6-ethyltetradecanoate.

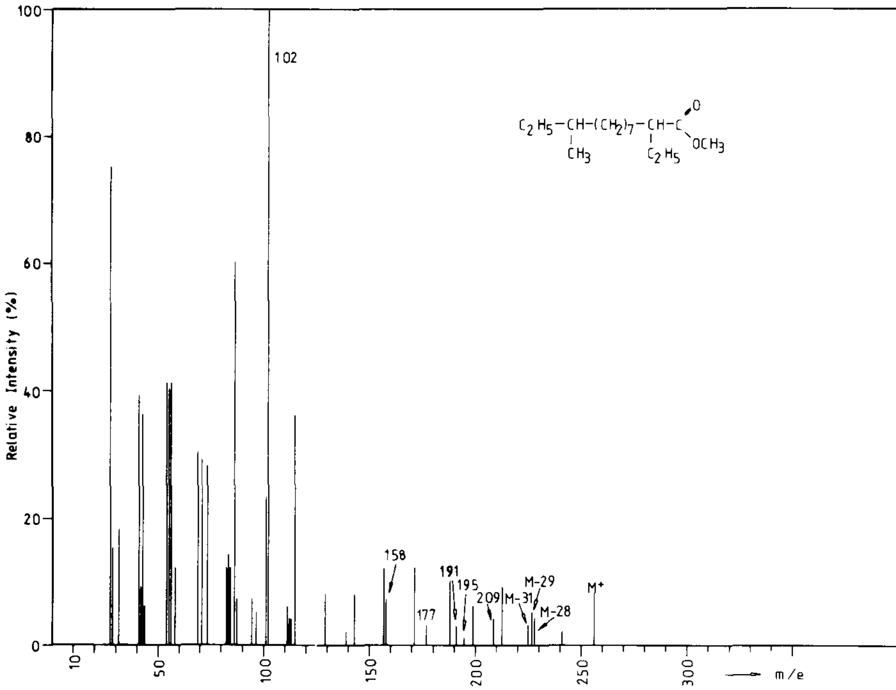

FIG. 4. Mass spectrum of methyl 2-ethyl-10-methyldodecanoate.

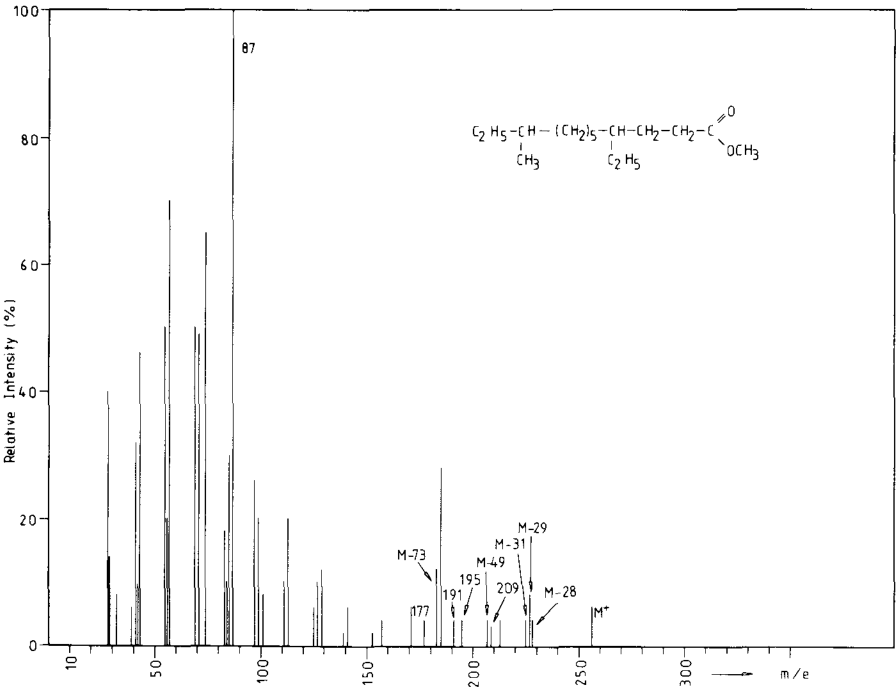

FIG. 5. Mass spectrum of methyl 4-ethyl-10-methyldodecanoate.

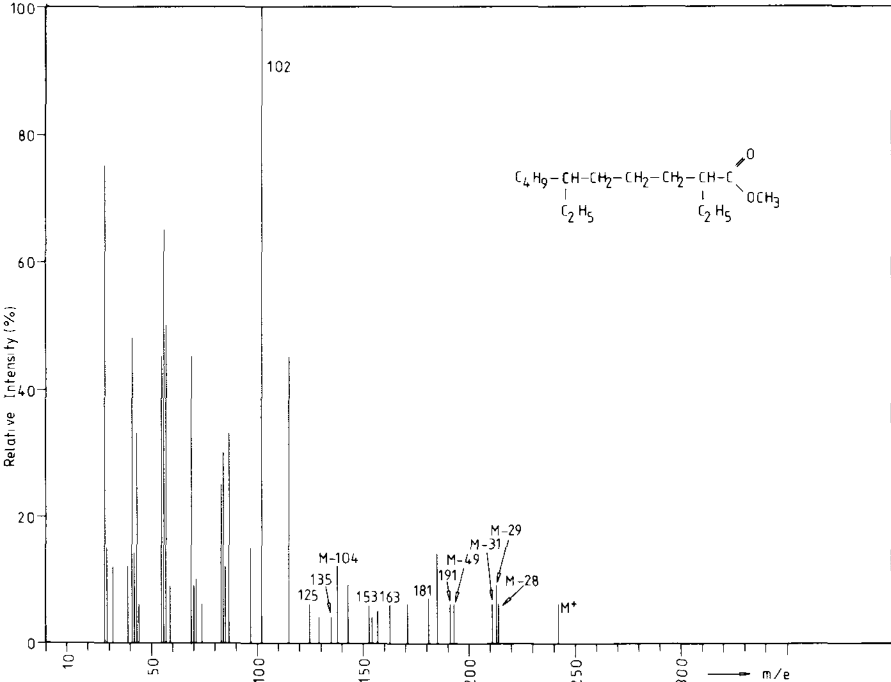

FIG. 6. Mass spectrum of methyl 2,6-diethyldecanoate.

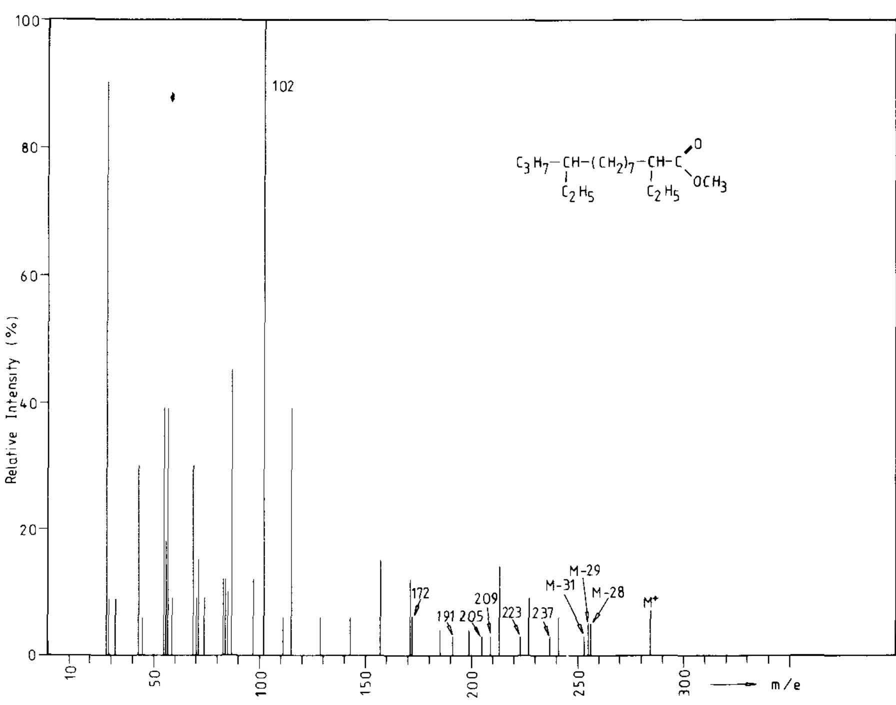

FIG. 7. Mass spectrum of methyl 2,10-diethyltridecanoate.

TABLE I

Composition of the Wax Constituents from the Uropygial Gland Secretion of the Hammerhead (*S. umbretta*)

	Fatty acids (%)	Alcohols (%)
Unbranched (total)	(11.5)	(87.1)
n-C_{10}	trace	---
n-C_{12}	0.2	---
n-C_{13}	0.3	---
n-C_{14}	1.1	---
n-C_{15}	---	0.1
n-C_{16}	5.0	5.4
n-C_{17}	---	6.6
n-C_{18}	4.9	75.0
Monomethyl-br. (total)	(62.2)	(12.2)
2-C_{12}	trace	---
2-C_{14}	0.2	---
2-C_{16}	6.9	0.7
2-C_{17}	1.4	---
4-C_9	trace	---
4-/6-C_{10}	0.1	---
4-/6-C_{11}	0.1	---
4-/6-C_{12}	0.5	---
4-/6-C_{13}	0.3	---
4-/6-C_{14}	6.9	---
4-/6-/8-/10-C_{15}	2.6	---
4-/6-/8-/10-/12-C_{16}	36.1	4.0
4-/6-/8-/10-/12-C_{17}	1.7	0.5
4-/6-/8-/10-/12-C_{18}	5.4	1.6
14-C_{16}	---	3.0
14-C_{17}	---	0.4
14-C_{18}	---	2.0
Dimethyl-br. (total)	(25.1)	(0.7)
2,6-C_{14}	0.6	---
2,6-C_{15}	1.3	---
2,10-C_{16}	1.9	---
2,14-C_{17}	0.3	---
4,6-C_{14}	2.5	—
4,8-C_{10}	0.1	---
4,10-C_{14}	2.7	---
4,10-C_{16}	4.0	---
4,12-C_{14}	2.7	---
4,12-C_{16}	2.1	---
4,14-C_{16}	1.5	0.7
4,14-C_{17}	0.3	---
6,10-C_{12}	0.7	---
6,10-/6,12-C_{14}	0.1	---
6,10/6,12-C_{16}	0.9	---
6,14-/10,14-C_{16}	3.4	---
Unidentified	1.2	---

MATERIAL AND METHODS

The uropygial glands were excised from freshly killed adult males and preserved in acetone. Extraction, purification, separation (thin layer and column chromatography) procedures were performed as published previously (5). Monoester waxes were obtained in case of both ibises and the hammerhead [*T. caudatus:* 56.4 (62.0) mg; *Th. aethiopica:* 111.9 mg; *S. umbretta:* 74 mg], whereas triglycerides were present in the marabou [*L. crumeniferus:* 105.3 (112.4) mg] (Data in parentheses are from a second specimen.)

Transesterification of monoester waxes and triglycerides was performed with 5% methanolic HCl, and the methanolysis products were purified by column chromatography on silica gel. Alcohols were oxidized with CrO_3/acetic acid in t-butanol/cyclohexane to the corresponding fatty acids which subsequently were esterified as above (5).

All constituents were identified by gas liquid chromatography (GLC) comparing ECL values (equivalent chain length) on a semi-log plot, and by mass spectrometry (MS). The ECL values found for methyl esters of 4-ethyl-branched acids (increment about + 1.4, depending on the chain length) were compared with authentic methyl 4-ethyloctanoate

TABLE II

Composition of the Wax Constituents from the Uropygial Gland Secretion of the Sacred Ibis (*Th. aethiopica*)

	Fatty acids[a] (%)	Alcohols (%)
2-Methyl-branched (total)	(46.9)	(54.7)
2-C_{10}	---	0.8
2-C_{11}	2.5	37.3
2-C_{12}	0.1	2.1
2-C_{13}	25.6	11.3
2-C_{14}	trace	trace
2-C_{15}	15.5	3.2
2-C_{16}	0.9	---
2-C_{17}	2.3	---
2,x-Dimethyl-branched (total)	(41.2)	(44.3)
2,6-C_9	---	0.2
2,6-C_{10}	---	1.0
2,6-C_{11}	1.6	20.0
2,6-C_{12}	0.2	0.3
2,6-C_{13}	12.3	1.6
2,6-C_{14}	0.5	---
2,8-C_{10}	---	0.7
2,8-C_{11}	0.9	11.3
2,8-C_{12}	0.1	0.3
2,8-C_{13}	5.8	3.2
2,8-C_{15}	1.7	---
2,10-C_{12}	0.5	0.6
2,10-C_{13}	11.9	5.1
2,10-C_{14}	0.7	---
2,10-C_{15}	2.0	---
2,12-C_{14}	0.4	---
2,12-C_{15}	2.6	---
2,x,y-trimethyl-br. (total)	(10.1)	(1.0)
2,4,8-C_{11}	2.3	---
2,6,8-C_{11}	---	0.5
2,6,10-C_{13}	7.8	0.5
Unidentified	1.8	---

[a]x and y = even-numbered.

resulting in an increment of + 1.43.

Mass spectra were recorded on Varian-MAT 111 (GNOM) instrument operating at 80 eV equipped with a glass connection between GC column and ion source. Glass columns, 10 m, (2 mm inner diameter) packed with Gas Chrom Q coated with 3% OV 101 were used, both for GC and combined GC-MS.

RESULTS

The patterns of wax constituents differ significantly among the four species investigated. The hammerhead possesses predominantly 2-, 4-, 6-, 8-, and 10-monomethyl- as well as lesser amounts of dimethyl-substituted acids. The alcohols are mainly unbranched (Table I). In the preen waxes of the sacred ibis, 2-methyl-branched and acids deriving from this type, e.g., 2,6-, 2,8-, 2,10-, 2,12-dimethyl- and 2,x,y-trimethyl-branched acids (x and y = even numbered), predominate. The structures of the alcohols are quite similar in this case (Table II). The buff-necked ibis shows unusual ester wax constituents (Table III) belonging to ethyl-branched homologous series. 2-Ethyl-substituted acids (Fig. 1) have been reported to occur in the preen waxes of Strigiformes (owls) (6), Paridae (tits) (7), Sphenisciformes (pengiuns) (8), and Podicipediformes (grebes) (9), but acids with ethyl-branches other than at C-2 have not been observed hitherto. An ethyl branch can be recognized from the intensity relation (M-29)>(M-31). The spectra readily can be misinterpreted since this intensity relation is also observed in (ω-2)-methyl-branched esters. Moreover, the ion series M-29$\rightarrow$ $\xrightarrow{-CH_3OH}$M-61 $\xrightarrow{-H_2O}$ M-79 is observed in both ester types as well, so that the ECL values must be carefully regarded.

Ethylene elimination (M-28) is observed only in 2-ethyl-substituted esters and not, or to a minor extent only, in esters with ethyl branches in the middle of the chain, e.g., 4-, 6-, 8-, or 10-ethyl-substituted esters. 4-Ethyl-

TABLE III

Composition of the Wax Constituents from the Uropygial Gland Secretion of the Buff-necked Ibis (*Th. caudatus*, Bodd.)[a]

	Fatty acids (%)	Alcohols (%)
Unbranched (total)	(2.1)	(55.7)
n-C_{15}	---	0.6
n-C_{16}	2.1	9.2
n-C_{17}	---	2.1
n-C_{18}	---	43.8
Monoethyl-branched (total)	(---)	(29.9)
12-C_{14}	---	0.6
12-C_{15}	---	4.6
13-C_{16}	---	20.7
15-C_{17}	---	4.0
Monoethyl-branched (total)	(50.8)	(9.0)
2-ethyl-C_{12}	7.5	0.6
2-ethyl-C_{14}	13.0	0.6
2-ethyl-C_{16}	1.5	---
4-ethyl-C_{12}	8.0	2.1
4-ethyl-C_{14}	9.0	1.5
4-ethyl-C_{16}	6.5	---
6-ethyl-C_{12}	0.9	---
6-ethyl-C_{14}	4.4	0.7
8-/10-ethyl-C_{12}	---	0.9
12-ethyl-C_{14}	---	0.2
12-ethyl-C_{16}	---	2.4
Dialkyl-branched (total)	(34.8)	(3.6)
2-ethyl-6-methyl-C_{12}	1.2	---
2-ethyl-10-methyl-C_{11}	2.8	---
2-ethyl-10-methyl-C_{12}	3.3	---
2-ethyl-14-methyl-C_{16}	3.9	---
2,6-diethyl-C_9	0.3	---
2,6-diethyl-C_{10}	2.7	---
2,10-diethyl-C_{13}	6.5	---
2,12-diethyl-C_{14}	2.7	---
4-ethyl-10-methyl-C_{11}	---	0.4
4-ethyl-10-methyl-C_{12}	11.4	1.6
4,6-diethyl-C_{11}	---	1.6
Unidentified	12.3	1.8

[a]The percentages of a second specimen differed only little and insignificantly.

substituted esters (Fig. 2) show small parent peaks, but an intense m/e 87, which is even more intense than the McLafferty ion at m/e 74. As reported for 4-methyl-substituted esters (10), intense (M-49)- and (M-73)-fragments are observed. M/e 115 is small, but m/e 129 is rather intense if compared with m/e 101 (due to the fragment which bears the substituent). Elimination of C-2/C-3/C-4 including the ethyl substituent at C-4 gives rise to the fragment (M-71) (being analogous to M-57 in case of 4-methyl-substituted esters). In contrast to the aforementioned 4-ethyl-branched esters, 4,4-dimethyl-substituted esters show no (M-73)- but an intense (M-15)-fragment, and m/e 129 being the base peak according to $(CH_3)_2\dot{C}\text{-}CH_2\text{-}CH_2\text{-}COOCH_3$ (11).

6-Ethyl-substituted esters (Fig. 3) show intense (M-76)-fragments which are generally observed in the mass spectra of 6-alkyl-substituted esters (10). The (M-29)-fragment is larger than (M-31), and two series are observed according to alkyl-elimination followed by methanol and water loss, namely (M-29) $\xrightarrow{-CH_3OH}$ (M-61) $\xrightarrow{-H_2O}$ (M-79), and more intense m/e 157 $\xrightarrow{-CH_3OH}$ m/e 125 $\xrightarrow{-H_2O}$ m/e 107 (weak).

2-Ethyl-10-methyl-branched esters (Fig. 4) can be recognized from the McLafferty ion m/e 102 and the intense (M-28)- and (M-29)-fragment. In case of methyl 2-ethyl-10-methyl dodecanoate, two ion series indicate the second branching position: m/e 241 $\xrightarrow{-CH_3OH}$ m/e 209 $\xrightarrow{-H_2O}$ m/e 191 and m/e 227 $\xrightarrow{-CH_3OH}$ m/e 195 $\xrightarrow{-H_2O}$ m/e 177, respectively.

In this ester, intense ions at m/e 158 are observed which correspond to m/e 144 ob-

TABLE IV

ECL Increments of Some Ethyl-substituted Methyl Esters

C-Atom substituent	Increment
2-ethyl-[a]	+ 1.10
4-ethyl-	+ 1.40
6-ethyl-	+ 1.35
(ω-2)-ethyl-	+ 1.65
2-ethyl-6-methyl-	+ 1.45
2-ethyl-(ω-2)-methyl-	+ 1.85
2,6-diethyl-	+ 2.48
2, (ω-2)-diethyl-	+ 2.80

[a]Valid for C_{10-14} acids; Increments depend a little on the chain length.

TABLE V

Composition of the Triglyceride Acids from the Uropygial Gland Secretion of the Marabou (*L. crumeniferus*)

Fatty acid	(%)
n-$C_{8:0}$	2.3 (2.4)[a]
n-$C_{10:0}$	19.8 (15.0)
n-$C_{12:0}$	21.5 (18.0)
n-$C_{14:0}$	4.7 (4.6)
n-$C_{16:0}$	17.5 (21.9)
n-$C_{18:0}$	6.8 (12.5)
n-$C_{16:1}$	2.4 (2.4)
n-$C_{18:1}$	25.0 (23.2)

[a]Data in parentheses are from a second specimen.

served in 2,10-dimethyl- and to m/e 130 observed in 10-methyl-substituted esters (7).

4-Ethyl-10-methyl-substituted esters (Fig. 5) show similar patterns for the second branch as the aforementioned. The 4-ethyl-substituent is recognized from m/e 87 (base peak), intense (M-29)-, (M-49)-, (M-71)-, and (M-73)-fragments as well as the relatively intense (M-28)-fragment.

2,6-Diethyl-branched esters (Fig. 6) show M-104 being characteristic for a second branch at C-6. Moreover, the ethyl substituent at C-6 can be recognized from the fragmentation pattern m/e 185 $\xrightarrow{-CH_3OH}$ m/e 153 $\xrightarrow{-H_2O}$ m/e 135. The ion series resulting from an elimination of the ethyl group (m/e 213/181/163) is also observed.

In 2,10-diethyl-substituted esters (Fig. 7), the first ethyl branch can be recognized from m/e 102 as well as from the intense (M-28)- and (M-29)-elimination. The second ethyl branch is indicated by the fragmentation pattern: m/e 255 $\xrightarrow{-CH_3OH}$ m/e 223 $\xrightarrow{-H_2O}$ m/e 205 and m/e 241 $\xrightarrow{-CH_3OH}$ 209 $\xrightarrow{-H_2O}$ m/e 191. Moreover,

TABLE VI

Composition of the Uropygial Gland Secretions from the Hitherto Investigated Ciconiiform Species

Lipid	Nycticorax nycticorax (3)	Ardea cinerea (2)	Ciconia ciconia (4)	Leptoptilos crumeniferus	Threskiornis aethiopica	Theristicus causatus	Scopus umbretta
Monoester waxes containing n-acids	+	+	+			(+)	+
Monoester waxes containing branched acids					+	+	+
Monoester waxes containing secondary alcohols	+						
Diester waxes			+				
Triglycerides		+	+	+			

a small but characteristic ion at m/e 172 is observed which seems to be analogous to m/e 158 observed in 2-ethyl-10-methyl-branched esters (see above).

Additional information can be drawn from the GC retention time. Some ECL increments of ethyl-branched methyl esters are given in Table IV.

In contrast to the above-mentioned species, the uropygial gland secretion of the marabou contains trigylcerides (Table V).

DISCUSSION

From the chemotaxonomic viewpoint, the order Ciconiiformes must be regarded as very heterogenous if the hitherto available patterns from a total of seven species are compared. The lipids show not only different types of constituents but even belong to different classes in some cases, e.g., monoester waxes (branches and unbranched), diester waxes, and triglycerides (Table VI). The preen wax constituents of *Th. aethiopica* show certain similarities with those of some Charadriiformes and Lariformes (1) and so do the constituents from *S. umbretta*. On the other hand, similarities between the Scopus wax and the preen wax pattern of Anseriformes species can be seen as well, but no relationship to herons and storks was observed which does not agree with results of electrophoretic investigations of egg white proteins (12). *Th. caudatus,* however, possess unique wax constituents, some of which have been observed in the preen waxes of *Pelecanus onocrotalus* (white pelican) (Jacob, unpublished results) and of some Podicipediformes (9). *L. crumeniferus,* possessing exclusively triglycerides, show relationship to Ciconia and Ardea, but many more analytical data from this order must be elaborated until serious systematically relevant conclusions can be drawn.

It remains, however, that the divergence among the preen wax patterns is unusual if compared with other orders, which, as to our experience, seem to indicate that the relationships in this taxon are more complex. It, therefore, should be considered as an open question whether the Ciconiiformes all derive from a common ancestor (as often stated) or whether at least some of them are convergent species.

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METHODS

Mass Fragmentographic Determination of Docosenoic Acid in Rapeseed Oils

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ABSTRACT

A highly sensitive and accurate reference method for determination of docosenoic acid (mainly erucic acid, 22:1n-9) in different rapeseed oils is described. A fixed amount of [1-^{14}C]erucic acid methyl ester (about 1 μg) is added to a fixed amount of oil. After treatment with sodium methoxide/methanol reagent and extraction with hexane, the amount of unlabeled erucic acid is determined from the ratio between the recordings at m/e 320 and m/e 322 obtained after analysis with a combined gas chromatograph-mass spectrometer equipped with an MID (multiple ion detector). The two ions used correspond to the M-32 peak in the mass spectrum of unlabeled and [1-^{14}C]labeled erucic acid methyl ester. The relative standard deviation of the method is about 1.8%. The method was compared with a gas chromatographic method for determination of erucic acid.

INTRODUCTION

Low erucic acid varieties of rapeseeds have been available on the market since the early 1970s. In order to establish correct values concerning the contents of erucic acid, accurate determination of erucic acid in rapeseed oils is of greatest importance. Hitherto gas liquid chromatography (GLC) has been considered to be the most accurate procedure for determination of the fatty acid content of oils. Thus, most routine methods for determination of erucic acid in fat and oils are based on GLC (1-4). The accuracy of these methods has not previously been evaluated with use of highly specific reference methods. As pointed out previously (5,6), mass fragmentography is well suited as a reference method in view of the high specificity and the possibility of correcting for losses in extractions and chromatographic steps with an internal standard which differs from the authentic compound by only a few mass units. In the present work, a mass fragmentographic method for the determination of docosenoic acid (mainly erucic acid, 22:1n-9) in rapeseed oils has been developed using [1-^{14}C]erucic acid as internal standard. The method has been compared with a routine method based on GLC.

MATERIALS AND METHODS

[1-^{14}C] Erucic Acid Methyl Ester

[1-^{14}C] Erucic acid was obtained from Cea Ire Sorin (Gif-Sur-Yvette, France) and had a specific radioactivity of 40 Ci/mol. The acid was converted to its methyl ester with H_2SO_4-methanol containing 2,2-dimethoxypropane (7). The material contained 85% ^{14}C-labeled molecules as determined by mass spectrometry (cf. Fig. 1). No impurities could be detected by radio-gas chromatography.

Trierucin

Trierucin was obtained from Nu-Chek-Prep, Inc., Elysian, MN, with a purity of more than 99% according to specifications.

Oils

The triacylglycerol fraction of cruciferae seed oils were the same as investigated earlier (4).

Other Compounds and Reagents

Behenic acid (22:0) and docosadienoic acid (22:2) were purchased from Supelco Inc., Bellefonte, PA, and sodium methoxide/methanol reagent (0.5 M) and t-butyldimethylchlorosilane (t-BDMCS) reagent (1.0 M t-BDMCS and 2.5 M imidazole dissolved in dimethylformamide) from Applied Science Labs, Inc., State College, PA.

Routine Laboratory Procedure

Erucic acid was determined by GLC as described earlier (4).

Preparation of Samples for Mass Fragmentography

The oils were dissolved in benzene to give an appropriate concentration. In the standard pro-

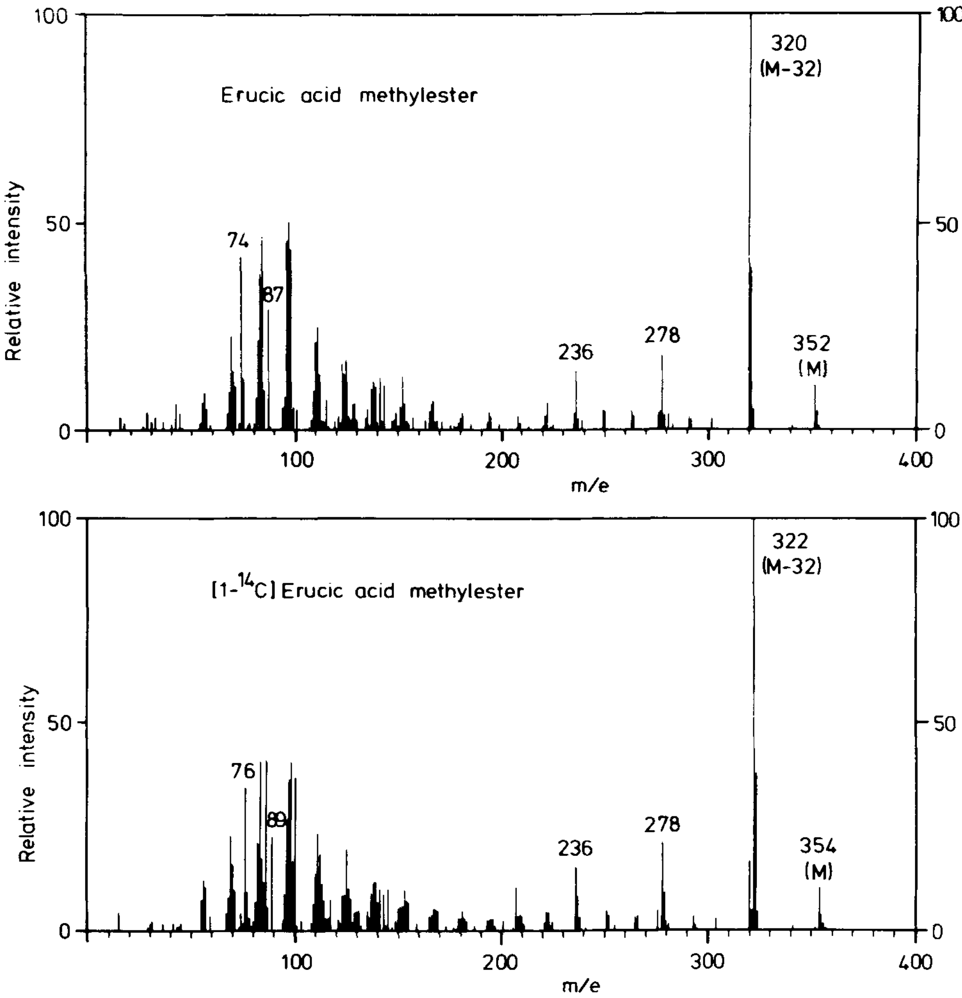
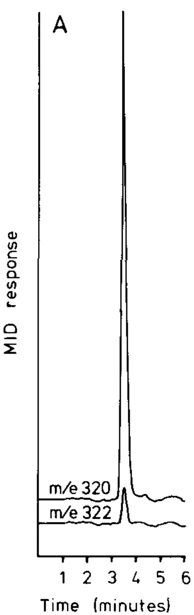


FIG. 1. Mass spectrum of unlabeled erucic acid methyl ester (upper spectrum) and [1-^{14}C] erucic acid methyl ester (lower spectrum).

cedure, 1 μg of [1-^{14}C] erucic acid methyl ester dissolved in 20 μl of benzene was added to a fixed volume of oil solution (ca. 20 μl, corresponding to 1-2 μg of erucic acid). In the preparation of the standard curve, the oil solution was substituted for benzene containing 0.1 to 3.4 μg of unlabeled trierucin. In the standard procedure, 100 μl of sodium methoxide/methanol reagent was added, and the mixture was allowed to stand at 60 C for 20 min. After cooling and addition of 0.5 ml of 0.1 M HCl, the methyl esters were extracted with hexane. The hexane phase was reduced to 50 μl under argon prior to gas chromatographic-mass spectrometric analysis.

In order to check the transesterification step used in the standard procedure, the sodium methoxide/methanol reagent was in some analyses substituted for 5 ml H_2SO_4-methanol (0.25:5.0, v/v) containing 100 μl of 2,2-dimethoxypropane (7). The [1-^{14}C] erucic acid methyl ester was in this case replaced by a fixed amount of [1-^{14}C] erucic acid. After incubation at 70 C for 2 hr, the samples were analyzed in the same way as the samples in the standard procedure.

Furthermore, the accuracy and specificity of the standard procedure were investigated by substituting the transesterification step with a saponfication step with subsequent conversion of the fatty acids to their t-butyldimethylsilyl (t-BDMS) derivatives. The saponifications were carried out in 0.5 ml of 0.5 M KOH-ethanol at 65 C for 30 min. After cooling, the mixture was acidified, and the fatty acids were extracted with hexane. The hexane phase was evaporated,



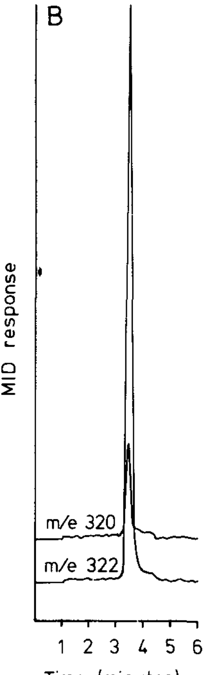


FIG. 2. A: MID recording of unlabeled erucic acid methyl ester and B: [1-^{14}C]erucic acid methyl ester. For experimental details, see Methods.

and the fatty acids were converted to their t-BDMS derivatives with 50 μl t-BDMCS reagent at 60 C for 15 min. After cooling and addition of 1 ml of 0.9% (w/v) NaCl, the derivatives were extracted with hexane. The hexane phase

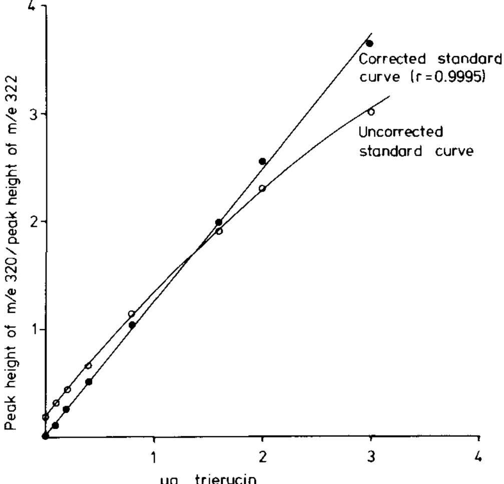


FIG. 3. Standard curve for determination of erucic acid in the range 0.1-3 μg. For experimental details, see Methods. The straight line is the standard curve corrected for contribution of unlabeled erucic acid methyl ester to m/e 322 and contribution of [1-^{14}C]erucic acid methyl ester to m/e 320 (cf. Results).

was reduced to 50 μl under argon prior to gas chromatographic-mass spectrometric analysis.

Mass Fragmentography

The extract, 2-6 μl, corresponding to about 5-15 nCi of [1-^{14}C]erucic acid, was analyzed by gas chromatography-mass spectrometry using an LKB 9000 instrument equipped with an MID (multiple ion detector). A 1.5% SE-30 column was used on Cromosorb W (80-100 mesh, 2 mm x 1.2 m). The carried gas was helium, and the flow rate was 30 ml/min. The temperature of the column was 225 C, and the temperature of the flash-heater was about 40 C above this. The temperatures of the ion source and separator were 290 C and 275 C, respectively. The electron energy was set to 20 eV, and the trap curred to 60 μA. The electron multiplier sensitivity was set to 180. In the standard procedure and in the procedure involving H_2SO_4-methanol as the transesterification medium, the first channel of the MID was focused on the ion at m/e 320 and the second at m/e 322. The two ions used are the base peaks and correspond to the M-32 peak in the mass spectrum of unlabeled and [1-^{14}C]-labeled erucic acid methyl ester, respectively. In the procedure using t-BDMS derivative of erucic acid, the first channel was focused on the ion at m/e 395 and the second at m/e 397. The two ions used are the base peaks and correspond to the M-57 peak in the mass spectrum of un-

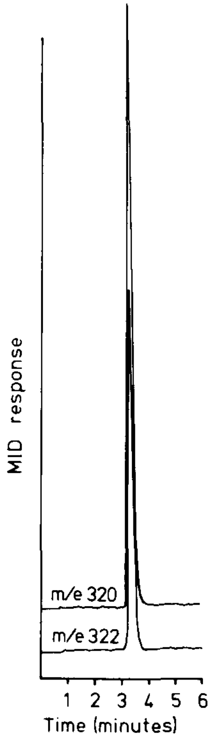


FIG. 4. MID recording of a transesterified rapeseed oil sample to which [1-^{14}C]erucic acid methyl ester had been added. For experimental details, see Methods.

labeled and [1-^{14}C]labeled t-butyldimethylsilyl erucic acid, respectively. The amplification used was always 900X for both channels. The filter settings were 0.5 Hz for both channels, and the measuring time was 20 msec. The MID recordings were made on UV paper, and the peak heights were measured.

RESULTS

The mass spectrum of the methyl ester of unlabeled and [1-^{14}C]labeled erucic acid is shown in Figure 1, and the MID recordings of the two compounds are shown in Figure 2. The ratio between the peaks at m/e 320 and m/e 322 obtained in MID recordings from transesterified standard mixtures of unlabeled trierucin together with 1 μg of [1-^{14}C]erucic acid methyl ester increased with increasing amounts of unlabeled erucic acid (Fig. 3). The increase in ratio, however was not linear with the amount of unlabeled erucic acid, and there was an intercept in the standard curve. This is due to the fact that unlabeled erucic acid methyl ester contributes to the recording at m/e 322 and that [1-^{14}C]erucic acid methyl ester contributes to the recording at m/e 320. After correction for these contributions, however, the ratio between the peak heights at m/e 320 and m/e 322 was linear with the amount of unlabeled erucic acid. The corrections were made according to the following formula:

TABLE I

Erucic Acid Content (weight %) of Different Cruciferae Seed Oils Determined by Different Methods.

Species and variety	GLC[a]	Mass fragmentography		
		NaOMe[b]	H_2SO_4/MeOH[c]	NaOH/t-BDMCS[d]
Brassica napus, v. "Oro"	0.69	0.65	0.68	0.73
Brassica napus, v. "Sinus"	12.0	12.5	12.8	11.6
Brassica napus, v. "SV-71"	24.0	24.1	--	23.7
Brassica napus, v. "Gulle"	35.7	35.0	36.0	33.9
Brassica napus, v. "Panter"	52.4	50.7	50.0	50.2
Relative standard deviation[e]		1.8%	1.6%	2.0%

[a]Determined by gas liquid chromatography.
[b]Determined by mass fragmentography involving sodium methoxide/methanol reagent (standard procedure).
[c]Determined by mass fragmentography involving H_2SO_4-methanol reagent.
[d]Determined by mass fragmentography involving saponification with subsequent derivatization with t-butyldimethylchlorosilane reagent.
[e]The relative standard deviation in % was calculated according to the following:

$$100 \sqrt{\frac{\Sigma \left(2 \frac{a-b}{a+b}\right)^2}{n-1}}$$

where a and b are duplicates and n is the total number of determinations.

$$A = \frac{B - C}{1 - \frac{B}{D}}$$

A = The corrected ratio between the peak height at m/e 320 and m/e 322. B = The uncorrected ratio between the peak heights at m/e 320 and m/e 322. C = The ratio between the peak height at m/e 320 and m/e 322 from [1-^{14}C]erucic acid methyl ester (cf. Fig. 2B). D = The ratio between the peak height at m/e 320 and m/e 322 from unlabeled erucic acid methyl ester (cf. Fig. 2A).

The stability of the instrument was investigated by injecting a sample every 2 hr for 12 hr. The peak height ratio was 0.510, and the coefficient of variation was found to be 1.2%.

In the determination of the concentration of erucic acid in a sample, the ratio between the peak heights was always corrected for these contributions and the corrected standard curve was used. Figure 4 shows a typical MID recording of a transesterified rapeseed oil sample to which [1-^{14}C]erucic acid methyl ester had been added. Table I shows a comparison between the erucic acid content in different cruciferae seed oils as determined by different methods. There were only small differences between the values obtained by the different methods. Regression analysis between the gas chromatographic method and the mass fragmentographic standard procedure gave a regression coefficient of 1.04 and a correlation coefficient of 0.9998. The relative standard deviation of the mass fragmentographic standard procedure was 1.8%.

In order to investigate the accuracy of the methods used, the erucic acid content of a synthetic triglyceride, 1,2-dioleoyl-3-erucoyl-*sn*-glycerol was determined. The value obtained was 34.9% (w/w) for all the three methods based on mass fragmentography. The value is somewhat less than the theoretical value, 36.0% (w/w);

The specificity of the method is based on the assumption that there are no contaminating compounds in the gas chromatographic peak corresponding to erucic acid that give ions at m/e 320 and m/e 322. There is the possibility that behenic acid (22:0) and docosadienoic acid (22:2) which are closely related to erucic acid interfere with the determination. To exclude this possibility, the methyl esters of these fatty acids were added to rapeseed oils before determination by the standard procedure. Table II shows that addition of behenic acid had a negligible influence on the results. Docosadienoic acid interfered to a larger extent than behenic acid, but the contribution was negligible in practice, in view of the very small amount of this fatty acid in rapeseed oil.

TABLE II

Determination of Erucic Acid by Mass Fragmentography after Addition of Known Amount of 22:0 and 22:2 to Different Oils.

	μg Erucic acid
Sample	0.82
Sample + 1 μg 22:0	0.83
Sample + 5 μg 22:0	0.85
Sample	1.07
Sample + 1 μg 22:2	1.13
Sample + 5 μg 22:2	1.35

DISCUSSION

It is evident that the present mass fragmentographic reference method for determination of docosenoic acid has a high accuracy and precision and may have applicability when there is a demand for specificity, accuracy, and precision. The method using sodium methoxide/methanol reagent was given priority since this method was less laborious than the other two methods tested and should under the conditions used have a higher specificity in presence of behenic acid (22:0) than the procedure using saponification with subsequent derivatization with t-BDMCS. This is due to the different fragmentation pattern for behenic and erucic acid methyl ester. Thus, behenic acid methyl ester gives the fragment (M-31) corresponding to m/e 323 and could not therefore, interfere with m/e 322 corresponding to (M-32) from [1-^{14}C]erucic acid methyl ester. On the contrary t-BDMS derivative of behenic acid has the same fragment, m/e 397, as the t-BDMS derivative of [1-^{14}C]erucic acid. Therefore, the presence of behenic acid in a sample could interfere with the determination of erucic acid and produce too low values.

The high correlation between results obtained with the mass fragmentographic method and the gas chromatographic method indicates that the gas chromatographic method for determination of erucic acid has sufficient accuracy. In agreement with what might be expected, somewhat lower values were obtained with the mass fragmentographic method than with the gas chromatographic method. The divergence of the regression coefficient from the theoretical value is probably due to lack of specificity with the gas chromatographic method.

It should be pointed out that the mass fragmentographic method under the conditions used only considers the total amount of doco-

senoic acid. Thus, erucic acid is not discriminated from other isomers of docosenoic acid. In our laboratory, work is in progress on the extension of the present method to include estimation of geometrical as well as positional isomers of docosenoic acid.

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COMMUNICATIONS

Δ9 Desaturase Activity in Normal Mouse Liver and Hepatoma SS1K

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ABSTRACT

The activity of Δ9 desaturase was determined in the microsomal fraction of normal mouse liver and hepatoma SS1K in the presence of the 105,000 x g supernatant. Neither hepatic nor hepatoma soluble fractions were able to modify the low desaturating capacity. Two enzymes from the microsomal electron transport chain associated with Δ9 desaturase, namely NADH-cytochrom b_5 reductase and NADH-cytochrome C reductase were also measured. The results indicate that the low Δ9 desaturase activity in hepatoma SS1K could be related to the reduced amount of desaturase.

INTRODUCTION

It has been demonstrated that several neoplasms are deficient in Δ9 desaturation (1-4) and that this alteration is independent of the tumor growth rate (2). Despite the low Δ9 desaturase activity, most of the hepatomas contain large amounts of octadecenoic fatty acids (5-8).

It has been reported that supplementation with supernatant increases desaturase activity (9,10), that free fatty acids from the soluble fraction influence the monounsaturating activity (11), and also that a cytoplasmic binding protein has a high affinity for fatty acids (12) and, in consequence, could have a possible physiological role in the transport and metabolism of fatty acids. It was therefore interesting to study the effect of 105,000 x g supernatant upon the activity of the Δ9 desaturase in hepatomas.

Moreover, since Δ9 desaturase is a multicomponent system correlated with the electron transport chain of microsomes (13-15), two enzymes from this chain were measured to test whether the alteration in the Δ9 desaturation in hepatomas is due to a decrease in the whole enzyme complex or only in the desaturase enzyme.

MATERIALS AND METHODS

The tumor is the K subline of a spontaneous hepatoma (second S) which was the first No. 1) transplanted in 1949 by J.W. Wilson, Brown University, Providence RI, into C3H/STW (first S) strain. It is fast growing and classified as a poorly differentiated hepato-cellular carcinoma (16). The hepatoma was maintained by implants in C3H/S male mice and was used for experiments 20 days after implants. Animals from the same breed were used as controls.

Animals were killed by decapitation, liver and hepatoma were removed, homogenized, and the microsomal and 105,000 x g supernatant fractions were isolated by differential centrifugation as described previously (17).

Microsomes and supernatant proteins were estimated by the biuret method (18). [1-^{14}C] Stearic acid (52.0 mCi/mmole, 99% radiochemically pure) were purchased from Radiochemical Center, Amersham, England.

The conditions of desaturase assay were as described previously (19), except that 25 mg supernatant protein in each incubation medium per 5 mg microsomal protein was added. After

TABLE I

Oxidative Desaturation of [1-^{14}C]Stearic Acid to [1-^{14}C]Oleic Acid by Microsomes from Normal Mouse Liver and Hepatoma SS1K[a]

Enzyme source microsomes	Supernatant (105,000 x g)	% Conversion C.18:0 C.18:0
Liver	Liver	22.2 ± 5.1 (4)
Liver	Hepatoma	27.7 ± 0.6 (4) N.S.
Hepatoma	Liver	6.5 ± 3.1 (5) P<0.001
Hepatoma	Hepatoma	4.6 ± 0.4 (5) P<0.001

[a]Number in parentheses indicates the number of individual samples in each group. Probability (P) values are related to normal liver. Data are the mean ± standard error.

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incubation, the mixture was saponified and the free fatty acids esterified. The conversion of [1-^{14}C]stearic acid to [1-^{14}C]oleic acid was measured as described previously (19). NADH-cytochrome b_5 reductase was assayed at 25 C measuring the NADH-ferricyanide reductase activity of the enzyme according to Strittmater (10). NADH-cytochrome C reductase activity was determined at 550 nm as was described by Rogers and Strittmater (21).

RESULTS AND DISCUSSION

Table I shows the in vitro activity of $\Delta 9$ desaturase when 105,000 x g supernatant was added to the incubation medium.

As it is clearly shown, no significant differences were observed in desaturation activity of normal liver microsomes when either hepatic or hepatoma supernatant was added to the incubation medium.

The microsomal desaturase activity of cancer tissue is not increased even when combined with the normal soluble fraction and remains as low as was described previously (2). Since the data reported here indicate that the level of $\Delta 9$ desaturase in cancer cells is not increased to hepatic level by the addition of soluble fraction, it is likely that the low $\Delta 9$ desaturation is related to an alteration in the microsomal desaturation. Supernatant has no role in stimulating the microsomal desaturase activity in either liver or hepatoma.

To test whether the altered $\Delta 9$ desaturase activity could represent an overall alteration in the electron transport chain of microsomes, two enzymes were measured: NADH-cytochrome b_5 reductase and NADH-cytochrome C reductase.

The NADH-cytochrome C reductase showed no differences between the two tissues under study. NADH-cytochrome b_5 reductase showed a reduction of 80% in its activity in cancer cells.

Despite the low activity of NADH-cytochrome b_5 reductase, this enzyme reaction does not appear to be rate limiting. The turnover number of this flavoprotein is ca. 29,000 moles of substrate per min per mole of enzyme (22). Besides, the desaturase enzyme has a turnover number of 21 (23). Thus, the 20% NADH-cytochrome b_5 reductase activity that remains in hepatoma could be adequate to carry out the overall desaturase reaction.

The presented results allow us to conclude that the low desaturase capacity of hepatoma could be to the low level of the $\Delta 9$ desaturase.

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Occurrence of Dolichol in Human Tissues

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ABSTRACT

The concentration of dolichol has been determined in various human tissues obtained at autopsy. The highest levels (~3000 μg/g wet weight) were found in testes. Liver and several other endocrine tissues contained about 1000 μg/g. Lower levels were present in other tissues examined. Only a small proportion of the total dolichol in human tissues was esterified to fatty acids.

INTRODUCTION

There is considerable interest in the role of dolichyl phosphate as a lipid intermediate in the biosynthesis of mammalian glycoproteins (1). Dolichol also exists in tissues as the free alcohol, or esterified to fatty acids, as well as in the form of glycosylated derivatives of dolichyl phosphate. In most tissues, the phosphorylated forms probably comprise only a very small proportion of the total dolichol content.

Little is known of the function and metabolism of the nonphosphorylated forms, which are found as a series of isoprenologues containing from 17-22 isoprene units, with differing amounts of the various homologues, depending on the source of the material (2). Earlier studies in our laboratory (3) showed that human pituitaries contain high levels of dolichol (~1400 μg/g wet weight), consisting largely of dolichol-19 (19 isoprene units), mainly in the unesterified form. These studies have now been extended to examine the level and degree of esterification of dolichol in other human tissues.

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MATERIALS AND METHODS

Normal human tissues, trimmed of adipose and connective tissues, were obtained from autopsies performed at University Hospital, London, Ontario, through the cooperation of the Department of Pathology. These were analyzed either immediately or after storage in the frozen state. Tissues were obtained from autopsies of 13 males and 8 females ranging in age from 23 to 95 years. The majority were males over 45 years of age.

Dolichol was identified by its behavior on thin layer chromatography (TLC) in two solvent systems (hexane-ether-acetic acid, 65:35:1, or chloroform) compared to that of a sample previously characterized as dolichol-19 by infrared, nuclear magnetic resonance, and mass spectroscopy (3). In several instances where there was sufficient sample, spectra were recorded and were found to be identical to the standard. All

TABLE I

Concentration of Dolichol and Dolichyl Esters in Human Tissues

Tissue	Total dolichol[a] (μg/g tissue wet weight)	% of Total dolichol esterified to fatty acids[b]
Testes	3226 ± 244 (9)	3
Adrenals	1273 ± 155 (7)	20
Liver	1226 ± 108 (11)	11
Thyroid	1145 ± 277 (7)	5
Pancreas	943 ± 148 (9)	20
Prostate	268 ± 70 (5)	--
Heart	262 ± 42 (7)	--
Kidney	240 ± 30 (13)	12
Spleen	161 ± 22 (11)	--
Lung	82 ± 35 (10)	--

[a]Values are mean ± SEM with number of samples analyzed shown in parentheses.
[b]Each value is the mean of results from two different analyses.

solvents and other chemicals were either reagent grade or conformed to ACS specifications.

Isolation of Dolichol

The nonsaponifiable lipid was isolated from each tissue sample by the method of Burgos et al. (4). This material was chromatographed on a column of Florisil (12 g, 1.2 x 20 cm), deactivated by 6% (w/v) water as described by Carroll (5). The fractions from the column were monitored by TLC on Silica Gel H, developed with hexane-ether-acetic acid (65:35:1). Dolichol was eluted with 15% ether in hexane (3), and the amount was determined gravimetrically. In most cases, it was possible to obtain enough tissue to isolate more than 5 mg of dolichol.

Separation of Esterified Dolichol from Free Dolichol

Total lipids were extracted from each tissue by the method of Folch et al. (6), dissolved in hexane and chromatographed directly on Florisil deactivated with 6% (w/v) water (5). On this column, phospholipids, glycolipids, and fatty acids are retained, while neutral lipids can be separated into their respective classes. Column fractions were monitored by TLC as described above. Dolichol esterified to fatty acids was eluted, together with cholesteryl esters, by 5% ether in hexane, while free dolichol was eluted with triglycerides, by 15% ether in hexane (3).

The column fractions containing esterified dolichol and free dolichol were individually treated with 15% KOH in ethanol at 50 C for 3 hr and the nonsaponifiable material recovered by extraction with petroleum ether. Soaps and glycerol were removed by washing with water. The nonsaponifiable lipid from the 5% ether in hexane fraction was again chromatographed on deactivated Florisil to separate dolichol from cholesterol. Analysis of the petroleum ether extract of the alkaline hydrolysis of the 15% ether in hexane fraction by TLC showed that it contained pure dolichol in most cases. Any impurities present were removed by Florisil chromatography, and the amount of dolichol was determined gravimetrically.

RESULTS AND DISCUSSION

The results of a survey of the dolichol content of ten human tissues are shown in Table I. It can be seen that liver and endocrine organs had considerably higher levels than other tissues examined. The highest levels were found in testes. Within this sampling, the levels of dolichol in different organs showed no particular trends with age or sex. The time elapsed between death and the collection of the tissue did not seem to affect the concentration of total dolichol.

The levels of total dolichol found in human liver and pituitary are 5-10 times higher than those reported for the corresponding tissues in other species (3,4,7,8). Human liver, which contains about 1200 μg/g, is an excellent source for the isolation of dolichol in quantity.

Mańkowski et al. (9) described the occurrence of small amounts of dolichol-11 in pig liver. The methods used in our studies did not permit the separation of short chain from long chain dolichols, but nuclear magnetic resonance indicated that short chain dolichol was present only in small amounts, if at all, in human tissues.

The percentage of dolichol esterified to fatty acids in six of the tissues is also shown in Table I. The small amounts involved (1-5 mg) made gravimetric determination rather inaccurate, but it is evident that only a relatively small portion of the total dolichol was esterified to fatty acids in the tissues analyzed.

It seems probable that dolichol is synthesized by an isoprenoid biosynthetic pathway, which gives rise to dolichyl pyrophosphate as the primary product (2), and that the free alcohol is formed by the action of tissue phosphatases. It will be of interest to see whether the higher levels of dolichol in liver and endocrine tissues of humans compared to other species are associated with correspondingly higher levels of the phosphorylated derivatives.

It is not known whether free dolichol or dolichol esterified to fatty acids have any significant biological functions. The lipophilic nature of these compounds suggests that they are probably associated with membranes and studies on their subcellular distribution are in agreement with this concept (2,10). The unusually large size of these molecules and the relatively high concentration in some human tissues suggest the possibility that they influence physical and biochemical properties of cellular membranes in these tissues.

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Effects of Storage Conditions on Rat Brain Ethanolamine Glycerophospholipids, Cerebrosides, and Cholesterol

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ABSTRACT

The effects of storage on rat brain lipid composition were studied in terms of ethanolamine glycerophospholipid, cerebrosides, and cholesterol. Rat brains were stored at several combinations of temperature and time. Storage conditions were: 2 hr at room temperature, 12 hr of refrigeration, and a sequence of both of these conditions. Two-dimensional thin layer chromatography followed by colorimetric analyses of eluted lipids were used to determine molar ratios of phosphatidylethanolamine, ethanolamine plasmalogen, lysophosphatidylethanolamine, and cerebrosides. Cholesterol was also determined. These studies revealed small but significant increases in lysophosphatidylethanolamine in all three cases. A slight increase was also noted in the apparent molar proportion of cholesterol.

INTRODUCTION

It has been known for sometime that brain phospholipids are generally stable under postmortem conditions. There is, however, little information available regarding possible small changes in lipid composition of brains stored at low temperatures and for varying lengths of time, as is often the case in studies using human brain. Rouser and Kritchevsky (1) reported that the sphingomyelin and major glycerophosphatide composition of bovine brain was unchanged after 48 hr at 24 C. Significant decreases were observed in the content of phosphatidic acid and diphosphatidylglycerol. The cerebroside content of human white matter was found by Leube and Lindlar (2) to remain unchanged after 24 days at room temperature. Uchimura et al. (3) found that 24 hr room temperature storage of rat brain resulted in no change in the distribution of ethanolamine phosphatides, phosphatidylcholine, sphingomyelin, cerebrosides, and gangliosides. The content of phosphatidylserine and sulfatide was approximately halved, while cholesterol appeared to increase by ca. 30%. Fewster et al. (4) could find no significant changes in ethanolamine glycerophospholipid composition of human or bovine brain stored 24 hr at room temperature.

The present study reports effects of storage on rat brain lipid composition, in terms of ethanolamine glycerophospholipids, cerebrosides, and cholesterol as molar percentages of total lipid phosphorus. Temperatures and duration of storage were chosen to provide information relevant to lipid compositional studies of human brain. Ethanolamine glycerophospholipids were chosen for this study because they represent a convenient and significant family of lipids: glycerophosphatides, plasmalogens, and lysoglycerophosphatides. Cerebrosides were studied because they represent major neural membrane sphingolipids as well as glycolipids, and cholesterol because of its key role as a membrane lipid.

MATERIALS AND METHODS

Four groups of six male Sprague-Dawley rats (COBS, Charles River, Wilmington, MA) were

TABLE I

Composition of Adult Rat Brain Lipids

Lipid	Mole % of TLP[a]
Ethanolamine glycerophospholipids	39.2 ± 1.0[b]
Ethanolamine plasmalogen[c]	22.1
Phosphatidylethanolamine[c]	17.1
Choline glycerophospholipids	33.5 ± 0.4
Phosphatidylserine	14.8 ± 0.3
Sphingomyelin	5.5 ± 0.2
Phosphatidylinositol	3.5 ± 0.2
Phosphatidic acid	2.4 ± 0.1
Diphosphatidylglycerol	1.0 ± 0.05
Lysophosphatidylethanolamine	0.36 ± 0.02
Unknown phospholipids at origin	0.42 ± 0.06
Total phospholipid recovery	102 ± 1.5
Cerebrosides[d]	24.4 ± 0.7
Cholesterol[d]	64.5 ± 0.6

[a]TLP = total lipid phosphorus.

[b]Values are given as averages ± SE of four determinations of choline glycerophospholipids, five of phosphatidylserine and of ethanolamine glycerophospholipids, and six of all others.

[c]Values for these are calculated from reactional TLC analyses (see text), from which it was determined that ethanolamine plasmalogen = 56.4 ± 0.6% of ethanolamine glycerophospholipids.

[d]For purposes of comparison with phospholipid composition, these values are expressed as mole ratios, where TLP = 100.

TABLE II

Storage Stability of Adult Rat Brain
Ethanolamine Glycerophospholipids, Cerebrosides, and Cholesterol

	Storage condition (Mole % of TLP[b])			
	0	2 hr Room temp[a]	12 hr Refrig[a]	2 hr room temp + 12 hr refrig
Ethanolamine glycerophospholipids	39.2 ± 1.0[c]	37.5 ± 0.5	40.6 ± 0.8	40.0 ± 0.6
Ethanolamine plasmalogen[d]	22.1	21.5	23.1	22.9
Phosphatidylethanolamine[d]	17.1	16.0	17.5	17.1
Lysophosphatidylethanolamine	0.36 ± 0.02	0.47 ± 0.06	0.44 ± 0.11	0.59 ± 0.06[e]**
Cerebrosides	24.4 ± 0.7	24.6 ± 0.03	24.5 ± 0.5	23.8 ± 0.2
Cholesterol	64.5 ± 0.6	66.7 ± 0.7*	66.9 ± 0.8*	66.1 ± 0.7

[a]Room temperature = 25.1-25.2 C; refrigerator temperature = 4.8-5.1 C.

[b]TLP = total lipid phosphorus.

[c]Values are given as averages ± SE of at least five determinations in each case.

[d]Values for these were calculated from reactional TLC analyses (see text), from which it was determined that ethanolamine plasmalogen was, successively, 56.4 ± 0.6, 57.2 ± 0.6, 57.0 ± 0.3, and 57.3 ± 0.4% of ethanolamine glycerophospholipids.

[e]Significant differences from zero time *($p < 0.05$); **($p < 0.01$).

used. Rats were adults, weighing 462-520 g. In order to minimize possible artifacts from the method of sacrifice, brains were removed as rapidly as possible after decapitation and were immediately subjected to the appropriate storage condition. Elapsed time between first sacrifice and storage of the sixth brain varied from 10-16 min. In the cases of the control and of the immediate-refrigeration groups, brains were placed on ice as soon as removed. Refrigerated brains were stored for 12 hr at 4.8-5.1 C in sealed plastic bags. Brains kept at room temperature were also stored in plastic bags. Room temperature varied between 25.1 and 25.2 C during the course of room temperature storage.

Total lipids were prepared from each group of brains by a modification of the method of Folch et al. (5). The chloroform-methanol 2:1 mixture used for extraction contained 0.01% 2,6-tert-butyl-4-methyl phenol as antioxidant, and 0.1 vol of 0.74% aqueous KCl was used for backwashing.

After concentration to small volume in vacuo, total lipid samples containing ca. 12 μg of phosphorus were separated into components, which were subsequently analyzed for phosphorus, according to the two-dimensional thin layer chromatographic method of Rouser et al. (6).

Separate aliquots of total lipids were used for analyses of ethanolamine plasmalogens by a modification of the two-dimensional reactional thin layer chromatographic method described by Horrocks and Sun (7). A nitrogen flushing chamber was used to remove solvent after development in the first dimension, and to remove residual HCl after reaction with HCl fumes. Development in the second dimension was accomplished with the same neutral solvent system used in the first dimension, chloroform-methanol-water, 140:60:8.

Cerebrosides were isolated from total lipid aliquots by thin layer chromatography (TLC) in chloroform-methanol-water, 140:60:8, followed by elution from aspirated silica gel bands using chloroform-methanol, 2:1. Water was then added to produce a two-phase system, chloroform-methanol-water, 8:4:3. After centrifugation, which left the silica gel in the upper phase, lower phases were evaporated under N_2 and assayed for galactolipid by reaction with anthrone reagent as described by Yamamoto and Rouser (8).

Aliquots of total lipid preparations were also analyzed for cholesterol. TLC in chloroform-benzene, 2:1, was used to separate cholesterol from other lipids, after which cholesterol-containing silica gel bands were aspirated. Cholesterol was eluted with chloroform-methanol-water, 8:4:3, and after evaporation under N_2, the lower phase was analyzed for cholesterol by the colorimetric method of Zlatkis and Zak (9).

One-dimensional TLC for both galactolipid and cholesterol analyses was carried out on 20 x 20 cm glass plates coated with 250 μ of Silica Gel G (E. Merck, Darmstadt, Germany). Cholesterol and cerebroside bands were visualized using Rhodamine G and iodine vapors, respectively.

RESULTS AND DISCUSSION

In order to insure that our data were consistent with compositional studies previously reported, zero time values were determined for all identifiable and quantifiable phospholipids, cerebrosides, and cholesterol. The results, shown in Table I, are in agreement with reports of rat brain composition in terms of phospholipids (10-13), cerebrosides (10,14), and cholesterol (10,13,15). For consistency as well as for comparison, all values are reported as mole % of total lipid phosphorus.

Table II displays the results of storage stability of ethanolamine glycerophospholipids, ethanolamine plasmalogen, lysophosphatidylethanolamine, cerebroside, and cholesterol. According to these data, ethanolamine glycerophospholipids and cerebrosides were quite stable under the storage conditions used, while small but significant increases occurred in the cases of lysophosphatidylethanolamine and cholesterol. The stabilities of ethanolamine glycerophospholipids and cerebrosides are consistent with earlier reports of the rather remarkable stabilities of these families of compounds under postmortem or autolysing conditions (1-4). We have additionally demonstrated that, under the conditions used, there is no measurable quantitative postmortem change in the ethanolamine plasmalogen and phosphatidylethanolamine components of ethanolamine glycerophospholipids in rat brain.

The observed increases in lysophosphatidylethanolamine upon storage at room temperature for 2 hr and upon storage for 12 hr in the refrigerator appear to be additive. Unfortunately, the unusually high standard error calculated in 12 hr refrigeration data makes it impossible to arrive at a conclusion on this point. The lysophosphatidylethanolamine increases are not reflected as decreases in ethanolamine glycerophospholipids probably because the ethanolamine glycerophospholipids measurements are an order of magnitude larger than those of lysophosphatidylethanolamine, and the small changes involved are not discernible within the correspondingly greater standard error of ethanolamine glycerophospholipid measurements. It is reasonable to suggest that the lysophosphatidylethanolamine increases are the result of action of hydrolytic enzymes. This is a change that would be generally expected in a tissue undergoing autolysis. Indeed, Rouser and Kritchevsky (1) have reported increased lysophosphatidylethanolamine as a postmortem change of human heart phospholipids.

In the case of cholesterol, the increases were the same for all three storage conditions, ca. 2 mole %, or about 3% of the original amount. There is insufficient cholesterol ester in adult brain to invoke hydrolysis as the reason for the apparent increase in cholesterol. It is possible that the increase represents a postmortem pertubation of the steady state, i.e., that biosynthetic systems did not shut down as rapidly as catabolic ones. Another explanation is that the same interfering substance was formed under all three conditions, possible from contaminating blood pigments, and that the observed increase is artifactual. In either of these, or in any case involving a dynamic explanation, the phenomenon is fully expressed by either 2 hr at room temperature or 12 hr of refrigeration, because these changes are not additive, and are in fact significantly greater in the first two conditions than in the combined case.

We conclude that room temperature storage of rat brains for as little as 2 hr produces small but significant changes in values obtained for lysophosphatidylethanolamine and cholesterol, reported as molar percentages of total lipid phosphorus.

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Steric Requirements for the Stimulation of Glycosyltransferase Activity by Lysophosphatidylcholine

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ABSTRACT

1-Palmitoyl-*sn*-glycerol-3-phosphocholine and 3-palmitoyl-*sn*-glycerol-1-phosphocholine have been found to be equipotent in the stimulation of membrane-bound glycosyltransferases in microsomes of rat intestinal villus cells. This indicates that the stimulatory effect of lysophosphatidylcholine is not stereospecific, but that it may be related to a specific detergent property dependent upon the peculiar balance of hydrophilic and hydrophobic components in the molecule.

INTRODUCTION

Many membrane-bound enzymes require specific phospholipid environments (1). As a result, investigations of lipid-protein interactions *in membranes* are essential to our understanding of membrane function. One approach to this problem has been to alter lipid composition and study the effect on membrane-bound enzymes. This has been carried out in recent years by enzyme action (1), by nutritional changes (2), and by altering the lipids of recombinant systems (3). For membrane proteins which have strong hydrophobic interactions with lipid, detergents have provided a convenient method for altering lipid composition. Most research in this area involves the use of neutral or weakly ionic detergents such as Tweens, Tritons, or bile salts. The basic need is to stimulate the native membrane environment. As Tanford and Reynolds (4) have pointed out, the *ultimate* ideal from the point of view of stimulation of the native environment is provided by small soluble phospholipid vesicles, which one could form from endogenous phospholipids to achieve almost perfect simulation. The next best approximation to the native environment is provided by lysophosphatides (4). Possessing a single hydrocarbon chain per polar head group, they form small micelles with a curved surface instead of extended bilayers (5).

One such lysophosphatide that has been used is lysophosphatidylcholine. It has been reported that 1-acyl-*sn*-glycerol-3-phosphocholine can stimulate galactosyltransferase and sialyltransferase activity in rat kidney (6) and liver (7,8) microsomes. It has also been demonstrated that the addition of lysophosphatidylcholine to isolated rat intestinal villus cells markedly stimulated the incorporation of [^{14}C]-glucosamine into glycoproteins (9). The question of the stereospecificity that may be involved in the association between proteins (or enzymes) and the phospholipid must be raised, and at present our knowledge of this is rudimentary. We recently reported (10) that the phosphocholine cytidyltransferase activity of the cytosol fractions of intestinal mucosa and liver could be stimulated to an equivalent degree by 1-palmitoyl-*sn*-glycerol-3-phosphocholine and by its enantiomer, 3-palmitoyl-*sn*-glycerol-1-phosphocholine, indicating a lack of stereospecificity in the stimulation. It is, therefore, of interest to determine the stereospecificity of the stimulation of membrane-bound enzymes by lysophosphatidylcholine. As an example of such an enzyme system, we have investigated the effects of enantiomeric lysophosphatidylcholines on the glycosyltransferase enzymes in microsomes of rat intestinal villus cells.

The present report provides the first demonstration that enantiomeric lysophosphatidylcholines are equipotent in the stimulation of membrane-bound glycosyltransferases.

MATERIALS AND METHODS

The enantiomeric lysophosphatidylcholines were prepared as previously described (10). All radioactive nucleotide sugars were purchased from New England Nuclear (Boston, MA). Desialized fetuin (DSF) and sialic acid and galactose-depleted fetuin (DSG-fetuin) were prepared by mild acid hydrolysis of fetuin (Sigma, St. Louis, MO) according to the method of Spiro (11). Microsomes were prepared from isolated rat intestinal villus cells (12) as described previously (13). Protein was determined by the method of Lowry et al. (14) using bovine serum albumin as standard.

The UDP-galactose, glycoprotein galactosyltransferase was assayed essentially as described by Mookerjea and Yung (7). A standard assay for galactosyltransferase contained intestinal villus cell microsomes 20 μl (0.5 mg protein);

TABLE I

Effect of Lysophosphatidylcholine and Nonionic Detergents on the Activities of Glycosyltransferases in Microsomes of Rat Intestinal Villus Cells

Additions	Source	Amount	Enzyme activity[a]		
			N-Acetylglucosaminyltransferase	Galactosyltransferase	Sialyltransferase
None	---	---	6.2 ± 0.7	63 ± 8	232 ± 29
Mixed acid 1-acyl-*sn*-glycerol-3-phosphocholine	Rat intestine	100 μg	29.4 ± 3.5	196 ± 29	1184 ± 188
		200 μg	50.2 ± 4.6	428 ± 56	2168 ± 284
Mixed acid 1-acyl-*sn*-glycerol-3-phosphocholine	Egg yolk	100 μg	31.7 ± 3.8	190 ± 21	1346 ± 168
		200 μg	48.4 ± 4.9	429 ± 52	2290 ± 270
1-Oleoyl-*sn*-glycerol-3-phosphocholine	Commercial	100 μg	30.1 ± 2.9	202 ± 26	1310 ± 148
		200 μg	47.6 ± 4.2	436 ± 59	2324 ± 244
Triton-X 100	Commercial	0.1 %	10.4 ± 1.4	96.8 ± 8.4	286 ± 36
		0.2 %	13.1 ± 1.8	147.4 ± 13.6	410 ± 48
Tween-80	Commercial	0.1 %	9.1 ± 0.8	88.2 ± 9.8	343 ± 41
		0.2 %	11.8 ± 1.1	127.6 ± 12.2	740 ± 68

[a]pmol/mg protein/hr ± SEM. The enzymes were assayed as described in Materials and Methods.

TABLE II

Stimulation of Glycosyltransferase Activity by Enantiomeric Lysophosphatidylcholines in Microsomes of Rat Intestinal Villus Cells

Additions	Amount (μg)	Enzyme activity[a]		
		N-Acetylglucosaminyltransferase	Galactosyltransferase	Sialyltransferase
None	---	6.2 ± 0.7	63 ± 8	232 ± 29
1 – Palmitoyl-*sn*-glycerol-3-phosphocholine	50	13.5 ± 1.2	116 ± 14	745 ± 86
	100	30.6 ± 2.9	188 ± 23	1236 ± 138
	200	48.8 ± 3.8	434 ± 52	2064 ± 242
3 – Palmitoyl-*sn*-glycerol-1-phosphocholine	50	14.5 ± 1.3	101 ± 12	722 ± 88
	100	31.9 ± 3.6	199 ± 16	1328 ± 146
	200	45.8 ± 4.4	458 ± 57	2127 ± 240
1 – Palmitoyl-*sn*-glycerol-3-phosphocholine	50	12.8 ± 1.6	106 ± 15	732 ± 87
plus	100	33.4 ± 3.9	220 ± 29	1488 ± 159
3 – Palmitoyl-*sn*-glycerol-1-phosphocholine (1:1 mixture)	200	50.8 ± 5.6	427 ± 66	2240 ± 256

[a]pmol/mg protein/hr ± SEM. The enzymes were assayed as described in Materials and Methods.

UDP-galactose-^{14}C, 60 nmol (45,000 cpm); 2-(N-morpholino) ethanesulfonic acid buffer (MES 12.5 μmol (pH 6); Mn Cl_2, 0.625 μmol; DSG-fetuin, 125 μg; in a total volume of 50 μl. The enzyme activity assayed as above was linear with time and protein concentration. N-Acetylglucosaminyltransferase and sialyltransferase activities were measured essentially with an identical microsomal incubation system as above, but with UDP-*N*-acetylglucosamine-^{14}C (9 nmol; 48,000 cpm) or CMP-sialic acid-^{14}C (10 nmol; 115,000 cpm) as substrates replacing labeled UDP-galactose. ATP (2 mM) was used in the assay to protect the labeled substrate from hydrolytic breakdown (7). α_1-Acid glycoprotein, depleted of sialic acid, galactose, and glucosamine by enzymatic procedures (15), was used as the acceptor for the assay of N-acetylglucosaminyltransferase, and DSF was used as acceptor for the assay of sialyltransferase activity. Mn^{2+} was omitted for the sialyltransferase assay (7). The results obtained are expressed as the mean ± SEM of five experiments. Intestinal villus cell and egg yolk lysophosphatidylcholines were prepared from their respective phosphatidylcholines by digestion with phospholipase A_2 as previously described (9).

RESULTS AND DISCUSSION

The effects of various lysophosphatidylcholines on the activities of several glycosyltransferases are shown in Table I. All three of the intestinal glycosyltransferases studied were observed to be stimulated 5 to 10-fold by the two naturally occurring choline lysophosphatides. These results are in good agreement with studies done in rat kidney (6) and rat liver (7,8) microsomes. Moreover, Table I also shows that the synthetic 1-oleoyl-*sn*-glycerol-3-phosphocholine had an equivalent stimulatory effect to the two naturally occurring lysophosphatides, and that a stimulatory effect could also be effected by two structurally nonrelated detergents.

To determine whether or not the stimulatory effect of lysophosphatylcholine was stereospecific or simply due to its detergent properties, it was decided to study the effects of enantiomeric lysophosphatidylcholines on the activities of the intestinal glycosyltransferases. Table II shows that 3-palmitoyl-*sn*-glycerol-1-phosphocholine is equipotent in stimulating the activities of the glycosyltransferases when either added alone or as a 1:1 mixture with its naturally occurring enantiomer. The sialyltransferase was then studied more closely to determine the effects of the enantiomeric lysophosphatidylcholines on its kinetic parameters. Normal bisubstrate kinetics were observed when the concentration of CMP-sialic acid was varied. The apparent Km for CMP-sialic acid was 72 μM in the absence of lysophosphatidylcholine and was essentially the same in the presence of 2 mM of either enantiomer. The Vmax in the absence of lysophosphatidylcholine was 28.6 pmol of sialic acid transferred to DSF/min/mg enzyme protein, and this was increased to 98.4 pmol of sialic acid transferred to DSF/min/mg enzyme protein in the presence of 2 mM of either of the enantiomeric lysophosphatidylcholines tested.

Lysophosphatidylcholine, which is a normal membrane component (16) and a naturally occurring detergent (17), belongs to a group of substances described by Haydon and Taylor (18) as wedge-shaped molecules. The present findings that both enantiomers are equipotent in the stimulation of membrane-bound glycosyltransferases indicate that the stimulatory effect of lysophosphatidylcholine is not stereospecific, but that it may be related to a specific detergent property dependent upon the peculiar balance of hydrophilic and hydrophobic components in the molecule, as well as to its wedged shape, which facilitates lipid-protein interaction. In a separate study (19), it has been demonstrated that enantiomeric lysophosphatidylcholines are also equipotent in the activation of guanylate cyclase in the microvillus membrane and in the deactivation of adenylate cyclase in the Golgi apparatus and basal-lateral membrane of the intestinal villus cell.

Taken in toto, the results from both the present and previous studies suggest that the detergent properties of lysophosphatidylcholine may provide the physical basis for a role by this monoacylphospholipid as a metabolic modulator in the intestinal villus cell. In vivo, lysophosphatiylcholine appears in the intestinal lumen ready for absorption into the villus cell following deacylation of dietary and biliary phosphatidylcholine by a calcium-stimulated phospholipase A (20). It can also be generated in the villus cell during the phosphoglyceride deacylation-reacylation process (21). Perturbation of these molecular processes by increasing either the luminal or intracellular concentration of calcium could alter the level of lysophosphatidylcholine in the villus cell membranes. Lysophosphatidylcholine absorbed by, or generated at, one part of the villus cell could subsequently diffuse rapidly through the cell membranes to modulate the activities of both membrane-bound and cytosolic enzymes in other parts of the cell, as well as possibly modifying general properties of the membrane

such as fluidity and permeability.

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Changes in Fatty Acid Composition of Cardiac Mitochondrial Phospholipids in Rats Fed Rapeseed Oil

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ABSTRACT

Male Wistar rats were fed rapeseed oil containing high or low levels or erucic acid for 20 weeks, and changes in the fatty acid composition of cardiac mitochondrial phospholipids were studied. Treatment with rapeseed oil containing 46.2% erucic acid showed incorporation of 22:1 (5.6%) into isolated cardiolipin from heart mitochondria. After high or low (3.7%) erucic rapeseed oil feeding, linolenic acid was slightly incorporated into cardiolipin. Moreover, both of these rapeseed oils induced a significant increase of linoleate-arachidonate ratio in phosphatidylethanolamine and phosphatidylcholine. This ratio was also significantly increased in fatty acids esterified to the β-position of these phospholipids. On the basis of such results, we have to consider the role of linolenic acid which is present at a high level in the different rapeseed oils used, as a possible inhibitor of heart microsomal enzymes involved in linoleate arachidonate conversion. Such alterations might account for mitochondrial fragility and myocardial lesions obtained in long term rapeseed oil feeding experiments.

INTRODUCTION

Considerable attention has been given to the origin of cardiac pathological lesions obtained in rats after a diet of rapeseed oil (RSO) containing high or low levels of erucic acid (22:1) (1). We have shown previously that phosphatidylethanolamine (PE), phosphatidylcholine (PC), and cardiolipin (CL) contents of heart were not modified in rats treated for 20 weeks with high or low erucic RSO (2). Nevertheless, after RSO feeding for various durations several authors have established the incorporation of 22:1 in phospholipids of rat heart and found a specific affinity of erucic acid to cardiolipin (2,3). Moreover, after 7 days of feeding, it has been shown that high or low erucic acid RSO induced a highly significant reduction in saturated fatty acids of rat heart mitochondrial lipids (4).

Based on recent reports, one could suggest some relationship between the acyl-moiety alterations of cardiac mitochondrial phospholipids and the pathological lesions observed in rat heart after a long or a short duration of RSO feeding (2,4). Specific phospholipids are required for catalytic activity of mitochondrial enzymes, and modifications of their acyl-moieties could induce a loss of metabolic function (5,6). Moreover, similar modifications of unsaturated fatty acids have been found after essential fatty acid deficiency or long term RSO feeding experiments in liver phospholipids (7,8).

In the present work, changes in the fatty acid composition or cardiac mitochondrial phospholipids have been studied in rats after feeding high or low erucic RSO for 20 weeks. Both of these RSOs induced a significant increase of linoleate-arachidonate ratio in PE and PC. In this way, the pathological lesions observed in myocardium after long term RSO feeding experiments could be related to a decrease of arachidonic acid which appears to be essential to structure and function of bio-

TABLE I

Fatty Acid Composition of Dietary Oils

Fatty acid	Peanut oil (PNO)	Rapeseed oil (RSO)	Primor rapeseed oil (PRO)
16:0	10.1	3.4	5.1
16:1	0.3	0.3	0.3
18:0	3.9	1.0	1.7
18:1	52.5	14.6	55.7
18:2	27.9	14.1	20.5
18:3	--	8.0	11.1
20:0	1.6	1.0	--
20:1	1.1	11.0	1.9
22:0	2.6	0.4	--
22:1	--	46.2	3.7

TABLE II

Relative Composition of Total Fatty Acids (moles %) of Cardiolipin (CL), Phosphatidylethanolamine (PE), Phosphatidylcholine (PC) in Mitochondria of Control and Treated Rat Hearts[a]

Fatty acid	CL			PE			PC		
	PNO	RSO	PRO	PNO	RSO	PRO	PNO	RSO	PRO
16:0	3.6 ± 2.3	2.4 ± 2.2	1.7 ± 0.5	9.9 ± 2.0	9.4 ± 2.9	10.2 ± 1.7	13.0 ± 1.9	12.8 ± 3.0	12.8 ± 1.9
16:1	1.2 ± 1.3	0.9 ± 0.8	1.0 ± 0.2	0.7 ± 0.3	1.1 ± 0.6	0.8 ± 0.4	0.5 ± 0.3	0.5 ± 0.1	0.4 ± 0.1
18:0	3.2 ± 2.3	2.0 ± 1.6	1.5 ± 1.3	32.3 ± 2.7	32.4 ± 6.6	34.8 ± 2.6	35.2 ± 3.0	35.0 ± 3.2	34.9 ± 2.1
18:1	11.9 ± 4.7	8.0 ± 3.6	11.0 ± 1.3	10.8 ± 2.4	17.4 ± 1.2	16.3 ± 1.1	7.3 ± 1.8	10.6 ± 1.0	11.5 ± 0.5
18:2	77.3 ± 10.6	76.2 ± 7.9	81.1 ± 3.7	6.7 ± 2.5	8.9 ± 1.1	6.0 ± 1.0	8.6 ± 3.1	16.0 ± 2.6	11.4 ± 0.9
18:3	--	1.8 ± 0.8	1.2 ± 0.2	--	0.1 ± 0.2	0.5 ± 0.1	--	0.3 ± 0.1	0.3 ± 0.1
20:1	--	1.7 ± 0.6	0.1 ± 0.1	--	2.2 ± 1.7	0.4 ± 0.1	--	1.3 ± 0.4	0.3 ± 0.1
20:4	2.8 ± 1.9	1.1 ± 0.7	1.0 ± 0.7	23.8 ± 3.2	17.2 ± 4.7	15.1 ± 2.6	32.7 ± 4.3	20.8 ± 4.4	23.9 ± 3.4
20:5	--	--	0.4 ± 0.3	--	1.0 ± 1.5	1.4 ± 1.8	--	0.4 ± 0.1	0.2 ± 0.1
22:1	--	5.6 ± 1.1	0.1 ± 0.1	--	0.7 ± 0.5	0.2 ± 0.1	--	0.5 ± 0.2	0.1 ± 0.1
22:5	Tr	0.1 ± 0.1	0.2 ± 0.2	2.7 ± 0.8	2.5 ± 1.3	3.3 ± 0.5	0.8 ± 0.2	0.7 ± 0.3	1.9 ± 0.3
22:6	Tr	0.2 ± 0.2	0.7 ± 0.5	13.1 ± 4.2	7.1 ± 2.6	11.0 ± 3.5	1.8 ± 1.2	1.1 ± 0.4	2.3 ± 0.9

[a]Results are expressed by the mean ± standard deviation of eight mitochondrial pools (each pool consisting of four rat hearts).

logical membranes (9).

MATERIALS AND METHODS

Three groups of 32 male Wistar rats, 4 weeks old, were fed for 20 weeks a diet containing 18% casein, 36% wheat starch, 4% salt mixture, 2% agar-agar, 25% sucrose, and 15% fat. The control group was fed peanut oil (PNO) and the other two groups high erucic (46.2%) RSO or low erucic (3.7%) primor oil (PRO) (Table I). After the animals were killed, the hearts of rats were immediately removed and homogenized gently in ice-cold medium containing 0.07 M sucrose, 0.21 M mannitol, and 1mM EGTA (pH = 7.4). Rat heart mitochondria were isolated by several centrifugations: twice at 9,000 x g for 7 min with resuspension of the pellet in the medium, then at 1,500 x g for 15 min to isolate mitochondria in the supernatant which was centrifuged at 9,000 x g for 10 min giving a pellet again resuspended and centrifuged at 7,500 x g for 7 min. The final pellets of mitochondria obtained from four hearts were collected. Total lipids from each pool were extracted by the procedure of Folch et al. (10), and the total lipid extract was stored in chloroform-BHT at -20 C for a maximum of 1 month.

Phospholipid separation using silicic acid column chromatography was carried out as described previously (2). PE, PC, and CL were then isolated by thin layer chromatography (TLC). The positioning of the fatty acids in PE and PC was determined by hydrolysis with phospholipase A_2 (*Crotalus adamenteus*). The methyl esters of fatty acids were obtained by transesterification of lipids with methanol-H_2SO_4 at 70 C (11). After extraction with heptane, the fatty acid methyl esters were separated and identified by gas liquid chromatography (GLC) using a capillary column of carbowax 20 M (50 m) at 200 C. The different peak areas were calculated by integrator. The results were expressed in mole percent.

RESULTS AND DISCUSSION

The relative composition of total fatty acids (Table II) confirmed the incorporation of erucic acid (5.6%) into isolated CL from heart mitochondria of rats fed high erucic RSO already described by Blomstrand and Svensson (3). Eicosenoic acid was also present (1.7%), but no differences in linoleic acid were found. After high or low erucic RSO feeding, linolenic acid was slightly incorporated (RSO = 1.8%, RPO = 1.2%) which could be related to dietary intake (Table I). The independent turnover of acyl groups (acylation-deacylation) might ex-

TABLE III

Linoleate Arachidonate Molar Ratios of Phosphatidylethanolamine (PE) and Phosphatidylcholine (PC) in Mitochondria of Control and Treated Rat Hearts[a]

		PNO	RSO	PRO	PNO vs. RSO	PNO vs. PRO	RSO vs. PRO
PE	Total fatty acids	0.27 ± 0.09	0.56 ± 0.20	0.41 ± 0.10	$p < 0.01$	$p < 0.05$	NS
	Fatty acids esterified to the β-position	0.27 ± 0.06	0.47 ± 0.18	0.38 ± 0.16	$p < 0.05$	NS	NS
PC	Total fatty acids	0.26 ± 0.11	0.71 ± 0.17	0.49 ± 0.10	$p << 0.001$	$p < 0.001$	$p < 0.01$
	Fatty acids esterified to the β-position	0.16 ± 0.07	0.46 ± 0.18	0.33 ± 0.18	$p < 0.01$	NS	NS

[a]Results are expressed by the mean ± standard deviation of eight mitochondrial pools (each pool consisting of four rat hearts).

plain the incorporation of 22:1 and 18:3 into this mitochondrial phospholipid (12,13). The presence of unusual fatty acids in CL might determine a specific inhibitory effect upon mitochondrial function.

As previously established (2), 22:1 was slightly incorporated into PE and PC after the high erucic RSO diet (Table II). Nevertheless, after high or low erucic RSO, a decrease of arachidonic acid percentages was observed in PE and PC without any inverse relationship between 20:4 and other polyene fatty acids (22:5, 22:6) which tended to decrease. Moreover, there was a highly significant increase of linoleate-arachidonate molar ratio in total fatty acids in PE and PC after high or low erucic RSO feeding (Table III). This ratio was also significantly increased in fatty acids esterified to the β-position of PE and PC in rats fed high erucic RSO. Similar results were obtained previously in total lipid extracts from liver and heart in rats after long duration RSO feeding with high or low levels of erucic acid (14).

A decrease of arachidonic acid has been observed in several conditions, as in essential fatty acid deficiency (7) or alcohol-treated animals (15) and was associated with mitochondrial fragility (16). In RSO feeding experiments, the conversion of 18:2 to 20:4 might decrease by some inhibition of elongation desaturation microsomal system. In vitro, chain elongation of linoleic acid has been found to be inhibited by other fatty acids like linolenic acid (17) which is present at a high level in RSOs used in the experiments (Table I). Moreover, the presence of 20:5 in PE and PC of treated animals might be related to elongation desaturation of 18:3 (18).

Some have also postulated that linolenic acid plays a role in the etiology of cardiac necrosis observed after high or low erucic RSO feeding (19). Since RSO does not seem to contain a simple cardiotoxic agent, on the basis of results obtained in this study we have to consider the role of 18:3 as a possible inhibitor of heart microsomal enzymes involved in linoleate arachidonate conversion. Such an increase of 18:2/20:4 ratio in heart mitochondria PE and PC might account for mitochondrial dysfunction and myocardial lesions observed in longterm RSO feeding experiments.

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Lipid Peroxidation: A Mechanism Involved in Acute Ethanol Toxicity as Demonstrated by In Vivo Pentane Production in the Rat

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ABSTRACT

The effect of a single dose of ethanol on lipid peroxidation in three groups of rats fed different amounts of vitamin E was determined by the measurement of pentane in the breath. All rats had increased pentane production above basal levels by 15 min following oral administration of 6 g ethanol/kg body wt. The increase in total pentane production during a 13-hr test period after intragastric administration of ethanol was greater in the rats fed the vitamin E-deficient diet than in the rats fed vitamin E-supplemented diets (α = 2P = 0.02). The results support the hypothesis that acute ethanol toxicity involves lipid peroxidation and further demonstrate the usefulness in toxicological studies of monitoring pentane as an index of lipid peroxidation in vivo.

INTRODUCTION

Acute administration of ethanol to rats with a nutritionally adequate diet induces liver injury caused by excessive accumulation of hepatic triglycerides (1). Di Luzio (2) demonstrated that pretreatment with antioxidants inhibited ethanol-induced fatty liver and suggested that lipid peroxidation is involved in the hepatotoxicity of ethanol. Further investigation by Di Luzio and Hartman (3) showed increased formation of malondialdehyde in liver homogenates of rats given ethanol orally. Evidence by Reitz (4) supports the hypothesis that formation of lipid peroxides is enhanced by ethanol consumption. He found rates of microsomal NADPH oxidation, oxygen consumption, and malondialdehyde formation in chronic ethanol-treated rats increased above those of the controls.

Recently, techniques for monitoring in vivo lipid peroxidation by analyzing expired air for ethane and pentane have been developed. Riely et al. (5) injected carbon tetrachloride, a known liver prooxidant, into mice with a resultant increase in expired ethane levels. Dillard et al. (6) showed that rats fed a vitamin E-deficient diet produced more ethane and pentane than rats supplemented with vitamin E. When rats fed a vitamin E-deficient diet were challenged for 1 hr with 1 ppm ozone, which is a known free radical inducer, ethane and pentane levels were elevated (7). The present study examined the effect of intragastric administration of an acute dose of ethanol on pentane production in the rat.

MATERIALS AND METHODS

Three groups of three male Sprague-Dawley rats (Simonsen Laboratories, Gilroy, CA) were fed various amounts of vitamin E. The first group of 8-month-old rats was fed a vitamin E-deficient Draper diet (8) since weaning. The second group of 8-month-old rats was fed the Draper diet with 40 i.u. dl-α-tocopherol acetate/kg diet for 3 months prior to testing. Previously, this group was fed the basal diet containing lower amounts of added vitamin E. The third group of 5-month-old rats was supplemented with 200 i.u. dl-α-tocopherol acetate/kg diet for 3 months after first being fed the vitamin E-deficient diet since weaning.

Each animal was tested for pentane production after being fasted for 12 hr. Collection of expired air from the rat and subsequent analysis for pentane were carried out according to the procedure of Dillard et al. (6). After a basal pentane level determination, the animal was administered 15.6 ml normal saline/kg body wt by stomach tube. Breath samples were collected at 0.5, 1, 3, 5, 7, 9, 11, and 13 hr after treatment. Pentane values at these times served as the control level for the animal. One week later, the same rat was again fasted, and its basal pentane level was determined. The rat was given a 50% solution of 95% ethanol in normal saline (v/v), 15.6 ml/kg body wt, by stomach tube. This corresponds to a dose of 6 g ethanol/kg body wt. Breath samples were obtained at 15, 30, and 60 min following treatment, and hourly thereafter for 13 hr. The animals were allowed to drink water ad

TABLE I

Total Pentane Production during the 13-hr Test Period

Group no.[a]	Diet	Nanomoles pentane/100 g body wt	
		Saline control	Ethanol treatment (increase over saline)
1	Vitamin E-deficient diet	9.9	5.5
		8.7	5.8
		10.6	2.4
2	40 i.u. vitamin E/kg	0.6	1.0
		0.4	0.5
		0.6	0.7
3	200 i.u. vitamin E/kg	0.4	0.7
		0.6	1.3
		0.6	0.7

[a]The data for group 1 when compared to the pooled data of groups 2 and 3 were significantly different ($\alpha = 2P = 0.02$) using the Mann-Whitney U test in both the saline and ethanol treatments.

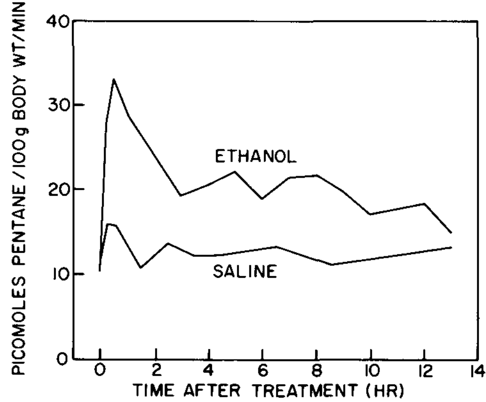

FIG. 1. Rate of pentane production following ethanol and saline treatment of a rat fed a vitamin E-deficient diet.

libitum during this time. The 13-hr breath sampling period was chosen for convenience. The expired pentane levels of the rats treated with ethanol did not return to basal levels within the 13-hr monitoring period. Pentane measurements taken at various intervals after the 13-hr period indicated a continuing decline toward basal pentane levels.

Upon completion of the study, the rats were killed, and the plasma of each rat was analyzed in triplicate for total tocopherol by the fluorometric method of Taylor et al. (9). The total tocopherol content of the three diets was similarly determined.

Since the variances in the groups were not homogeneous, as determined by the Bartlett's têst, parametric statistics were not used. For this reason, the Mann-Whitney U test, a nonparametric statistical analysis, was used to determine differences in pentane values between groups.

RESULTS AND DISCUSSION

Saline administration alone caused no increase in the production of pentane. Table I lists the total pentane production during the 13-hr period following saline treatment. The rats fed a vitamin E-deficient diet had higher basal pentane levels than rats supplemented with vitamin E ($\alpha = 2P = 0.02$). This relationship has been demonstrated previously for younger rats (6). Vitamin E in the diet protects against lipid peroxidation and consequently lowers basal pentane production levels.

All nine rats, when treated with ethanol, displayed a 1.7- to 3.4-fold increase in breath pentane production above basal levels within 15 min. The rate of pentane production, in general, reached the highest level during the first hour after ethanol treatment and decreased slowly thereafter. As an example, a characteristic curve of ethanol-induced pentane production by one of the animals is shown in Figure 1.

Total pentane production after an acute dose of ethanol was given is listed in Table I. In this table, ethanol-induced pentane production is expressed as the difference between the total pentane expired over the 13-hr period following ethanol administration and the total pentane expired over the 13-hr period after saline administration for each animal. Following the ethanol dose, the rats fed the vitamin E-deficient diet had an increase in total pentane production of 5.6 times ($\alpha = 2P = 0.02$) greater than the increase found in the six vitamin E-

supplemented rats over the entire 13-hr monitoring period. No significant difference was noted between the rats supplemented with 40 i.u. and 200 i.u. vitamin E/kg diet.

The average values for plasma total tocopherol of rats fed the vitamin E-deficient diet, rats supplemented with 40 i.u. vitamin E/kg diet, and rats supplemented with 200 i.u. vitamin E/kg were 1.1, 4.4, and 12.8 mg/100 ml, respectively. The Draper vitamin E-deficient diet, the 40 i.u. vitamin E-supplemented diet, and the 200 i.u. vitamin E-supplemented diet had corresponding total tocopherol values of 7, 45, and 204 mg/kg.

Pentane is derived from autoxidized linoleic acid (10). When preformed linoleate hydroperoxide was decomposed in vitro, 1.3 mol % pentane was produced (11). Techniques for measurement of ethane, a decomposition product of linolenate hydroperoxide (11), and pentane have now been developed to study toxicological agents whose mechanism of action is lipid peroxidation (7,12). Recently, Köster and his co-workers (13) demonstrated increased exhalation of ethane when rats were orally treated with 5 g ethanol/kg body wt.

The technique used to measure breath pentane levels is adapted easily to the study of many pathological conditions. A wide range of environmental, chemical, and dietary factors that effect in vivo lipid peroxidation can be examined. Since the procedure is noninvasive, each subject serves as its own control. In mixed populations, this eliminates many variables, such as age, sex, and genetic makeup.

The proposal that acute ethanol toxicity occurs via lipid peroxidation has been questioned by Hashimoto and Recknagel (14). They were unable to find increased formation of diene conjugation in rat livers following ethanol administration. Our data, showing increased pentane production following a single dose of ethanol and an inhibition of pentane formation when the antioxidant vitamin E is provided in the diet, support the hypothesis that lipid peroxidation is a mechanism involved in acute ethanol toxicity.

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Prostaglandin Metabolism by Human Testis

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ABSTRACT

Human testis preparations appear to carry out both the synthesis and turnover of prostaglandins. Prostaglandins and prostaglandin metabolites were extracted from whole tissue. Testis microsomes converted both endogenous and exogenous substrate to prostaglandins. Microsomal prostaglandin biosynthesis was inhibited by indomethacin. Prostaglandin E_2-9-ketoreductase was present in both the cytosolic and microsomal fractions of human testis. Prostaglandin metabolism by human testis has not previously been reported.

INTRODUCTION

Although prostaglandins occur in human semen (1,2) and have recently been shown to be formed by homogenates of human seminal vesicle (3), their presence in and synthesis by human testis has not yet been documented. We have observed that prostaglandin biosynthesis and catabolism occur in rat testis and are studying the synthesis, turnover, and function of these compounds in this tissue (4-6). Human testis contains the fatty acid biosynthetic precursors of prostaglandins (7,8). This paper reports studies which indicate that endogenous fatty acids, as well as exogenous arachidonate serve as substrates for the synthesis of prostaglandins in human testis and that prostaglandin E_2-9-ketoreductase activity is found in this tissue.

EXPERIMENTAL PROCEDURES

Human testicular tissue was obtained from a 66-year-old male with prostatic cancer immediately after orchidectomy. The patient has not received steroid treatment, and the neoplasia did not involve the testis. The tissue was packed in 0.15 M NaCl in an ice chest, and tissue preparations were initiated 45 min after surgery. A portion of the tissue was fixed in Bouin's solution for histological studies. Prostaglandin metabolism was determined in (a) extracts of whole tissue, (b) microsomes, and (c) cytosol. Extraction of whole tissue: 1.5 g samples of tissue were homogenized in 7 ml saline-citrate (0.15 M NaCl, 0.02 M Na citrate, pH 3.5), in the presence and absence of indomethacin (10 μg/ml). $[^3H]$-$PGF_{1\alpha}$ (1400 cpm) was added as an internal standard, and the prostaglandin fraction was extracted with ethyl acetate and partitioned into classes on small columns of silicic acid (9). Microsomal prostaglandin synthesis: Microsomes were prepared from the supernatant of testicular tissue homogenized in 0.1 M phosphate buffer sucrose, pH 7.0, (1/3, w/v), and centrifuged at 10,000 x g for 10 min. The pellet was resuspended and centrifuged at 105,000 x g for 45 min; this process was repeated. Microsomal prostaglandin synthesis was determined in 2 ml systems containing 2 mg microsomal protein and 12.5 μg arachidonic acid in 0.06 M phosphate buffer, pH 8.5, and incubated for 30 min at 33 C. Reactions were stopped with the addition of citric acid (0.1 ml, 1.5 M). ^3H-$PGF_{1\alpha}$ (2,000 cpm) was added to monitor recovery. Prostaglandins were extracted with ethyl acetate, washed to neutrality with water, and partitioned into classes on small columns of silicic acid. The PGE effluent was treated with KOH, and PGB formed was extracted with ethyl acetate. Prostaglandins in the column effluents were quantified by radioimmunoassay using antisera for PGB, $PGF_{2\alpha}$,

TABLE I

Prostaglandins Extracted from Whole Human Testis

Prostaglandin	Indomethacin present	Indomethacin absent	Difference
	(ng/g tissue)		
PGE	2.15	3.18	1.03
PGF	1;48	5.10	3.62
DKF	0.55	1.05	0.50
PGF/PGE	0.94	1.93	3.50

TABLE II

Prostaglandin Synthesis by Human Testis Microsomes

Addition	Specific activity[a]			
	Endogenous		Exogenous	
	PGF	PGE	PGF	PGE
None	1.01	0.83	2.27	1.84
Heated supernatant	1.73	0.79	2.74	0.54
Indomethacin	0.25	0.32	0.22	0.65
Glutathione and epinephrine	1.21	2.05	3.42	4.64

[a]Specific activity is the net synthesis of prostaglandin expressed as ng/mg microsomal protein equivalent to 1 g of testis/30 min. Recovery of microsomal protein was 3.6 mg/g tissue. Microsomes (1.2 mg protein) were incubated for 0 and 30 min at 33 C in the presence and absence of arachidonic acid. Additions to the reactions were 0.1 ml of supernate boiled for 3 min, indomethacin (10 μg/ml), 1 mM glutathione, and 0.2 mM epinephrine.

and 13,14-dihydro-15-keto $PGF_{2\alpha}$ ($DKF_{2\alpha}$) and ^{3}H-labeled PGB, $PGF_{2\alpha}$ or $DKF_{2\alpha}$. Several aliquots of each sample showing a dose response were used. Standard curves, 20-200 pg of appropriate prostaglandin, were run with each assay. Sample values were calculated from the standard curves which were plotted on log probit paper. Prostaglandin E_2-9-ketoreductase: Testicular cytosol (105,000 x g supernatant) was prepared from tissue homogenized in 0.092 M phosphate buffer, 0.1 mM dithiothreitol. The reactions were carried out for 30 min at 37 C in 250 μl systems containing 5 mM pyridine nucleotide (NADH or NADPH), and either 0.8 mg of cytosolic or 1.21 mg of microsomal protein. Control reactions were done using heat inactivated enzyme. The reactions were stopped by boiling for 3 min, and the $PGF_{2\alpha}$ formed was determined by radioimmunoassay of diluted aliquots of the inactivated reaction systems. Protein was assayed by the method of Lowry et al. (10) using serum albumin as a standard.

RESULTS AND DISCUSSION

Prostaglandins are rapidly synthesized and released during handling and homogenization of a number of tissues; however, this has not been demonstrated for human testis. When tissue is processed in the presence of inhibitors of prostaglandin synthesis, such as indomethacin, biosynthesis of prostaglandins is inhibited. The differences in the concentrations of prostaglandins extracted from tissues in the presence and absence of indomethacin is a reflection of net synthesis and turnover. Samples of human testis were homogenized in the presence and absence of indomethacin, and the prostaglandins were extracted, partitioned into classes, and quantified by radioimmunoassay. The results indicate that there is a net synthesis of prostaglandin E (PGE), prostaglandin F (PGF), and 13,14-dihydro-15-keto PGF (DKF) from endogenous substrate (Table I). Human testis lipids contain significant concentrations of both 20:3n6, 57%, and 20:4n6, 12-13% (7,8), thus the fatty acid precursors of both the 1-series and 2-series of prostaglandins are potentially available as substrates. The formation of the prostaglandin metabolite, 13,14-dihydro-15-keto PGF, indicates that prostaglandin 13,14-reductase and prostaglandin 15-hydroxy-dehydrogenase activities are present in human testis. Prostaglandins produced from endogenous substrate are identified as PGE, PGF, and DKF, as the antisera used do not distinguish between the 1- and 2-series.

The subcellular localization of the prostaglandin synthetase complex of a variety of tissues has been shown to be in the endoplasmic reticulum. Microsomes of rat testes synthesize prostaglandins (5), and microsomes isolated from human testis also synthesize prostaglandin (Table II). A net synthesis of both PGE and PGF from endogenous substrate and a stimulation of biosynthesis in the presence of arachidonic acid is observed. Indomethacin, an inhibitor of prostaglandin cyclooxygenase, inhibits prostaglandin biosynthesis by human testis microsomes. Addition of glutathione and epinephrine stimulate the formation of PGE from both endogenous and exogenous precursor. Addition of heated 105,000 x g supernatant from testis homogenate to the microsomal system (heated supernatant addition) stimulates the synthesis of PGF, but not PGE, in contrast to the observations on microsomes from other tissues, in which addition of cytosol tends to increase the formation of PGE. Addition of the heated supernatant to rat testis microsomes incubated in the presence of arachidonate, results in an increase in PGE_2, but has no effect on $PGF_{2\alpha}$ (5). Prostaglandin synthesis by microsomes of adult rat testis is

TABLE III

Prostaglandin E-9-Ketoreductase Activity of Human Testis

Fraction	Coenzyme 5 mM	Other addition	ng $F_{2\alpha}$ formed	Specific activity[a]
Cytosol	NADH	---	299	11.2
	NADPH	---	1279	47.9
	NADPH*	---	1688	63.2
	NADPH*	PPi	2311	86.6
Microsome	NADH	---	67	1.8
	NADPH	---	294	8.1
	NADPH*	---	384	10.6

[a]Specific activity is expressed as ng/mg protein/min. Pyrophosphate (PPi) concentration was 10 mM. *Incubations were carried out in the presence of an NADPH generating system (0.1 M glucose-6-phosphate and 2 units of glucose-6-phosphate dehydrogenase).

characterized by a higher level of $PGF_{2\alpha}$ than PGE_2 (6). The ratio, PGF/PGE, for the human testis microsomes in this study is similar to that which we have observed in preparations of rat prepubertal testis (6).

Prostaglandin E-9-ketoreductase, an enzyme that converts PGE to PGF and which has a high specific activity in rat testis (11), is also present in human testis (Table III). As in rat testis, the enzyme is found both in the cytosolic and microsomal fractions. The soluble enzyme has a six-fold higher specific activity than the microsomal enzyme. NADPH functions more efficiently as a cofactor than NADH in the reaction, and pyrophosphate stimulates the activity. Although the specific activity of human testicular cytosolic ketoreductase is relatively high compared to other tissues of the rat (12), it is only about 25% of that routinely observed in similar preparations from rat testis (11).

The occurrence of prostaglandins and prostaglandin metabolites in the tissue, the demonstration of microsomal synthesis, and the presence of prostaglandin E-9-ketoreductase activity, provide evidence that human testis metabolizes prostaglandins. Synthesis and catabolism of prostaglandins by human testis has not been previously reported. Prostaglandins were not detected when homogenates of human testis were incubated with $[1\text{-}^{14}C]$-20:3n6 (3). In our own studies on prostaglandin biosynthesis by rat testis, we have not been successful in demonstrating synthesis by homgenates of the tissue, but can readily demonstrate synthesis by extracting whole tissue (4) or incubating segments of whole testis (13) or isolated microsomes (5). Both the low prostaglandin biosynthetic capability and the apparent high activity of the metabolizing enzymes in rat testis are probably factors that make it difficult to measure net synthesis of prostablandins in a homogenate of the whole tissue. Similar problems may be encountered in such preparations from human testis.

Since prostaglandins appear to be produced by human testis, they are expected to have a function in this tissue. The biological significance of prostaglandins in human testis will require further study, as little is known at this time in regard to the synthesis, turnover, and function of prostaglandins in the male reproductive tract of humans or any other mammalian species. The concentrations of prostaglandins in human seminal plasma are higher than those reported from most tissues (1), and decreased levels of seminal prostaglandins have been reported in men with low fertility (2). Homogenates of human seminal vesicle carry out the synthesis of PGE_1 (3), indicating seminal vesicle is a source of prostaglandins of human semen. Prostaglandins that are found in human seminal plasma could originate in the testis. That testicular fluid in the ram has been found to have high concentration of $PGF_{2\alpha}$ (14) may be significant in this context.

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Characterization of a Prostaglandin-Like Metabolite of Linolenic Acid Produced by a Flaxseed Extract

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ABSTRACT

One of the products formed upon incubation of linolenic acid (*cis*9,12,15-octadecatrienoic acid) with an extract of flaxseed acetone powder has been characterized as 8-[2-(*cis*-pent-2'-enyl)-3-oxo-*cis*-cyclopent-4-enyl] octanoic acid. The cyclopentenone ring structure of this acid is analogous to that of the A-type prostaglandins produced in mammalian systems.

The direct oxygenation of polyunsaturated fatty acids in plant tissues is catalyzed by lipoxygenase, and the resultant hydroperoxide products have been characteristized and extensively investigated (1). Plant metabolism of these hydroperoxides yields α- and γ-ketols by hydroperoxide isomerase enzymes (2,3), 12 carbon-oxo-acids by hydroperoxide lyase enzymes (4), and a divinyl ether acid (5). This communication reports the structure of a novel, cyclic fatty acid produced on incubation of α-linolenic acid with a flaxseed extract.

MATERIALS AND METHODS

Chemicals

Linolenic acid (99% pure) was obtained from Nu-Chek Prep Inc. (Elysian, MN), N-Methyl-N-nitroso-*p*-toluenesulfonamide from Sigma Chemical Co. (St. Louis, MO), 2% methoxyamine HCl in pyridine and N,O-*bis*-(trimethylsilyl)-trifluoroacetamide from Pierce Chemical Co. (Rockford, IL), sodium borohydride from Fisher Scientific Co. (Fairlawn, NJ), platinum oxide from Matheson, Choleman and Bell (Norwood, OH), 10% palladium on charcoal from Sargent-Welch (Skokie, IL), 3% OV-210 on 100/120 mesh Gas-Chrom Q from Appied Science Laboratories, Inc. (State College, PA), and Anasil HF silica gel thin-layer plates from Analabs, Inc. (North Haven, CT).

Incubation and Isolation

Acetone powder (2 g) of flaxseed (*Linum usitatissimum* L., var. Linott) was extracted with 20 ml of 0.05 M potassium phosphate buffer, pH 7.0, at 4 C for 45 min. The extract was centrifuged at 12,000 x *g* for 15 min. The supernatant was decanted and used immediately. Linolenic acid substrate solution was prepared according to the method of Surrey (6). Substrate solution (20 ml) was added to 400 ml of 0.05 M potassium phosphate buffer, pH 7.0, and 10 ml of the flax extract. After 90 min at 25 C, 300 ml of chloroform-methanol (2:1, v/v) was added, the mixture acidified to pH 3.0 with 1 M citric acid solution, and the flask flushed with nitrogen gas. After 30 min, 200 ml of chloroform was added. Ninety minutes later, the organic phase was removed and dried with anhydrous sodium sulfate. The solvent was evaporated at 40 C under reduced pressure and the residue dissolved in a minimum amount of diethyl ether.

Purification

The crude ether extract was spotted on thin layer silica gel plates and developed three times in chloroform-acetic acid (100:2, v/v) solvent. The band at R_f=0.66 (band A) was marked by viewing under ultraviolet light (254 nm), scraped from the plate, and eluted from the gel with diethyl ether. This band is just ahead of the α-ketol band (band B), the major product of the incubation. Esterification was accomplished with diazomethane. The methylated products were injected onto a 180 cm glass column packed with 3% OV-210 on Gas Chrom Q, 100/120 mesh. The column was programmed from 190 C to 240 C at 2°/min.

Reductions

A 2 mg portion of the material from the purified ether extract was dissolved in 50 ml methanol, 50 mg of sodium borohydride was added, and the flask quickly flushed with nitrogen. After 60 min, the mixture was acidified with acetic acid. The methanol was removed under vacuum at 40 C, diethyl ether added, and the ether layer washed four times with water. The ether layer was then dried over anhydrous sodium sulfate. The material, ca. 2 mg, was dissolved in 10 ml methanol, 100 mg of 10% palladium/charcoal or 20 mg platinum oxide were added, and hydrogen bubbled into the solution at 24 C for 2 hr. The catalyst was separated by centrifugation.

PHYTONOIC ACID

12-OXO-cis-10,15-PHYTODIENOIC ACID

FIG. 1. Carbon sketeton and numbering system for phytonoic acid and structure of 12-oxo-*cis*-10,15-phytodienoic acid.

Derivatization

Methyl oximes were prepared, following evaporation of the ether solvent, by reacting 0.5 ml of methoxyamine reagent with the sample for 16 hr at 24 C, under nitrogen. Most of the excess reagent was removed by evaporation and the remainder by dilution with diethyl ether and washing with water. Trimethylsilyl ether derivatives were prepared by reacting 1 ml of N,O-*bis*-(trimethylsilyl)-trifluoroacetamide with ca. 2 mg of sample. Reaction was complete after 2 hr at 24 C.

Spectral Analyses

Infrared spectra were obtained on a Perkin Elmer 337 spectrophotometer and ultraviolet spectra with a Beckman DK-2 spectrophotometer. Mass spectra were obtained on both a Varian/MAT CH-5DF mass spectrometer and a Hewlett-Packard 5992A GC/MS system. Both were operated at an electron potential of 70 eV. NMR spectra were obtained with a Varian XL-100 instrument.

RESULTS AND DISCUSSION

The isolation of the metabolites in pure form was difficult to achieve. We frequently observed distinct bands on thin layer plates which exhibited two to five components when analyzed by gas chromatography. Band A, for which infrared and NMR data are reported, contained three components as shown by gas chromatography. Two of these components, with carbon numbers 24.13 and 24.53, comprised 95% of the sample and produced identical mass spectra. We believe these two components are geometric or stereoisomers.

The infrared spectra gave no evidence for the presence of *trans* double bonds or hydroxyl groups. The unreduced methyl ester yielded strong absorption at 1735 cm^{-1} (ester carbonyl) and 1705 cm^{-1} (conjugated, five-membered ring carbonyl) (7). The reduced, free acid form showed strong absorptions at 1710 cm^{-1} (acid carbonyl) and 1745 cm^{-1} (saturated five-membered ring carbonyl). The data clearly indicated the presence of a five-membered ring containing a carbonyl group in conjugation with a double bond. We propose the name phytonoic acid for the 18 carbon fatty acid containing a five-membered ring between 9 and 13 (Fig. 1).

The mass spectra of the unreduced products were consistent with the presence of a cyclopentenone ring structure. The mass spectra showed peaks at m/e 306 (molecular ion), m/e 275 (M-31), m/e 238 (M-C_5H_8), m/e 206 [M-(C_5H_8 + CH_3OH)], m/e 149 [M-$(CH_2)_7COOCH_3$)], and a base peak m/e 95 (C_6H_7O). These fragments are consistent with the structure of the methyl ester of 12-oxo-*cis*-10,15-phytodienoic acid (Fig. 1). In this instance, it was impossible to differentiate between the presence of a ring and a double bond by mass spectrometry because of identical masses and similar fragmentation patterns. The presence and location of the ring became evident after reduction of the double bonds.

Mass spectral analysis of the hydrogenated methyl ester products indicated a molecular

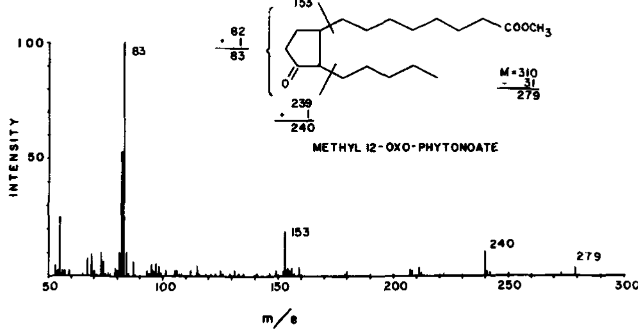

FIG. 2. Mass spectral fragmentation pattern of methyl-12-oxo-phytonoate.

weight of 310 and ring closure between carbons 9 and 13. The mass spectrum showed peaks at m/e 279 (M-31), m/e 240 (M-C_5H_{10}), m/e 153 [M-$(CH_2)_7COOCH_3$)], and a base peak at m/e 83 (C_5H_7O), indicating the structure was methyl 12-oxo-phytonoate (Fig. 2). Reaction of the latter product with methoxyamine produced a material whose mass spectrum showed peaks at m/e 308 (M-31), m/e 269 (M-C_5H_{10}), m/e 182 [M-$(CH_2)_7COOCH_3$)], and a base peak at m/e 112 ($C_6H_{10}NO$), indicating the compound was methyl 12-methoxime-phytonoate. Reduction of methyl 12-oxo-phytonoate with sodium borohydride followed by preparation of the trimehtylsilyl ether with N,O-*bis*-(trimethylsilyl)-trifluoroacetamide yielded a product whose mass spectrum contained peaks at m/e 384 (M), m/e 369 (M-15), m/e 337 (M-47), m/e 294 (M-90), m/e 224, m/e 129, and m/e 73 (TMS), indicating methyl 12-trimethylsiloxyphytonoate.

The ultraviolet absorption maximum of the band A material was at 222 nm, indicative of a carbonyl group conjugated with a double bond. Direct chemical evidence for the location of the oxo group has not yet been obtained. However, we believe it is on carbon number 12 based on the appearance of the mass fragment at m/e 240 (Fig. 3). We believe this fragment arises by H transfer to the oxo group by a McLafferty rearrangement when fragmentation occurs between carbons 13 and 14. The fragment at m/e 153 indicates cleavage between carbons 8 and 9 without evidence of H transfer. The assignment of the oxo group at carbon 12 is also supported by NMR analysis. The NMR spectrum in the olefin region (doublet of doublets at 7.71 δ, doublet of doublets at 5.17 δ, multiplet at 5.39 δ) is similar to that of prostaglandin A_1 (9). The spectra also indicated the presence of a terminal ethyl group, consistent with the location of the double bond at C_{15}.

Our results indicate that the structure of the metabolite found in band A was 8-[2-(*cis*-pent-2′-enyl)-3-oxo-*cis*-cyclopent-4-enyl] octanoic acid, designated here as 12-oxo-*cis*-10,15-phytodienoic acid (Fig. 1). It was produced enzymatically by incubation of α-linolenic acid with a flaxseed acetone powder extract and constituted approximately 25% of the total incubation products. Its five-membered ring with an oxo group, and a double bond is similar to that found in the A-type prostaglandins (8). It differs from mammalian prostaglandins in that it contains 18 carbons instead of 20, lacks hydroxyl groups, and has double bonds in different positions. We do not know if 12-oxo-*cis*-10,15-phytodienoic acid possesses any of the physiological effects of mammalian prostaglandins. Its function and mechanism of formation in plant tissue are unknown. Structural similarities suggest that 12-oxo-*cis*-10,15-phytodienoic acid might be a precursor in the biosynthesis of jasmone, jasmonic acid (10), or cucurbic acid (11) (Fig. 3). Additional studies on the formation and metabolism of cyclic fatty acids in plants are underway in our laboratory.

CH_3

O

JASMONE

CH_2COOH

O

JASMONIC ACID

CH_2COOH

OH

CUCURBIC ACID

FIG. 3. Structures of jasmone, jasmonic acid, and cucurbic acid.

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Lipid Composition and Biosynthesis of Human Omental Tissue

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ABSTRACT

Two groups of five males each were selected for total lipid analyses of their omental tissue. One of these groups had been subjected to a severe caloric restriction and had undergone total weight reduction of about 20%. The other group served as control. Both of these groups of patients required elective surgical procedures during which it was possible to obtain small samples of omental tissue, adipose pad, and/or mesenteric tissue. Total lipid analyses were performed on all of the materials. A distinctive positional distribution of the acyl groups was maintained in the triglycerides of omental tissue for all the patients regardless of dietary state. Patients under caloric restriction showed a reduction in their total triglyceride content, a reduction in their content of unsaturated fatty acyl groups, and a relative increase in phospholipid content. The de novo lipid biosynthetic capacity of omental tissues, as determined by 1-[^{14}C]-acetate incorporation, showed an inverse proportionality to the lipid content of the samples. Omental tissue is biosynthetically a very versatile material capable of yielding rapidly many types of fatty acids. This ability, among others, could account for the usefulness of omental tissue as a supporting base in many types of restorative surgery.

INTRODUCTION

Omental tissues have been used in surgical techniques as aids in transplants. This tissue has been used as a pouch or fold to mechanically hold the transplanted tissues and to increase collateral circulation to them in areas of the body where poor circulation exists (1-11).

Information as to the lipid characteristics of human omental tissue is scarce. The in vitro lipid biogenetic capacity of this tissue from [^{14}C]-acetate substrate is unknown.

It was the purpose of this investigation to determine the lipid composition and biosynthetic capacity of human omental tissue of individuals considered to be in a normal dietary condition and those that have been under weight reduction. Fresh biopsy materials from various individuals were used for the biosynthetic work. Fresh postmortem materials were used for the chemical analyses. The results are compared to those observed for adipose tissue. By use of pancreatic lipase and the Vander Wal (12), Coleman and Felton (13), and Gunstone (14) equations, positional distribution of saturated and unsaturated acyl groups of the triglycerides was determined and found to be nonrandom.

MATERIALS AND METHODS

Patient Material

Fifteen biopsy specimens were obtained through the kindness of Drs. E. Rosato, R. Felix, and S. Dudrick of the Surgical Department of the Veterans Administration Hospital-University of Pennsylvania, and Philadelphia General Hospital, Philadelphia, PA. All of the patients were 40-to-45-year-old males who had been carefully checked for their nutritional state. No patients were undergoing chemotherapy. For at least 2 months prior to biopsy, each patient was on (15,16) either a standard 2400-2500 calorie diet (250 g carbohydrate, 100 g protein, and 110 g fat) or a 1200-1300 calorie weight reduction diet (120 g carbohydrate, 65 g protein, and 55 g fat) (17).

Five additional samples of omentum were obtained through the courtesy of Drs. S. Bornstein, P. Skerrett, and A. Tore of the Pathology Department of the Veterans Administration Hospital-University of Pennsylvania, and Philadelphia General Hospital, Philadelphia, PA. These were fresh postmortem specimens obtained from patients that did not have any diabetic or similar metabolic disorders.

All human investigation was carried out according to the Declaration of Helsinki.

Lipid Analyses

All lipids or solutions containing lipids were kept under nitrogen at all times. Also, when stored, lipids were kept under nitrogen at -20 C in the presence of approximately 5 μg of butylated hydroxytoluene (BHT) per mg of lipid. Lipid standards were purchased from Applied Sciences, State College, PA, and were tested for purity by duplicate chromatography, as described below. In no cases did the level of impurities exceed 1%, and in most instances, no detectable impurities were present. The tissue specimens (100-500 mg) were chopped finely and homogenized, following which they were extracted with 100 ml of chloroform-methanol

(2:1, v/v) (18). Two extractions were required. Repeated extractions did not yield additional lipid material. One aliquot of the extract was subjected to silicic acid (Bio-Sil A, 100-200 mesh) column chromatography to separate neutral lipids (by chloroform elution) from phospholipids (by methanol elution). Individual lipid families were obtained from the separated neutral lipids and phospholipids by chromatography on thin layer plates. Silica Gel G thin layer chromatography (TLC) plates were developed with petroleum ether-ethyl ether-glacial acetic acid (90:10:1, v/v/v) for separation of neutral lipids. Polar lipids were separated by TLC on Silica Gel H plated using chloroform-methanol-glacial acetic acid-water (200:120:25:15, v/v/v/v/) as the developing system. Known amounts of authentic standards were spotted on alongside of each experimental sample. Iodine vapor was used for identification of lipid areas. The appropriate areas of the individual lipid was then scraped off and placed in individual test tubes containing 2 ml of conc. sulfuric acid. The tubes were heated to 200 ± 2 C for exactly 15 min. The test tubes were then cooled, immersed in ice, and 3 ml of water was then added to each tube. The test tubes were centrifuged for a few minutes at 3000 rpm to remove the silica gel, and each solution was decanted into a separate quartz cuvette. The charred lipids were quantitatively assayed by use of a Beckman DB spectrometer set at 375 nM and compared with known standards obtained in the same manner (19,20). The total sum of the charring data of all fractions were considered equal to 100% (21,22).

For analysis of the total fatty acid composition of each triglyceride fraction, a known aliquot of the stock solution was spotted on a plate and developed in the systems used to assay for neutral lipids as described above. The resulting triglyceride fractions were scraped from the plate and used for the preparation of methyl esters. These were prepared by trans-esterification with 14% boron trifluoride.

The resulting methyl esters were assayed by gas liquid chromatography (GLC) using a 6 ft 10% diethylene glycol succinate polyester column maintained at 20 lb pressure of argon. Temperature programming was at 1 C per min starting at 140 C until 190 C was reached. The area corresponding to each fatty acid ester was measured by triangulation. Known standards were assayed in the same manner. For each sample, the sum of all the areas of the peaks was considered equal to 100%. Conditions have been discribed more thoroughly elsewhere (23,24).

Protein

For determination after extraction of lipids, the residue was isolated by centrifugation and washed with 5 ml of cold 5% trichloroacetic acid (TCA). Five ml of 5% TCA were added to the remaining residue, and the mixture was heated at 90 C for 15 min. After cooling, the solution was filtered. The soluble material was transferred to a volumetric flask for use as the stock solution for the assay of protein. The biuret technique was utilized for protein assays (25,26). These were done in 1 ml aliquots of the stock solution. Bovine serum albumin (ICN-Nutritional Biochemicals, Cleveland, OH) was used as the standard.

Dimethyl-acetal Derivatives

For the determination of total ethanolamine-containing plasmalogens, the method of Dittmer and Wells (27) was used. A fraction of the total phosphatidylethanolamine was used to prepare the dimethyl-acetal derivatives. These were then chromatographed on Silica Gel G plates with toluene-diethyl ether-acetic acid (90:10:1, by vol) as solvent. Standards were used to determine R_f values. Material was detected with I_2 vapor. The plates were scraped, and the extracts were rechromatographed in the same system. The material obtained from the scrapings was assayed for total content of the dimethyl-acetal derivative by charring with H_2SO_4 (21) by comparison with a standard (28). Another portion of the total ethanolamine phospholipids was used to prepare the N-dinitrophenyl O-methyl derivative by the method of Renkonen (29). The areas corresponding to the methylated dinitrophenyl derivatives of O-(acylalkenylglycerophosphoryl) ethanolamine and (acylalkylglycerophosphoryl) ethanolamine were scraped. The material obtained from the TLC scrapings was extracted into chloroform and placed in a small volumetric flask. Portions were taken for the assays. (a) UV spectrophotometry at 236 nm was used to quantify the derivatives. (b) Another portion was evaporated to dryness and charred. The acid hydrolysis method of Viswanathan (30) was used to hydrolyze the O-(acylalk-1-enylglycerophosphoryl)ethanolamine. The hydrolyzed and unhydrolyzed materials were then separated in the second dimension. This was followed by alkaline methanolysis of the resulting O-(alk-1-enylglycerophosphoryl) ethanolamine and unhydrolyzed diacylethanolamine phospholipids, which permitted separation and quantitative determination by the use of an internal methyl ester standard and GLC (31,32).

TABLE I

Changes in % Lipid Composition of Omental Tissues[a]

	"Under caloric restriction"	"Control"
Neutral lipids		
Free fatty acids	trace	0.41 ± 0.10
Mono- and diglycerides	trace	1.21 ± 0.05
Triglycerides	70.55 ± 2.35	93.58 ± 3.52
Cholesterol	0.4 ± 0.03	0.36 ± 0.01
Cholesteryl esters	0.04 ± 0.03	1.00 ± 0.08
Methyl esters of acids	0.02 ± 0.01	0.20 ± 0.02
Unidentified	0.69	1.71
Total neutral lipids	71.70	98.47
Polar lipids		
Phosphatidylcholine	9.79 ± 0.8	0.80 ± 0.05
Phosphatidylserine and inositol	3.54 ± 0.3	0.10 ± 0.02
(nonplasmalogen) phosphatidylethanolamine	6.00 ± 0.5	0.21 ± 0.01
(plasmalogen) phosphatidylethanolamine	1.02 ± 0.2	0.05 ± 0.01
Sphingomyelins	7.75 ± 0.9	0.21 ± 0.02
Lysophosphatidylcholine and ethanolamine	trace	0.02 0.01
Unidentified	.20	0.14
Total polar lipids	28.30	1.53

[a]Averages of five samples in duplicate ± standard error of the mean.

Analysis of 2-Monoglycerides

To 50 mg of triglyceride (obtained after TLC), in a 5 ml screw-cap vial (45 x 15 mm), 9 mg of pancreatin (Steapsin-ICN-Nutritional Biochemical, Cleveland, OH) were added. The standard procedure (33,34) developed for this analysis was then followed. The recovered fatty acids were weighed, transferred quantitatively to a 50 ml volumetric flask with ether, made up to volume, and reserved for fractionation and analysis. A 5 ml aliquot of the ether solution was treated with diazomethane directly to convert the free acids to their methyl esters. A known weight of internal standard (15:0 methyl ester) was added, and the mixture was subjected to GLC analysis (22,23,25).

Biosynthesis

Homogenates were prepared after mincing the tissue in two-and-a-half volumes of buffer containing K_2HPO_4, 0.067 M; KH_2PO_4, 0.042 M; $MgCl_2$, 0.006 M and nicotinamide, 0.03 M at pH 7.0 at 0 C. The mince was then homogenized quickly in a loose-fitting Potter-Elvehjem glass homogenizer (36). The mixture was centrifuged for 7 min at 500 g to remove unbroken cells, nuclei, and cell debris. For each reaction, 5 mg of 500 g supernatant protein was added to a flask containing 1 mg of 1-[^{14}C]-acetate (1 mCi/mMole) and in pH 7.2 buffer. Final volume of each reaction was 5 ml. The flasks were incubated at 37 C for 3 hr with gentle shaking; the gaseous phase was 100% oxygen (37). After incubation, 2 mg of a carrier mixture of fatty acids (16:0, 16:1, 18:0, and 18:1) was added to each flask. Solid pellets of metaphosphoric acid were added to bring the mixture to pH 2. The material was extracted continuously with ether for 24 hr. The ether extract was evaporated to dryness and placed in a desiccator over KOH. One ml of 10% acetic acid was added to each residue which was again dried in a desiccator over KOH. Each residue was dissolved in absolute ether in a 1 ml volumetric flask. Aliquots were taken for radioassay.

Radioactivity Assays

Sodium 1-[^{14}C]-acetate (specific activity 15 mCi/mMole) was obtained from New England Nuclear Corp., Boston, MA. A Packard Tri-Carb Spectrometer was used for determination of radioactivity. Radioactive assay of the GLC products was by oxidation and subsequent assay in a proportional radioactivity counter (Packard 894). Counting was to ± 3% SD (23,38).

RESULTS AND DISCUSSION

The total lipid compositions are shown in Table I; a major loss of triglycerides is seen to have taken place in patients undergoing a severe caloric restriction. Table I also shows the neu-

TABLE II

% Fatty Acid of Triglycerides[a] (Major Fatty Acids)[b]

Fatty acids	Omentum (under caloric restriction)	Omentum (control)	Adipose (control)
14:0	5.3 ± 0.3	4.0 ± 0.5	3.6 ± 0.7
16:0	28.3 ± 1.4	20.2 ± 2.4	20.7 ± 2.6
18:0	3.7 ± 0.4	7.7 ± 1.3	4.1 ± 1.1
16:1	18.5 ± 0.9	5.0 ± 1.6	7.5 ± 2.1
18:1	42.9 ± 3.9	55.5 ± 4.2	54.5 ± 5.2
18:2	1.0 ± 0.3	9.5 ± 2.1	7.7 ± 1.9

TABLE III

% Triglyceride Structures [Distribution of Acyl Groups Stearate(s), Octadeceneoate(O), and Octadecadienoate (L).] (For Five Specimens) (12-14)

	Omentum (Under caloric restriction)	Mesenteric (control)	Omentum (control)	Adipose (control)
SSS	3.0	2.5	2.1	1.3
SSO	6.5	6.1	6.0	3.9
SOS	30.5	21.1	18.3	17.9
SSL	.6	.7	.9	---
SLS	.6	3.8	3.7	1.5
OOS	33.0	25.0	18.3	27.8
OSO	7.0	7.1	8.3	6.1
LLS	---	.5	.9	.5
LSL	---	.1	.2	---
SOL	---	2.9	3.7	2.4
SLO	---	4.6	4.9	4.7
LSO	---	.8	1.1	.5
LLO	---	.8	1.0	.8
LOL	---	.5	.7	.4
OOL	.5	3.5	5.0	3.6
OLO	.8	5.3	6.9	7.3
OOO	18.1	14.7	18.0	21.7
LLL	---	---	---	---

[a]S = Stearate; O = Octadeceneoate; L = Octadecadienoate.

tral and polar lipids for these two groups of samples of omentum. The patients undergoing severe caloric restrictions (with a 20-30% weight loss) showed a drop from 93 to 70% in their triglyceride content. To balance this, we see a relatively higher percentage of polar material.

It is interesting to note that, in relation to the wet weight, the omentum yielded 40-50% of total lipids while adipose tissue yielded 50-70%. In both cases, the predominant component was triglyceride fat.

In Table II, the percentage fatty acid composition of the triglyceride fraction is shown for the two types of omental tissue assayed and for adipose tissue, used here as a reference. In this table, the main acyl components are shown to be the same in all three samples except that the normal omentum contains a larger concentration of octadecadienoate. The concentration of this acid drops in patients under caloric restrictions while octadecenoate increases.

Table III shows the positional distribution of the three major acyl groups (stearate, octadecenoate, and octadecadienoate) in the triglycerides of adipose tissue, normal omentum, omentum after caloric restrictions, and mesenteric tissue of normal individuals. Dioctadecenoyl-stearoyl triglyceride was found to be the largest component of all tissues. In the normal omentum, large concentrations of trioleate as well as stearoyl-octadecenoyl-stearoyl triglyceride were found. Patients under caloric restrictions showed a different acyl distribution in their triglycerides.

In Table IV, the relationship of saturated and unsaturated fatty acyl groups to their positional distribution in the triglycerides is presented. These assays, as well as those mentioned above, were made by use of pancreatic lipase,

TABLE IV

% Triglyceride Structures [Distribution of Acyl Groups Saturated(S) and Unsaturated (U)] (For Five Specimens) (12-14)

	SSS	SUS	SSU	USU	SUU	UUU
Statistical random distribution (based on analyses of acyl group)	3.3	19.4	7.3	7.3	42.6	23.4
Found in "control" omentum triglycerides	2.1	22.5	6.7	9.4	27.2	32.1
Found in "under caloric restriction" triglycerides	3.0	31.1	6.5	7.0	33.0	19.4
Found in "control" mesenteric triglycerides	2.5	24.9	6.8	8.0	33.0	24.8
Found in "control" adipose tissue triglyceride	2.4	18.9	6.0	4.9	41.0	26.8

TABLE V

Specific Activity of Recovered Mixtures of Fatty Acids Obtained from 1-[^{14}C]-acetate by Homogenates of Tissues (dpm/mgC x 10^{-3})/mg Protein

Tissue	Average of five tests ± SEM	% Protein[a]
Adipose "control"	314 ± 19	8.2 ± 2.3
Omentum "control"	394 ± 17	8.8 ± 2.1
Omentum "under caloric restriction"	489 ± 12	10.3 ± 2.0
Mesenteric "control"	517 ± 21	8.4 ± 2.7

[a]Wet weight of sample.

and the results for distribution were calculated according to Vander Wal's "1,3-Random 2-Random" rule (12-14). As can be seen from the data, the "control" omentum tissue was highest in its content of triglycerides with three unsaturated acyl groups. Patients under caloric restrictions show the greatest reduction of these unsaturated triglycerides. Omentum showed a definite acyl positional distribution, suggesting that the triglyceride composition is somewhat positionally directed. The adipose tissue triglycerides are close to 1,3-random-2-random distribution.

The results of de novo biosynthesis of fatty acids from 1-[^{14}C]-acetate are shown in Table V. The results are reported for equal quantities of protein. The incubation mixtures were not fortified with cofactors, so the results of acetate incorporation more fully represent the in situ situation. As could be expected the omentum from patients under caloric restrictions showed the highest incorporation of 1-[^{14}C]-acetate, approximately 20% more than the omentum preparations from the "control" patients. For comparison, the incorporation of acetate in the adipose tissue of "controls" is shown. This is very close to the value shown by the omentum tissue of "control" patients. All of these results suggest a reverse proportionality of acetate incorporation to the total lipid triglyceride content of every sample.

The patients that underwent dietary restrictions showed a drop in total triglycerides, especially those having unsaturated acyl groups, and a relative increase in phospholipids.

The composition of the polar lipids indicated that phosphatidylcholine was the major phospholipid component. This was followed closely by sphingomyelin. The phosphatidylethanolamine fractions were separated into: (a) O-(acylalk-1-enylglycerophosphoryl) ethanolamine (plasmalogens), (b) diacylglycerophosphoryl ethanolamine (diacylphosphatidylethanolamine).

A very small amount (less than 0.09%) of O-(acylalkylglycerophosphoryl) ethanolamine

was sometimes obtained during these separations; since this amount varied with minor technique changes it was not included in these analysis. It is interesting to note the high content of plasmalogen in the sample, representing a little over 10 % of the total phosphatidylethanolamine fraction.

The acyl composition of the triglycerides shows a higher content of unsaturated groups in the triglycerides of omental tissue than in those of adipose tissues. By the use of pancreatic lipase, the frequency of the different types of acyl groups in triglycerides was determined. The positional distribution of unsaturated acyl group in omental tissue triglycerides was found to be non-random and slightly different from that of other tissues.

The biosynthetic capacity of the tissues indicates their ability to utilize acetate in a fashion inversely proportionaly to their total lipid content.

The obtained data indicate that omental tissues are capable of synthesizing triglyceride with acyl groups arranged in a slightly different pattern than those found in other tissues. This tissue was also very sensitive to dietary manipulations. These factors may contribute to the high success shown by this tissue as a pedicle for grafts and an aid in the vascularization of graft tissue in general restorative and plastic surgery.

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Biosynthesis of Hydrocarbons in Insects: Decarboxylation of Long Chain Acids to *n*-Alkanes in *Periplaneta*

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ABSTRACT

The biosynthetic pathway of *n*-alkanes was investigated in the cockroaches *Periplaneta americana* and *Periplaneta fuliginosa*. Both sodium [1-^{14}C] acetate and randomly tritiated long chain fatty acids were incorporated into the cuticular hydrocarbons of both species. The relative incorporation of acetate into each component of the hydrocarbon fraction was about the same as the relative amount of each component in the fraction. In contrast, [R-^{3}H] hexacosanoic acid was preferentially incorporated into *n*-pentacosane in *P. americana* and [R-^{3}H] tetracosanoic acid into *n*-tricosane in *P. fuliginosa*. Long chain ketones and secondary alcohols, likely intermediates in the proposed condensation-reduction pathway for *n*-alkane biosynthesis, were not incorporated into hydrocarbon. Results from experiments with dual labeled lauric aicd were also not consistent with the condensation-reduction pathway. The demonstration of the direct decarboxylation of long chain fatty acids to *n*-alkanes one carbon unit shorter and the lack of incorporation of proposed intermediates of a condensation-reduction pathway constitute the strongest evidence to date that insects utilize an elongation-decarboxylation pathway for *n*-alkane biosynthesis.

INTRODUCTION

Hydrocarbons are common constituents of the cuticular lipids of plants and insects (1,2) and are also often found in bacteria (3). The biosynthesis of hydrocarbons has received considerable attention in plants and microorganisms, with only a few studies reported using insects as the experimental organism. The two most considered pathways for *n*-alkane biosynthesis are the elongation-decarboxylation pathway (4) and the condensation-reduction pathway (3). Kolattukudy and co-workers (4-6) have presented convincing evidence that in plants *n*-alkanes arise from fatty acid elongation followed by decarboxylation. In bacteria, Albro and Dittmer (7-10) have proposed a modification of the condensation-reduction pathway in which a fatty acid and the aliphatic portion of the alk-1-enyl-ether of a neutral plasmalogen condense head-to-head with the loss of a carboxyl group. The resulting alkene can then be reduced to an alkane (7-10). The work reported in this paper strongly supports an elongation-decarboxylation pathway for *n*-alkane biosynthesis in insects.

MATERIALS AND METHODS

Sodium [1-^{14}C] acetate (58 mCi/mmol) and [1-^{14}C] lauric acid (10 mCi/mmol) were purchased from New England Nuclear, Boston, MA. [R-^{3}H] 12-Tricosanone (abbreviation used: [R-^{3}H], randomly tritiated) and [R-^{3}H] 12-, 11-, 10-, 8-, and 7-tricosanols (specific activities of 50 to 500 mCi/mmol) were prepared by Wilzbach tritiation and were a gift from Dr. Larry L. Jackson, Montana State University, Bozeman, MT. They were purified by thin layer chromatography (TLC) developed in hexane-chloroform (50:50) prior to use. [R-^{3}H]-Hexacosanoic acid (5.3 Ci/mmol), [R-^{3}H]-tetracosanoic acid (4.1 Ci/mmol), and [R-^{3}H] lauric acid (10 mCi/mmol) were prepared by the Wilzbach tritiation of 5 mg of material with 2-4 Ci of tritium gas by ICN Pharmaceuticals, Irvine, CA. The tritiated fatty acids were purified prior to use by repetitive TLC double developed first in hexane, then in hexane-diethyl ether-formic acid (40:10:1). Purity of the isolated samples was verified by analytical TLC in the same solvent system. The methyl esters of the long chain acids were prepared by the method of Schlenk and Gellerman (11). Radio gas liquid chromatography (radio-GLC) of the labeled substrates was accomplished as described below.

Colonies of *P. americana* and *P. fuliginosa* were reared in metal containers on a diet of ground dry dog food and an agar-water (1:99) gel fed ad lib. Middle instar nymphs were used in all studies except where noted.

Labeled substrates in 2 to 10 μl of solvent were injected just beneath the integument between the fifth and sixth abdominal sternae. Labeled acetate was injected in a water solution and the labeled fatty acids, ketone, and secondary alcohols in a Tween 20 emulsion. The in-

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TABLE I

Major Cuticular Hydrocarbons of *P. americana* and *P. fulinginosa* Middle Instar Nymphs[a]

Hydrocarbon	Percent composition	
	P. americana	*P. fuliginosa*
n-Tricosane	--	15
11-Methyltricosane	—	1
3-Methyltricosane	--	14
12-Methyltetracosane	—	1
n-Pentacosane	16	tr
13-Methylpentacosane	—	57
3-Methylpentacosane	13	--
n-Heptacosane	1	7
cis,cis-6,9-Heptacosadiene	70	---
n-Nonacosane	tr	3

[a]Hydrocarbon components were identified by comparison of GLC traces to those reported by Jackson (14,15). In addition, the *n*-alkanes were co-chromatographed with standards and were included in 5 Å molecular sieves. The structures of the branched alkanes were verified by mass spectrometry. Three groups of six to nine insects per group were analyzed for each species.

sects were kept at room temperature and sacrificed after 12 hr except where noted.

Cuticular lipids were extracted by immersion of the insects in two 5 ml portions of hexane for 5 min each. Extracts were combined and taken to about 1 ml under nitrogen. Lipid samples in hexane were transferred to a Pasteur pipet containing ca. 50 mg BioSil A, and hydrocarbon was isolated by elution with 8 ml hexane (12).

Samples were transferred in hexane to counting vials, the solvent removed under nitrogen, and 10 ml of a fluor solution (0.4% PPO in toluene) added. Samples were assayed for radioactivity in a Packard Tri-Carb liquid scintillation counter at 73% efficiency for carbon-14 and 50% efficiency for tritium. All data points are the average of three to six experiments.

The saturated and alkadiene hydrocarbon components of *P. americana* were separated by TLC developed in hexane. Material was visualized with Rhodamine 6G, scraped into scintillation vials, and fluor solution with 4% cabosil was added. The branched and straight chain components were separated by 5Å molecular sieve as described earlier (13).

Radio-GLC was performed on a 6 ft x 1/8 in. 3% SE-30 on Gas Chrom Q column programmed for 180 to 300 C at 4° per min. A 9:1 stream splitter was used, and samples collected in Pasteur pipets. Material was washed into counting vials with 10 ml of fluor solution. Analytical GLC was performed on the same column programmed from 150 C to 300 C at 8° per min. Quantitation was obtained with either disc or electronic integration.

RESULTS

Hydrocarbon Composition

The major cuticular hydrocarbons of adult *P. americana* are *n*-pentacosane (10%), 3-methylpentacosane (16%), and *cis,cis*-6,9-heptacosadiene (70%) (14). A similar hydrocarbon composition was observed in the nymphs used in this study (Table I). The cuticular hydrocarbons of *P. fuliginosa* nymphs are somewhat more complex and are different quantitatively than those from the adult insect (15). In the nymphs used in this study, *n*-alkanes comprise 25% of the hydrocarbons, 3-methylalkanes 14%, and internally branched monomethylalkanes 59% (Table I). *n*-Tricosane is the major *n*-alkane, comprising 15% of the cuticular hydrocarbons of this insect. Several groups of insects from the third through the seventh instars from both species were examined, and little quantitative difference in the hydrocarbon composition among different groups was observed.

Incorporation of Labeled Acetate and Long Chain Acids into Hydrocarbon

Both labeled acetate and long chain fatty acids were incorporated into the cuticular hydrocarbon of *P. americana* and *P. fuliginosa* (Table II). No significant difference was observed in the incorporation of either acetate or the long chain acids into hydrocarbon among the various aged nymphs, and middle instar nymphs were used for all further studies with both species.

The in vivo incorporation of [R-^{3}H] tetracosanoic acid into the hydrocarbon of *P. fuliginosa* showed an increased incorporation of

TABLE II

Incorporation of [1-^{14}C] Acetate, [R-^{3}H] Tetracosanoic Acid, and [R-^{3}H] Hexacosanoic Acid into the Cuticular Hydrocarbons of *P. fuliginosa* and *P. americana*[a]

Experimental organism	Percent incorporated into hydrocarbon		
	Na acetate	Tetracosanoic acid	Hexacosanoic acid
P. fuliginosa			
Early instar nymphs	0.93 ± 0.12	0.20 ± 0.10	---
Middle instar nymphs	0.97 ± 0.52	0.34 ± 0.14	---
Late instar nymphs	0.77 ± 0.30	0.35 ± 0.20	---
Adults	0.42 ± 0.04	0.20 ± 0.04	---
P. americana			
Early instar nymphs	0.67 ± 0.14	---	0.32 ± 0.03
Late instar nymphs	0.78 ± 0.06	---	0.36 ± 0.06

[a]Each value represents the mean ± SD of three experimental groups. Three to six insects used in each experimental group.

label for at least 24 hr (Fig. 1). Similar data were obtained for the incorporation of [R-^{3}H] hexacosanoic acid into the hydrocarbon of *P. americana*. At longer time periods, the variance in incorporation among different groups increased considerably, and the amount of label incorporated into hydrocarbon leveled off and even decreased slightly in some experiments.

Incorporation of Hexacosanic Acid into *n*-Pentacosane

Separation of the saturated and unsaturated hydrocarbons from *P. americana* by TLC showed that the label from [1-^{14}C] acetate was distributed about equally between both classes, with 54 ± 7% of the label in the alkane fraction and 46 ± 7% in the alkadiene fraction. In contrast, 91 ±3% of the label from [R-^{3}H] hexacosanoic acid that was incorporated into hydrocarbon was found in the alkane fraction, which is comprised primarily of *n*-pentacosane and 3-methylpentacosane. Separation of the methyl branched hydrocarbons from the normal hydrocarbons by 5 Å molecular sieves showed that about three-fourths of the label from [1-^{14}C]-acetate that was incorporated into hydrocarbon was present in the straight chain components. In contrast, over 90% of the label from [R-^{3}H]hexacosanoic acid that was incorporated into hydrocarbon was present in the straight chain fraction, which is comprised primarily of *n*-pentacosane and *cis,cis*-6,9-heptacosadiene.

The selective incorporation of [R-^{3}H] hexacosanoic acid into hydrocarbon fractions that contained *n*-pentacosane prompted a closer examination of the labeled hydrocarbons resulting from the incorporation of [1-^{14}C]-acetate and [R-^{3}H] hexacosanoic acid. The results of radio-GLC showed that labeled acetate

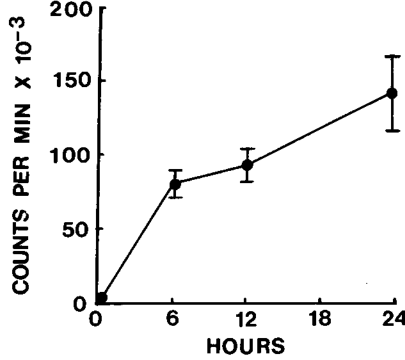

FIG. 1. In vivo incorporation of [R-^{3}H] tetracosanoic acid into cuticular hydrocarbon of *P. fuliginosa* with time. Eighteen μCi of [R-^{3}H] tetracosanoic acid were used in each experiment. Error bars represent the SD of three experimental groups consisting of six insects per group.

was incorporated into each hydrocarbon component in about the same proportion as the percentage composition of hydrocarbon (Fig. 2). [R-^{3}H] Hexacosanoic acid, however, was incorporated almost exclusively into *n*-pentacosane (Fig. 3).

Incorporation of Tetracosanoic Acid into *n*-Tricosane

Separation of the branched and normal alkanes from *P. fuliginosa* after the incorporation of labeled acetate showed that 34 ± 3% of the label that was incorporated into hydrocarbon was found in the *n*-alkane fraction, which comprises 25% of the cuticular hydrocarbons. In contrast, 64 ± 3% of the label from [R-^{3}H]-tetracosanoic acid that was incorporated into hydrocarbon was found in the normal alkanes.

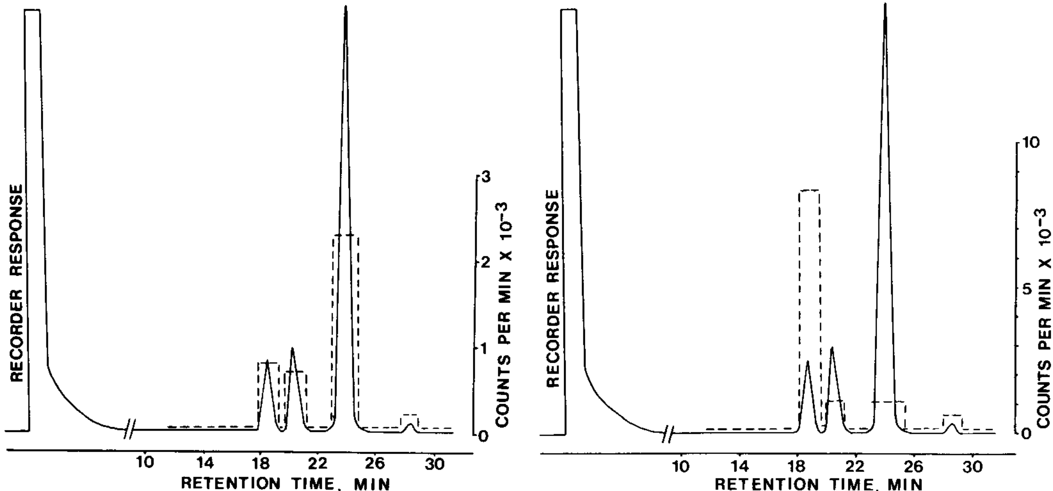

FIG. 2. Radio-GLC of the hydrocarbon fraction isolated after injection of sodium [1-^{14}C]acetate into *P. americana* nymphs. Hydrocarbon components are (A) *n*-pentacosane; (B) 3-methylpentacosane; (C) *cis,cis*-6,9-heptacosadiene; and (D) *n*-nonacosane. GLC was performed on a 6 ft x 1/8 in. 3% SE-30 column programmed from 180 C to 300 C at 4° per min. The solid line is the GLC trace and the dashed line represents recovered radioactivity.

FIG. 3. Radio-GLC of the hydrocarbon fraction isolated after injection of [R-^{3}H]hexacosanoic acid into *P. americana* nymphs. Hydrocarbon components are (A) *n*-pentacosane; (B) 3-methylpentacosane; (C) *cis,cis*-6,9-heptacosadiene; and (D) *n*-nonacosane. GLC was performed on a 6 ft x 1/8 in. 3% SE-30 column programmed from 180 C to 300 C at 4° per min. The solid line is the GLC trace and the dashed line represents recovered radioactivity.

Radio-GLC of the hydrocarbons from *P. fuliginosa* after the incorporation of [1-^{14}C]-acetate showed that the label was distributed in each hydrocarbon component in about the same proportion as the relative composition of each component (12). In contrast, [R-^{3}H]-tetracosanoic acid was preferentially incorporated into *n*-tricosane. Forty percent of the radioactivity from [R-^{3}H]tetracosanoic acid that was incorporated into hydrocarbon was recovered in the fraction corresponding to *n*-tricosane, compared to 18% in 3-methyltricosane, 3% in 12-methyltetracosane, 21% in 13-methylpentacosane, 12% in *n*-heptacosane, and 5% in *n*-nonacosane.

Experiments to Test the Condensation-Reduction Pathway

If *n*-tricosane was formed by a pathway which involved the condensation of two molecules of lauric acid with the loss of a carboxyl group, a decrease to about one-half in the carbon-14/tritium ratio of hydrocarbon formed from dual labeled [1-^{14}C] and [R-^{3}H]lauric acid would be expected. However, when lauric acid with a carbon-14/tritium ratio of 1.0 was injected into *P. fuliginosa*, the hydrocarbon isolated after 6, 12, or 24 hr had a carbon-14/tritium ratio of 2.1 to 2.4 (Fig. 4). Radio-GLC of the hydrocarbon fraction showed that the *n*-tricosane had essentially the same carbon-14/tritium ratio as did the other hydrocarbon components.

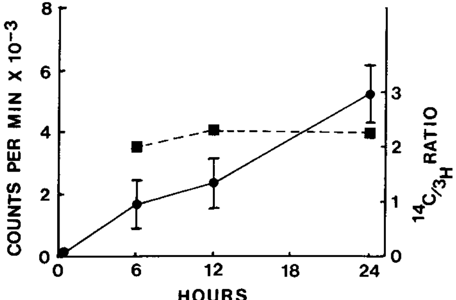

FIG. 4. Incorporation of dual labeled [1-^{14}C] and [R-^{3}H]lauric acid into the cuticular hydrocarbon of *P. fuliginosa* with time. The solid line represents the incorporation of carbon-14 with time, and the dashed line the carbon-14/tritium ratio. Error bars indicate the SD of three experimental groups consisting of three to six insects per group.

The increased carbon-14/tritium ratio observed in the hydrocarbon fraction compared to the dual labeled substrate could be due to the β-oxidation of lauric acid to acetate, in which much of the tritium would be lost, and the subsequent incorporation of acetate into hydrocarbon.

To further explore the possibility that a condensation-reduction pathway might be

operative, likely intermediates of this pathway were obtianed, and their incorporation into the hydrocarbons of *P. fuliginosa* was studied. The results showed that neither tritium labeled 12-tricosanone nor symmetrical or unsymmetrical secondary alcohols served as efficient precursors to hydrocarbon (Table III). The unsymmetrical secondary alcohols were used to detect the possiblility of two nonidentical fatty acids (C_{10} + C_{14} or C_8 + C_{16}) condensing to yield unsymmetrical ketones and secondary alcohols (10- or 8-tricosanol) which could then be reduced to hydrocarbon. No such reduction was detected.

TABLE III

Incorporation of Labeled 12-Tricosanone and 12-, 11-, 10-, 8-, and 7-Tricosanols into Cuticular Hydrocarbons of *P. fuliginosa*[a]

Substrate	Percent incorporated
[R-^{3}H] 12-tricosanone	<0.001
[R-^{3}H] 12-tricosanol	<0.001
[R-^{3}H] 11-tricosanol	0.004
[R-^{3}H] 10-tricosanol	0.080
[R-^{3}H] 8-tricosanol	<0.001
[R-^{3}H] 7-tricosanol	0.004

[a]Each value represents the mean of three experimental groups. Each group contained six insects.

DISCUSSION

The results presented in this paper showing the direct decarboxylation of hexacosanoic acid to n-pentacosane and of tetracosanoic acid to n-tricosane constitute the first direct evidence that hydrocarbon biosynthesis occurs via an elongation-decarboxylation pathway in insects. This conclusion is in agreement with four lines of indirect evidence that have been reported using insects as the experimental organism: (a) structural relationships between n-alkanes and primary alcohols (2,16) indicate that both arise from common, very long chain acid precursors; (b) the structures of the secondary alcohols in grasshoppers (16,17), in which hydroxyl groups are found on both odd and even numbered carbons, suggest that they do not arise from a condensation-reduction pathway; (c) the nonconversion of labeled secondary alcohols and ketones to alkanes (18); and (d) the unidirectional metabolism of n-alkanes to secondary alcohols (18,19). The results of this study also support the proposed pathways for the biosynthesis of the 2-methylalkanes (20), 3-methylalkanes (13,21), and the internally branched monomethylalkanes (21) in insects, in which it is proposed that the branching methyl groups are incorporated during chain elongation prior to decarboxylation.

The lack of incorporation of 12-tricosanone and 12-, 11-, 10-, 8-, and 7-tricosanols into hydrocarbon, along with the data indicating that the carboxyl carbon of lauric acid was not lost during incorporation into n-tricosane, strongly suggest that the classical condensation-reduction pathways does not contribute to n-alkane formation in *P. fuliginosa*. In addition, if a condensation-reduction pathway was responsible for n-alkane biosynthesis in *P. americana* in which fatty acids condensed and were then reduced to hydrocarbon, it would require the condensation of two 13 carbon acids followed by reduction to yield n-pentacosane. Although α-oxidation systems have not been extensively studied in insects, large amounts of odd chain fatty acids are not common to insect cuticular lipids (2).

The evidence presented in this paper suggests that alkane biosynthesis in insects is similar to that reported for plants. Studies in plants have demonstrated that labeled C_2 to C_{24} acids were incorporated into very long chain fatty acids, and that acids of C_{24} and C_{32} were decarboxylated to n-alkanes one carbon unit shorter (4). Kolattukudy (22) has proposed an elongation-decarboxylation complex in plants which elongates 16 carbon acids to 30 and 32 carbon acids which are then decarboxylated to C_{29} and C_{31} alkanes. Plants often contain very long chain fatty acids corresponding in chain length to the major alkanes. Kolattukudy has suggested that these very long chain fatty acids are released from the elongation-decarboxylation system, and are then incorporated into fatty aldehydes, fatty alcohols, wax esters, and polar lipids (4).

In contrast to the situation in plants, very long chain fatty acids with carbon numbers in the same range as the usual cuticular hydrocarbons are not found in most insects (2). In an examination of the fatty acids of *P. americana*, no such very long chain acids were observed in either the cuticular lipids, epidermal tissue, or fat body tissue (Blomquist and Major, unpublished results). This suggests that an efficient decarboxylation system is present, and that the relatively low metabolic rate for exogenous long chain acids may be due to their difficulty in reaching the enzyme system because of their insolubility, or a tight coupling between the elongation and decarboxylation systems, perhaps involving bound intermediates.

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The Acylation of 1-Palmitylglycerol 3-Phosphate with *Cis* and *Trans* C-16 to C-22 Monoenoic Fatty Acids in Rat Liver Microsomes

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ABSTRACT

The configurational specificity of acyl-CoA:1-palmitylglycerol 3-phosphate acyltransferase from rat liver microsomes was investigated with *cis* and *trans* C-16, C-18, C-20, and C-22 monoenoic and saturated fatty acyl-CoA. Oleyl-CoA was transferred three times more readily than elaidyl-CoA. Elaidyl-CoA was more inhibitory than oleyl-CoA, especially at low protein concentrations, but did not show this effect after the addition of 1 mg/ml bovine serum albumin (BSA). BSA permitted linearity of the acyltransferase over a wide range of protein concentrations and did not seem to affect the configurational specificity of the acyltransferase. The specificity of the enzyme preparation was in the following decreasing order: 18:1 *cis* > 16:1 *cis* ≃ 16:0 > 18:0 ≃ 16:1 *trans* > 18:1 *trans* > 20:1 *cis* > 20:1 *trans*. The enzyme preparation did not react with *cis* or *trans* 22:1 acyl-CoA.

INTRODUCTION

Trans fatty acids are found in small concentrations in dairy products (1,2), but most of them in the diet arise from the hydrogenation of fat. For example, *trans* fatty acids may constitute as much as 60% of the total unsaturated fatty acids found in partially hydrogenated fat (3). The increasing consumption of margarines, shortenings, cooking and salad oils which have been subjected to partial hydrogenation has stimulated investigations of the nutritional and biological effects of these unnatural fatty acids.

In vivo, *trans* fatty acids have been found to be depositied mainly in external positions of tissue triglycerides and phospholipids (4-9). They were, therefore, thought to replace saturated fatty acids in membranes. Since acyltransferases may control the specific positioning of fatty acids in lipids, studies were undertaken by Lands and colleagues (10-12) to see if these enzymes could discriminate between *cis* and *trans* isomers. The enzymes studied by these authors were those from the deacylation-reacylation cycle, with 1- and 2-acylglycerol 3-phosphorylcholine (1- and 2-acyl-GPC) as lipid acceptors (11). At position 2 of the 1-acyl-GPC, the enzyme did not distinguish between oleyl- and elaidyl-CoA, but it did between elaidyl- and stearyl-CoA. In contrast, at position 1 of the 2-acyl-GPC, oleyl-CoA was almost excluded, whereas elaidyl-CoA as well as stearyl-CoA were readily acylated. In the present investigation, the configurational specificity of 1-palmitylglycerol 3-phosphate (PGP) acyltransferase(s) from rat liver microsomes has been studied with some saturated and *cis* and *trans* monoenoic C-16 to C-22 acyl-CoA substrates.

MATERIALS AND METHODS

1-Palmitylglycerol 3-phosphate and elaidic acid (>99%) were the products of Serdary Research Laboratories Inc., London, Ontario. CoA, oleyl-CoA, stearyl-CoA, and palmityl-CoA were purchased from P-L Biochemicals, Inc. (Milwaukee, WI). Palmitoleyl-CoA, palmitelaidyl-CoA, *cis* 11-eicosenoyl-CoA, *trans* 11-eicosenoyl-CoA, *cis* 13-docosenoyl-CoA, and *trans* 13-docosenoyl-CoA were prepared for another study (13).

Elaidyl-CoA was synthesized from pure elaidic acid and CoA according to the method of Reitz et al. (14) which is a modification of the Seubert (15) procedure.

Albumin, fatty acid free, was purchased from Sigma Chemical Co. (St. Louis, MO) and DTNB, from Aldrich Chemical Co. (Milwaukee, WI).

Preparation of Microsomes

Rat liver microsomes were prepared according to modification of Barden and Cleland (16). Male adult Sprague-Dawley rats were killed by decapitation, and the livers were excised and perfused in cold 0.9% NaCl containing 1 mM EDTA. They were then cut into small pieces and homogenized in 3.5 volumes of 0.25 M sucrose, 1 mM Tris pH 7.4, and 10 mM EDTA with a Potter-Elvehjem apparatus. The particles sedimenting between 15,00 x g (15 min) and 48,000 x g (90 min) were suspended in 20 mM Tris, 1 mM EDTA, kept at 0-5 C, and used within 5 days. In some cases, the microsomes were quickly frozen in an acetone-dry ice mixture and kept for weeks without significant loss of activity.

Measurements of Enzyme Activity

The spectrophotometric assay of Lands and

TABLE I

Acyltransferase Activity with Different Concentrations of Acyl-CoA Substrates

Acyl CoA (μM)	Acyltransferase activity (mμ moles/min/mg protein) 16:0	18:0	18:1 *cis*	18:1 *trans*
2.5	31	17	56	22
5	36	22	58	23
10	39	26	61	24
15	36	25	60	22
20	37	24	60	21
25	37	18	—	—
30	29	13	61	9
40	31	—	54	0

TABLE II

Concentration Effects of Acyl-CoA on Acyltransferase Activity at Low Microsomal Protein Concentration, with or without Albumin

Acyl CoA (μM)	Albumin	Acyltransferase activity (mμ moles/min/mg protein) 18:1 *cis*	18:1 *trans*
2.5	–	34	13
5	–	50	18
10	–	53	12
15	–	55	9
20	–	47	6
30	–	24	0
40	–	9	0
2.5	+	38	15
5	+	52	21
10	+	59	20
15	+	58	21
20	+	59	23
30	+	60	23
40	+	59	23

Hart (17) using DTNB to measure the release of CoA SH from fatty acyl CoA was conducted at 30 C with a Gilford multiple sample absorbance recorder. Unless specifically indicated, the assay contained in a total volume of 0.4 ml: 72 mM Tris-HCl buffer pH 7.4, 1 mM DTNB, 0.17 mg/ml of microsomal protein, 60 μm palmityl-glycerol 3-phosphate, and 10 μM acyl-CoA. The reaction was started with the addition of acyl-CoA after 1 min preincubation of other components. A control was also run without the lipid acceptor. The reaction rates were measured, and the control, representing the hydrolase reaction, was subtracted to give the net acyltransferase reaction. The hydrolase reaction was usually no more than 10% of the acyltransferase reaction. Protein content was estimated according to Lowry et al. (18).

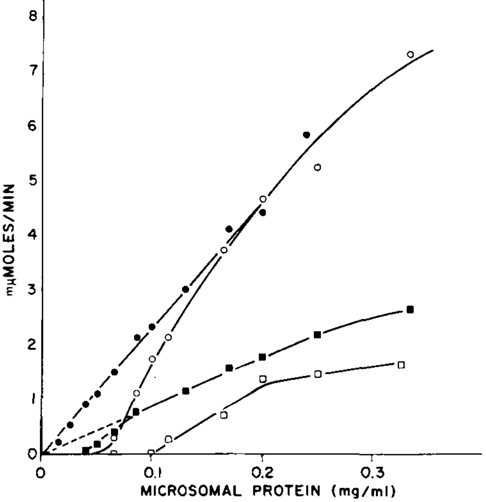

FIG. 1. Protein concentration curves. Standard incubation conditions have been used except that microsomal protein has been added as indicated in the figure and acyl-CoA as following: •, oleyl-CoA 10 μM; ○, oleyl-CoA 20 μM; ■, elaidyl-CoA 10 μM; □, elaidyl-CoA 20 μM.

RESULTS

Substrate Concentration Curves

At a protein concentration of 0.17 mg/ml, all four acyl-CoA tested gave a maximum velocity over a short concentration range (see Table I). Elaidyl- and stearyl-CoA exhibited similar behavior and were inhibitory at relatively low concentrations.

From maximal velocities, it can be seen that oleyl-CoA was acylated about three times faster than elaidyl- or stearyl-CoA. Palmityl-CoA gave an intermediate value.

Preliminary results with 0.1 mg/ml protein and 25 μM acyl-CoA gave more than six-fold activity with oleyl- than with elaidyl-CoA. An investigation of the enzyme velocity with varying oleyl- and elaidyl-CoA concentrations (Table II) revealed that maximum velocity without albumin was not obtained with elaidyl-CoA at a protein concentration of 0.1 mg/ml and that 25 μM acyl-CoA was inhibitory in both cases, particularly elaidyl-CoA. These adverse effects were not seen with 1 mg/ml albumin in the incubation system. In subsequent experiments, the protein was either adjusted to 0.17 mg/ml and the acyl-CoA to 10 μM or albumin was added.

Linearity of Acyltransferase Reaction with Protein

Results from Tables I and II were indicative

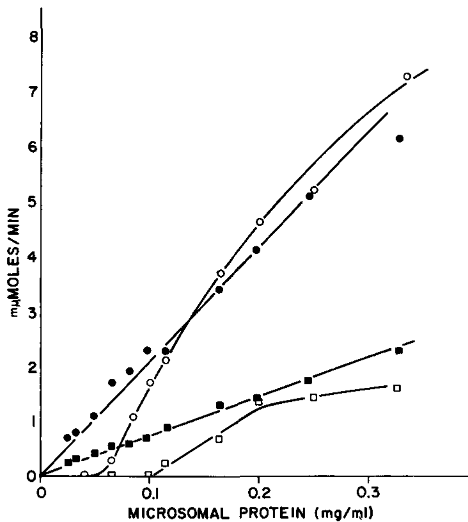

FIG. 2. Effect of albumin on protein concentration curves. Standard incubation conditions have been used except that microsomal protein has been added as indicated in the figure. When indicated, albumin was present at the concentration of 1 mg/ml. •, oleyl-CoA 20 μM with albumin; ○, oleyl-CoA 20 μM without albumin; ■, elaidyl-CoA 20 μM with albumin; □, elaidyl-CoA 20 μM without albumin.

of a nonlinear relationship between acyltransferase activity and protein concentration at high acyl-CoA concentrations. We then determined the effect of protein concentration at two levels of oleyl- and elaidyl-CoA. The results are shown in Figure 1. At 10 μM oleyl-CoA, the acyl transfer seemed to be linear with protein concentration up to about 0.25 mg/ml. At the same concentration, elaidyl-CoA gave a sigmoid curve with a linear region between 0.1 and 0.25 mg/ml. At 20 μM, both acyl-CoA substrates gave sigmoid curves, especially elaidyl-CoA. Here again, the inhibition was more severe with elaidyl-CoA.

With the addition of 1 mg/ml albumin in our system, we observed a straightening out of the protein curves with both oleyl- and elaidyl-CoA at 20 μM (see Fig. 2). The result was especially convincing with elaidyl-CoA and showed that albumin was very efficient in binding inhibitory elaidyl-CoA micelles.

On the other end, albumin did not seem to alter the maximum velocity of either acyl-CoA at saturating concentrations (see Table II). Oleyl-CoA was still acylated about three times faster than elaidyl-CoA.

Effect of Palmitylglycerol 3-Phosphate

In all the experiments previously cited, palmitylglycerol 3-phosphate was used at a concentration of 60 μM Table III shows that this concentration was sufficient for saturation with both oleyl- and elaidyl-CoA at 10 μM.

TABLE III

Effect of Palmitylglycerol 3-Phosphate on Acyltransferase Activity

1-Palmitylglycerol 3-phosphate (μm)	Acyltransferase activity 18:1 *cis*	18:1 *trans*
	(mμ moles/min/mg protein)	
10	30	12
15	44	15
20	50	16
40	57	17
60	60	20
80	61	14
100	57	17

TABLE IV

Acyltransferase Relative Activity with Some Saturated and *cis* and *trans* Monounsaturated Acyl-CoA

Acyl-CoA	Acyltransferase relative activity[a]
16:0	70.7 ± 1.3 (6)
16:1 *cis*	75.7 ± 1.6 (7)
16:1 *trans*	44.9 ± 1.6 (7)
18:0	45.0 ± 2.1 (7)
18:1 *cis*	100
18:1 *trans*	32.0 ± 0.9 (11)
20:1 *cis*	12.5 ± 0.8 (4)
20:1 *trans*	5.3 ± 0.3 (4)
22:1 *cis*	0
22:1 *trans*	0

[a]The absolute activity for each acyl-CoA varied significantly with different batches of microsomes, but the variation of the relative activity was small enough to allow useful comparison among various acyl-CoA. Figures presented are means of relative activity ± standard error of the mean. The number of batches of microsomes used are in parentheses.

Specificity with Saturated and *cis* and *trans* Monounsaturated Acyl-CoA

Under optimal conditions (as determined with palmityl-, stearyl-, oleyl-, and elaidyl-CoA), we compared the initial velocity of 1-palmitylglycerol 3-phosphate acyltransferase(s) with some saturated and *cis* and *trans* monounsaturated C-16 to C-22 acyl-CoA (Table IV). The enzyme seemed to prefer oleyl-CoA to palmitoleyl- and palmityl-CoA, both of which were acylated at about the same rate. Stearyl-CoA and palmitelaidyl-CoA gave similar activity, followed by elaidyl-CoA which was

acylated at about one-third the rate of oleyl-CoA. The *cis* and *trans* 11-eicosenoyl-CoA were much less active, and the *cis* and *trans* 13-docosenoyl-CoA were unreactive under our conditions.

DISCUSSION

The inhibition of 1-acylglycerol 3-phosphate acyltransferase(s) with moderate acyl-CoA concentrations has been previously reported (17,19). The degree of inhibition observed varied with the type of acyl-CoA, being more pronounced with saturated (stearyl-CoA) than with unsaturated (oleyl- and linoleyl-CoA) (17) and correlating with the critical micelle concentration of the acyl-CoA (16). In the present study, elaidyl-CoA has been shown to cause an inhibition, the intensity and behavior of which were similar to that of stearyl-CoA. This inhibition with elaidyl-CoA has also been shown to be relieved by bovine serum albumin and thus is not likely to be due to a specific enzyme-substrate interaction but more likely to surfactant properties as previously shown with common acyl-CoA (16,17,19).

The susceptibility of this enzyme to inhibition by acyl-CoA concentrations led Lands and Hart (17) to suggest that the level of acyl-CoA could play a role in the specificity of 1-acylglycerol 3-phosphate acyltransferase(s) in vivo. In this respect, our study suggests that elaidyl-CoA as well as stearyl-CoA acylation at position 2 could be depressed by high concentrations of these thioesters. Moreover, we observed that elaidyl-CoA at noninhibiting concentrations could also impair or compete with oleyl-CoA acylation in vitro since a lower total activity was obtained with both acyl-CoA substrates together than with the sum of individual activities (unpublished results).

Our results indicate that the 1-acylglycerol 3-phosphate acyltransferase(s) is sensitive to the configuration of the double bond and thus to the shape of the molecule since the acyltransferase velocity with elaidyl-CoA is three times less than with oleyl-CoA and, in that respect resembles stearyl-CoA. This contrasts with 1-acyl-GPC acyltransferase(s) but resembles 2-acyl-GPC acyltransferase(s) (10,11). However, the enzyme does not equate the *trans* fatty acyl-CoA to a saturated acyl-CoA since the relative activity with stearyl-CoA, although in the same range, is significantly higher than with elaidyl-CoA. Moreover, in keeping with the observation of Hill and Lands (19), the chain length is also important in conjunction with the double bond configuration. It was apparent that more enzymic discrimination occurred between the *cis* and *trans* monoenoic substrates of C-18 than of C-16 chain lengths; likewise, between the *cis* monoenoic and saturated substrates.

These observations suggest that the most suitable substrate for 1-acylglycerol 3-phosphate acyltransferase(s) is a symmetrical *cis*-9 configuration of 18 carbons chain length. A lower saturated chain length such as palmitic acid might partially fit the enzymic structure. Palmitelaidic acid might also fit but to a lesser extent because the *trans* double bond is likely to impair flexibility. Higher chain lengths than 18, even with a *cis* configuration, could hardly fit that structure because of linear steric hindrance.

Recently, Sgoutas et al. (20) found that various isomeric *cis*-octadecenoic acids were preferentially incorporated at position 2 of triglycerides and phosphatidylcholines when the double bond was close to n-9, and the molecule presented a symmetrical shape. When the double bond was near either end or when it was elaidic acid, the incorporation was predominantly at position 1.

The failure to obtain acylation with either isomer of docosenoic acid is consistent with previous results (19) and agrees with the low level of incorporation of these fatty acids into tissue phospholipids (21).

The present findings demonstrate that the configuration of the fatty acid substrate may be the major factor of specificity of 1-palmitylglycerol 3-phosphate acyltransferase(s). This is also consistent with in vivo results of *trans* fatty acids mainly acylated at position 1 of the phosphoglycerides.

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Plasma Membrane Phospholipid, Cholesterol and Fatty Acyl Composition of Differentiated and Undifferentiated L_6 Myoblasts

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ABSTRACT

The lipid composition of plasma membranes isolated from differentialted and undifferentiated L_6 myoblasts has been compared. In general, the plasma membranes of differentiated L_6 myoblasts have a higher cholesterol to phospholipid molar ratio than plasma membranes of undifferentiated cells. Differentiated L_6 myoblasts have increased relative amounts of phosphatidylethanolamine and phosphatidylcholine in their plasma membrane and a decreased relative amount of sphingomyelin when compared with the plasma membranes of undifferentiated myoblasts. In addition, preliminary results show that differentiated L_6 myoblasts plasma membrane phospholipid shows differences in the fatty acyl composition, specifically there appears to be relatively more 17:0 and 24:1 and less 16:1 and 18:1 than in plasma membrane phospholipids of undifferentiated L_6 myoblasts. These observations indicate that significant changes in plasma membrane lipid composition occur during myoblast differentiation. The role that changes in lipid composition play in control of cellular differentiation, however, remains to be elucidated.

INTRODUCTION

L_6 myoblasts serve as an in vitro model to study the control of cell differentiation (1,2). Postconfluent L_6 myoblasts show morphological and enzymatic evidence of cell differentiation. Muscle specific enzymes are induced (1,3,4), muscle specific proteins are synthesized (1,5-7), and myoblasts fuse to form myotubules (1,7,8). Myoblast differentiation can be promoted by factors that have plasma membrane receptors, such as insulin (9), and it has been suggested that modulation in plasma membrane structure and function may act as a signal to initial the differentiation process (9). Studies originally suggested that plasma membrane fusion which results in myotubule formation served as this signal, but more recent studies do not support this hypothesis (10,11). It appears more likely that the plasma membrane may influence cell differentiation by more subtle changes in membrane organization and function.

Since previous studies have shown a correlation between changes in plasma membrane lipid composition, cell *transformation*, and growth control (12-13), we have attempted to determine whether changes in plasma membrane lipid composition also occur during cell differentiation. Our results show that plasma membranes isoalted from differentiated L_6 myoblasts show differences in the cholesterol/phospholipid ratio, in the relative phospholipid composition, and in phospholipid-fatty acyl composition compared to the plasma membranes of undifferentiated L_6 myoblasts.

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MATERIALS AND METHODS

Cell Lines

The L_6 myoblast cell line (kindly supplied by Dr. David Shubert) used in these experiments were undifferentiated when grown to low cell densities and showed optimum morphological differentiation following culture for 14 days in a postconfluent state without medium change. Greater than 50% of differentiated myoblasts fused to form myotubes. Differentiated cultures also showed an increase in intracellular creatinine phosphokinase (CPK) levels. Undifferentiated (75% confluenct) L_6 myoblasts have a CPK activity of approximately 200 mU/mg cell protein, while differentiated (100% confluence for 14 days) L_6 myoblasts showed a CPK activity of 2500 mU/mg cell protein. CPK activity was determined using the CPK STAT PAC assay (Calbiochem, La Jolla, CA).

L_6 myoblasts were grown in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum. Stock cultures of L_6 myoblasts were maintained at low density, i.e., less than 60% confluent. For experiments described in this study, L_6 myoblasts were passed into 490 cm^2 roller bottles (Corning, NY) containing 35 ml of medium which had been pulsed with CO_2. For each experiment, plasma membranes were isolated from 15 roller bottles of undifferentiated (75% confluent) or differentiated (100% confluent for 14 days) L_6 myoblasts.

The L_6 myoblast cell lines used in these studies were found to be free of mycoplasma contamination by periodic cultural analysis (Flow Laboratories, Rockville, MD) and by transmission and scanning electron microscopy.

Plasma Membrane Isolation

The tissue culture medium was decanted, and the cell monolayers were washed three times in 30 ml of isotonic phosphate buffered saline (pH 7.4) containing 0.75 mM calcium and 0.5 mM magnesium (CMPBS). Cell monolayers were then induced to shed plasma membrane vesicles by exposure to 35 ml of the vesiculant solution containing 25 mM formaldehyde and 2 w M dithiothreitol prepared in CMPBS as recently described (14). After incubation for 2 hr at 37 C, the vesiculant solution containing plasma membrane vesicles was decanted, and the plasma membrane vesicles were sedimented by centrifugation at 30,000 x g for 30 min at 4 C. The resulting translucent pellet was washed three times in 30 ml of CMPBS. After each wash, the membranes were gently resuspended leaving any whole cell contamination as a dense white aggregate at the bottom of the tube. The final translucent plasma membrane pellet was examined by phase microscopy to assure that it was free of intact cells. This pellet, resuspended in distilled water and brought to a known volume, was designated the aqueous plasma membrane suspension. It was shown to represent a purified plasma membrane preparation by marker enzyme assays. The results of these studies established that L_6 myoblast plasma membrane vesicles show an 8.5-fold enrichment in 5′ nucleotidase activity, a plasma membrane marker enzyme, and no detectable endoplasmic reticulum-NADH cytochrome C reductase activity and no mitochondrial-succinic dehydrogenase activity (Scott, submitted for publication).

Protein determinations were done by the Lowry procedure (15) or fluorescamine method (16) using bovine serum albumin as a standard. All protein determinations used sodium dodecyl sulfate solubilization (17).

Lipid Analysis

Extraction of lipid from aliquots of the aqueous plasma membrane suspension was performed with chloroform-methanol (18). The chloroform and methanol used was distilled-in-glass grade (Burdick & Jackson Laboratories, Inc., Muskegon, MI). The chloroform-methanol extracted material was assayed for total cholesterol (19) and total lipid phosphorus (20). Phospholipids were separated on Silica Gel 60 plates (E. Merck, Darmstadt, Germany) by two dimensional thin layer chromatography (TLC) (21). When phospholipid classes were to be quantitated (20), the lipids were visualized by placing the plate in I_2 vapor (22). The total phospholipid fraction was isolated by one dimensional TLC and visualized under a UV light after spraying the plate with 2′7′ dichlorofluorescein in methanol (21). Plasma membrane phospholipid fatty acid methyl esters, prepared as previously described (21), were analyzed by gas liquid chromatography (GLC) on a 6 ft x 2 mm ID glass column packed with 10% SP2330 on 100/120 chromosorb WAW (Supelco, Inc., Bellefonte, PA). Fatty acids were identified by comparison of retention times with those of commercially available fatty acid methyl ester standard mixtures (NuCheck Prep, Elysian, MN) by using a Hewlett Packard Digital Integrator Model 3380A interfaced to the electrometer of a Beckman gas chromatograph Model GC-65. All analyses were performed in triplicate on two separate plasma membrane preparations except for fatty acyl analysis. For these studies, duplicate analyses were performed.

All lipid analyses were performed on extracts of purified intact L_6 plasma membrane vesicles. However, since intact vesicles contain soluble cytosol components not associated with the plasma membrane, it was first necessary to establish that the soluble fraction was essentially lipid free. We, therefore, compared the lipid composition of intact plasma membrane vesicles and purified plasma membrane fragments obtained from intact vesicles by disruption of intact vesicles by hypotonic lysis and nitrogen decompression (250 psi for 5 min) with resedimentation at 250,000 x g for 1 hr at 4 C. The results show that the 250,000 x g purified plasma membrane fragments contain greater than 97% of the vesicle lipid phosphorous and greater than 91% of the vesicle total choelsterol. The soluble fraction is, therefore, relatively lipid depleted.

RESULTS AND DISCUSSION

Significant differences in the cholesterol and phospholipid composition were identified in plasma membranes isolated from differentiated and undifferentiated L_6 myoblasts. In particular, the plasma membranes of differentiated L_6 myoblasts had a choelsterol/phospholipid ratio of 1.04 ± 0.08, whereas the ratio in membranes isolated from undifferentiated L_6 was significantly lower ($P > 0.005$, i.e., 0.733 ± 0.018.

Differences in the phospholipid content

TABLE I

Phospholipid Composition of Differentiated and Undifferentiated L_6 Myoblast Plasma Membranes

	Undifferentiated L_6 Myoblast	Differentiated L_6 Myoblast
Phosphatidylethanolamine	15.0 ± 1.4	19.6 ± 1.2[a]
Phosphatidylcholine	42.2 ± 1.8	48.4 ± 0.8[a]
Phosphatidylserine	11.1 ± 1.6	9.6 ± 1.6
Phosphatidylinositol	4.1 ± 1.3	2.6 ± 1.0
Sphingomyelin	27.6 ± 2.0	19.8 ± 2.7[a]

[a]Values expressed as mole percent; value indicated (a) show statistically significant differences between undifferentiated and differentiated myoblasts with P > .005.

TABLE II

Differences in the Fatty Acyl Composition of Differentiated and Undifferentiated L_6 Myoblast Plasma Membrane Phospholipids

	Undifferentiated	Differentiated
14:0	2.2	0.9
15:0	2.9	2.9
16:0	33.7	29.2
16:1	9.2	1.8
17:0	TR	6.1
18:0	10.8	14.1
18:1	24.4	18.7
18:2	3.2	TR
18:3	2.7	TR
$20:3^{5,8,11}$	TR	4.7
$20:3^{8,11,14}$	TR	2.3
20:4	1.2	2.8
22:5	TR	TR
22:6	3.5	TR
24:0	3.4	3.7
24:1	2.6	7.7

between differentiated and undifferentiated cells was also apparent in an analysis of the relative composition of individual classes of phospholipids in the plasma membrane (Table I). Differentiated L_6 myoblasts contain relatively more phosphatidylethanolamine and phosphatidylcholine and relatively less sphingomyelin than undifferentiated L_6 myoblasts.

Experiments were also performed to determine whether there are differences in the phospholipid fatty acyl composition of the plasma membranes of differentiated and undifferentiated L_6 myoblasts (Table II). The results show that the plasma membrane phospholipids of differentiated L_6 myoblasts contain relatively less 16:1 and 18:1 and relatively more 17:0 and 24:1 than plasma membrane phospholipids of undifferentiated cells.

No comparable data on lipid composition of the plasma membrane of differentiated and undifferentiated muscle cells have been previously reported, but Prives and Shinitzky (23) recently showed by fluorescence polarization techniques that differentiated chick myoblasts have increased membrane microviscosity compared to undifferentiated myoblasts. Our analytical data which show that the plasma membranes of differentiated L_6 myoblasts have a higher cholesterol/phospholipid molar ratio comapred to plasma membrane of undifferentiated myoblasts support their conclusions (24). The studies reported in this paper also suggest that there may also be important differences in phospholipid and fatty acyl composition of the plasma membranes of undifferentiated and differentiated cells. It will be most important (a) to expand these studies to an analysis of other differentiated cell systems; (b) to characterize the fatty acyl composition of individual phospholipids; and (c) to initiate studies on the role of modulation in plasma membrane lipid composition in the control of membrane function that occurs during differentiation.

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Absorption of Synthetic, Stereochemically Defined Acylglycerols in the Rat

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ABSTRACT

The stereochemistry of fat digestion and absorption was investigated in rats with thoracic duct fistulas, after feeding synthetic triacylglycerol or alkyldiacylglycerol. After feeding 1,2-dilauroyl-3-oleoyl-*sn*-glycerol, dilauroyloleoylglycerol and lauroyldioleoylglycerol were the most abundant chyle triacylglycerols. Positional analysis of the fatty acid distribution and the absence of optical activity indicated that the following structures dominated: *rac*-1,2-dilauroyl-3-oleoylglycerol and *rac*-1,3-dioleoyl-2-lauroylglycerol. Therefore, the triacylglycerol resynthesized from 2-lauroylglycerol (precursor to 60% of chyle triacylglycerol) and other precursors was essentially racemic. Chyle phospholipids contained largely endogenous fatty acids, and the proportion of lauric acid was very low. A racemic mixture of 1,2-di[^{3}H]oleoyl-3-tetradecyl-*sn*-glycerol and 1-tetradecyl-2,3-di[^{14}C]oleoyl-*sn*-glycerol was absorbed to a lower degree than triacylglycerol. The appearance of oleic acid with different labels in chyle and intestinal lipids did not differ, indicating the absence of stereospecificity in fat digestion. Possible explanations for the low absorption are discussed.

INTRODUCTION

Fat absorption has been extensively studied by feeding animals different fats and measuring the appearance of fat in chyle and faeces (1-3). Most often the fatty acid composition of the dietary fat used is given, but only in few studies the dietary fat has been characterized stereochemically (4,5).

Studies from several laboratories have indicated that pancreatic lipase degrades triacylglycerols without stereospecificity (6-8), which would indicate that fatty acids at positions 1 and 3 are treated identically in the intestinal lumen. Discrimination between positions 1 and 3 may, however, be exerted at several steps during the resynthesis of acylglycerols within the intestinal mucosa (9-11). Depending on these and other selectivities, chyle triacylglycerols may differ from the fed triacylglycerols. Since such comparisons are very difficult in experiments where natural fats are fed, we have instead used synthetic acylglycerols which are defined stereochemically. Our previous studies showed that the stereochemistry of acylglycerols can be assigned by optical rotatory dispersion (ORD) (12,13), and, therefore, chyle triacylglycerols were analyzed by this technique.

1-Alkyl-2,3-diacyl-*sn*-glycerols occur in nature and have also been useful in studies on the substrate specificity of lipases (7,14). To further elucidate the stereochemistry of fat absorption, we have also fed enantiomers of alkyldiacylglycerol and studied whether or not the enantiomers were metabolized in the same way.

MATERIALS AND METHODS

Preparation of Acylglycerols

1,2-Di[^{3}H]oleoyl-3-0-tetradecyl-*sn*-glycerol and 1-0-tetradecyl-2,3-di[^{14}C]oleoyl-*sn*-glycerol were synthesized as described previously (14). 1,2-Dilauroyl-3-oleoyl-*sn*-glycerol was prepared as follows.

1,2-Isopropylidene-*sn*-glycerol was prepared from D-mannitol according to the modified procedure of Fischer and Baer (15,16). Acylation with oleoylchloride and subsequent removal of the protecting group gave 3-oleoyl-*sn*-glycerol (17). Further details on the synthetic procedures are given elsewhere (18).

1,2-Dilauroyl-3-oleoyl-*sn*-glycerol was prepared in 47% yield from 3-oleoyl-*sn*-glycerol. A solution of the monoglyceride in benzene was acylated under anhydrous conditions with lauroylchloride (10% excess), in the presence of pyridine. The mixture was stirred at room temperature for 72 hr. It was then diluted with diethyl ether, washed with water, dried, and the solvent was removed in vacuo. The residue was purified on a column of neutral alumina in benzene. The elutant was removed, and the product was dried under high vacuum at 70 C for 3 hr. The purity of the final product was checked by thin layer chromatography (TLC) and gas liquid chromatography (GLC) (1.5% OV 1 on Anachrome ABS, 50 cm steel column). Determination of the positional fatty acid distribution (19,20) showed that position 2 contained 96.5% lauric acid.

n_D^{20} : 1.4608 Calc. (%) C 74.97 H 11.74 0 13.31.
Found (%) C 75.00 H 11.68 0 13.27.
$[M]_{300}$ = +4.5 (benzene.

TABLE I

Fatty Acid Composition in Chyle Lipids after Feeding Rats 0.5 ml of 1,2-Dilauroyl-3-oleoyl-*sn*-glycerol

	Fatty acid composition (mol %)							
	12:0	16:0	16:1	18:0	18:1	18:2	18:3	20:4
Triacylglycerol	57.0	4.2	0.3	0.6	35.7	1.6	0.6	—
Fatty acid	38.5	14.4	—	7.2	31.5	4.6	3.8	—
Diacylglycerol	65.1	5.8	—	1.2	26.7	1.2	—	—
Phosphatidylcholine	3.7	28.9	—	11.8	33.0	19.9	1.1	1.5
Phosphatidylethanolamine	0.5	13.5	—	38.1	31.8	10.4	1.1	4.7
Lysophosphatidylcholine + sphingomyelin	1.4	42.3	—	32.3	19.1	2.7	1.5	0.7

ORD measurements were obtained using a Cary 60 spectropolarimeter.

Animals

Male Sprague-Dawley rats weighing 200-300 g were fasted overnight, and then the thoracic duct was cannulated as described by Bollman et al. (21). The rats were allowed to recover from the operation for 1 or 2 days, and then synthetic acylglycerol was fed through a gastric fistula. Chyle was collected on ice for the time periods indicated. When the intestine and the intestinal contents were recovered at sacrifice, the intestine was rinsed free of its contents with 2 mM sodium deoxycholate.

Lipid Extraction and Analysis

Chyle was extracted according to Bligh and Dyer (22). Other samples were homogenized in 50 ml chloroform-methanol (1:1), and the extracts were then washed with water. The lipids were dissolved in petroleum ether and separated on silicic acid columns using mixtures of petroleum ether and diethyl ether as eluents. Polar lipids were eluted with chloroform-methanol (1:1) and pure methanol. Further isolation and also analytical separation of lipids were performed by TLC on Silica Gel H. For neutral lipids, the solvent was petroleum ether-diethyl ether-acetic acid (80:20:1) and for polar lipids, chloroform-methanol-acetic acid-water (65:25:4:4). The lipids were either extracted or directly scraped into liquid scintillation vials (23) or directly transesterified in 2% H_2SO_4 in methanol. In the latter case, an internal standard of pentadecanoic acid was added, and the methyl esters were analyzed by GLC as described previously (24). Tetradecylglycerol was analyzed simultaneously after treatment of the transesterified samples with acetic anhydride-pyridine (1:1) for 3 hr at room temperature.

RESULTS

Experiments with 1,2-Dilauroyl-3-oleoyl-*sn*-glycerol

In two experiments where 450 mg of this substance was fed to rats, 291 mg and 175 mg lipid were recovered in the chyle during 6 hr. The proportion of triacylglycerol was 87.7 and 77.5%, respectively. The fatty acid composition in different lipid classes was very similar in both experiments, and the data from one experiment are shown in Table I. In triacylglycerol, fatty acid, and diacylglycerol, the fatty acids from the administered fat dominated. In triacylglycerol, the ratio of lauric acid to oleic acid was somewhat lower (1.3-1.6) than that in the given lipid (2.0). Other fatty acids which must be endogenous amounted to 7.3% in triacylglycerol, and palmitic acid was most abundant. The fatty acid composition of phospholipids was completely different, and lauric acid was practically excluded. The low proportion of lauric acid may be related to the inhibition of phospholipid synthesis caused by this acid, at least in liver preparations (23). Also previous studies have shown that endogenous fatty acids constitute a large proportion in chyle phospholipids (25,26).

Trace amounts of [^{14}C]lauric acid and [^{3}H]oleic acid were fed together with 1,2-dilauroyl-3-oleoyl-*sn*-glycerol. The distribution of radioactivity and fatty acid mass between the secondary and the two primary positions in isolated triacylglycerol was determined by pancreatic lipase hydrolysis (Table II). The proportions of [^{3}H]oleic acid and oleic acid mass at position 2 were identical, indicating that oleic acid split off from position 3 had equilibrated with the added free [^{3}H]oleic acid. Only 13.1% of [^{14}C]lauric acid in triacylglycerol was located in position 2, whereas 44% of the lauric acid mass was at this position. It can be calculated that the specific radioactivity of lauric acid at position 2 was only 19.2% of that at the primary positions ($100 \cdot 13.1 \cdot 56.0/44.0 \cdot 86.9$). This indicated that 80.8% of the triacylglycerols containing lauric acid at position 2, i.e., 60.8% of total chyle triacylglycerol, had been synthesized from 2-lauroyl-glycerol formed in the intestinal lumen.

TABLE II

Positional Analysis of Chyle Triacylglycerol after Feeding Rats 0.5 ml of 1,2-Dilauroyl-3-oleoyl-*sn*-glycerol[a]

	Fatty acid					
	12:0	16:0	16:1	18:0	18:1	18:2
Fatty acid composition at position 2	75.3	4.2	0.1	0.6	17.2	2.6
Proportion of each fatty acid at position 2 (%)	44	33	(11)	33	16	54
Proportion of [^{14}C]12:0 and [^{3}H]18:1 at position 2 (%)	13.1				16.1	

[a]Triacylglycerol was treated with pancreatic lipase, and fatty acid composition and specific radioactivity were determined in triacylglycerol and monoacylglycerol.

TABLE III

Recovery of Lipids in Chyle and Intestine after Feeding Rats Equal Amounts of 1-Tetradecyl-2,3-di[^{14}C]oleoyl-*sn*-glycerol and 1,2-Di[^{3}H]oleoyl-3-tetradecyl-*sn*-glycerol[a]

Expt.	Recovery of lipids and lipid ^{3}H	Chyle 0-7 hr	Chyle 7-24 hr	Chyle 24-30 hr	Small bowel contents	Small bowel wall	Colon + contents	Faeces
I	Lipids (mg)	49	77					271
	Lipid ^{3}H (%)	6.1	2.3					42.5[a]
II	Lipids (mg)	65	139	34	9	516	197	291
	Lipid ^{3}H (%)	8.8	9.7	0.2	0.4	1.0	7.6	10.1

[a]In Expt. I, 423 mg of the racemic mixture was fed, in the Expt. II, 415 mg.
[b]Includes the content from the whole intestine.

Gas chromatography of intact triacylglycerols gave four major peaks with the carbon numbers 36 (11.3%), 42 (38.9%), 48 (32.0%), and 54 (17.7%). These peaks represent molecules containing three residues of lauric acid or oleic acid, or combinations between these fatty acids. From the fatty acid compositions in position 2 and in total triacylglycerol, it could be calculated that a random distribution of fatty acids between positions 1 and 3 would yield 47.9% lauric acid and 47.6% C_{18} fatty acid in both positions. If the fatty acids are randomly combined, the triacylglycerol species mentioned above would constitute 87% of total with the following distribution: 36 (19.8%), 42 (44.7%), 48 (30.2%), and 54 (5.3%). An uneven distribution of lauric acid and oleic acid between positions 1 and 3 would increase the proportion of the species with carbon number 42 at the expense of the other three species, which increases the similarity between the found and calculated distribution only for the species with carbon number 36. An uneven distribution of fatty acids between positions 1 and 3 would give rise to optically active triacylglycerols. However, no optical activity was recorded which indicates that most of the triacylglycerol (>90%) was racemic. (5-10% Optically active material in this mixture with an activity corresponding to that of 1,2-dilauroyl-3-oleoyl-*sn*-glycerol would easily have been detected by the sensitive ORD instrument.)

Since the difference between the found and calculated triacylglycerol composition was not due to the formation of optically active triacylglycerol, alternate explanations must be sought. A possible pool of triacylglycerol formed from endogenous fatty acids may contribute to the high proportion of the species with carbon number 54. The low proportion of trilauroylglycerol (carbon number 36) may reflect a slower formation rate for this species due to its higher melting point (33-46 C for different crystal forms). Reduced synthesis of saturated molecules has been observed in liver for phospholipids but so far not for triacylglycerols (23).

TABLE IV

Lipis Composition in Chyle and Intestine after Feeding Equal Amounts of 1-Tetradecyl-2,3-di[^{14}C]oleoyl-*sn*-glycerol and 1,2-Di[^{3}H]oleoyl-3-tetradecyl-*sn*-glycerol[a]

Lipid fraction	Chyle		Colon + contents	Faeces
	0-7 hr	7-24 hr		
		Total fatty acid (% of recovered)		
CE	1.7 (0.6)	2.3 (3.7)	0.9	6.2 (2.1)[b]
ADG	15.0 (9.7)	10.8 (3.1)	1.2	10.8 (5.6)
TG	55.9 (67.7)	57.1 (70.9)	62.9	1.1 (1.3)
FA	4.0 (4.7)	3.5 (4.0)	18.5	71.3 (78.0)
DG	4.7 (3.6)	2.8 (1.6)	3.1	6.5 (10.7)
S	18.7 (13.7)	23.6 (16.7)	13.4	4.1 (2.3)
		% of lipid ^{3}H		
CE	1.5 (1.8)	1.2 (1.3)	2.0	7.8 (1.9)
ADG	18.4 (12.0)	21.5 (10.6)	7.6	9.3 (7.3)
TG	67.9 (69.8)	65.3 (74.0)	5.1	1.2 (1.6)
FA	3.6 (5.0)	3.4 (3.6)	51.1	52.3 (58.6)
DG	5.4 (5.6)	3.6 (2.3)	27.9	19.3 (23.7)
S	3.2 (5.8)	5.2 (8.2)	6.4	10.1 (7.0)
		Oleic acid (% of total fatty acid)		
CE	36.3 (41.9)	28.7 (22.1)	42.7	50.3 (64.0)
ADG	78.2 (79.3)	71.5 (56.7)	76.6	46.1 (91.9)
TG	69.0 (68.1)	43.1 (22.7)	36.7	38.9 (34.0)
FA	54.6 (52.9)	38.2 (27.9)	14.2	29.5 (50.1)
DG	75.8 (76.6)	51.0 (26.0)	64.7	80.5 (94.2)
S	13.6 (16.7)	13.8 (25.4)	20.5	32.6 (30.0)

[a]The percentage distribution of total fatty acids and lipid ^{3}H in each lipid extract is given. Also the proportion of oleic acid in each sample is shown. Data are from Expt. I (within parentheses) and Expt. II. Abbreviations: CE, cholesteryl ester; ADG, alkyldiacylglycerol; TG, triacylglycerol; FA, fatty acid; DG, diacylglycerol; S, polar lipids

[b]In Expt. I, faeces plus intestinal contents were analyzed together.

Experiments with Tetradecyl-dioleoylglycerol

A racemic mixture containing equal amounts of 1-tetradecyl-2,3,-di-[^{14}C] oleoyl-*sn*-glycerol and 1,2-di[^{3}H] oleoyl-3-tetradecyl-*sn*-glycerol was fed to rats. Chyle and faeces were collected, and at sacrifice the intestine and its contents were subjected to lipid analysis. In all analyses, the ratio of ^{3}H to ^{14}C never did deviate significantly from that in the original mixture, indicating that oleic-acid-containing compounds were metabolized in the same way, irrespective of whether the fatty acid originated from positions 1 plus 2 or positions 2 plus 3. Chyle contained relatively much smaller amounts of lipids in these experiments than in those where triacylglycerol was fed (Table III), indicating that alkyldiacylglycerol and its degradation products were not efficiently absorbed in the intestine. A large amount of radio activity was recovered in the intestinal contents and faeces, but still approximately half of the administered dose remained unaccounted for. Most of the lipid radioactivity in chyle was located in triacylglycerol, but significant amounts were also found in alkyldiacylglycerol (Table IV). In the intestinal contents, on the other hand, most of the lipid radioactivity was in free fatty acid, and the diacylglycerol fraction was next in magnitude. The distribution of lipid radioactivity was essentially similar to the distribution of fatty acid mass. Oleic acid was the dominating fatty acid in most lipids (Table IV). In chyle, the proportion of oleic acid was considerably higher during the first 7 hr of sampling than during the following day. Again, the polar lipid fraction contained largely endogenous fatty acids.

The distribution of tetradecylglycerol in different lipids in chyle and intestinal contents was determined by gas chromatography after transesterification in methanol and subsequent acetylation. In chyle, 55-79% of total tetradecylglycerol was recovered in alkyldiacylglycerol, and the rest was mainly in the alkylacylglycerol fraction. In intestinal contents or faeces, 69-92% was recovered as free tetradecylglycerol. The rest was mainly in alkylacylgly-

cerol. The amount of alkyldiacylglycerol recovered from chyle was insufficient for stereochemical assessment by ORD. It is probably optically active since 1-alkyl ether is acylated in intestinal mucosa but not the *sn*-3-isomer (10,11).

DISCUSSION

Our results with stereospecifically synthesized triacylglycerol can be interpreted according to present knowledge of triacylglycerol digestion and absorption (1-3). Lauric acid at position 2 of 1,2-dilauroyl-3-oleoyl-*sn*-glycerol was absorbed largely as 2-monoacylglycerol, whereas the fatty acids at the primary positions evidently were absorbed as free fatty acids. Bloom et al. (27) found that only 15-55% of absorbed lauric acid (fed as free acid) was transported in the chyle, whereas 84-95% of absorbed stearic acid was found in chyle. Lauric acid from oral 1,2-dilauroyl-3-oleoyl-*sn*-glycerol was transported in the chyle to a larger degree, since the ratio of laurate/oleate in the fed triacylglycerol (2.0) decreased only to 1.3-1.6 in chyle triacylglycerol. This is probably due to the location of half of the lauric acid in position 2. A larger proportion of 2-lauroylglycerol than of free lauric acid would thus be directed into chyle triacylglycerol. Also lauric acid from a fed butter oil distillate was better incorporated into intestinal mucosa triacylglycerol than lauric acid from the corresponding hydrolysate (5). These observations will be physiologically important if lauric acid transported via portal blood or via chyle has different metabolic fates and physiological effects. Interestingly, most of the lauric acid in coconut oil is located in position 2, but in several other palmae seed fats, most of it is in the primary positions (28).

Feeding of synthetic triacylglycerol permits a reliable estimation of the contribution by endogenous fatty acids in chyle lipid synthesis. The contribution was 7% of total fatty acid both in total triacylglycerol and in position 2, although the role of endogenous oleate is not known. Laurate is practically excluded from phospholipids, which mostly contain endogenous fatty acids, in agreement with previous results (25,26). This may reflect the low utilization of 2-monoacylglycerol for phospholipid synthesis (29) and also a more defined substrate specificity in phospholipid synthesis than in triacylglycerol synthesis, as observed in liver (23). This points to the importance of phospholipid composition for chylomicron structure.

In cell-free systems from intestine (9,30) and liver (30,31), 2-monoacylglycerol is stereospecifically acylated at position 1, and the resulting 1,2-diacyl-*sn*-glycerol is further acylated to triacylglycerol. Although this sequence makes the synthesis of optically active triacylglycerol possible, the stereospecificity is not absolute, and in vivo both 1,2- and 2,3-diacyl-*sn*-glycerols are found in the intestinal mucosa after a fat meal (4). With the synthetic triacylglycerol fed in the present experiments, chyle triacylglycerol was essentially racemic. Most of it was formed from 2-monoacylglycerol, but the nature of the intermediate diacylglycerol is not known. When a fat containing a broader spectrum of fatty acid was fed, some of the fatty acids were asymmetrically distributed between positions 1 and 3 in intestinal mucosa triacylglycerol (5), but the evaluation of this type of data is complicated by the occurrence of a variable amount of endogenous fatty acids.

Different enantiomers of tetradecyldioleoylglycerol were digested and absorbed similarly. No discrimination between [^{3}H]oleate (from positions 1 and 2) and [^{14}C]oleate (from positions 2 and 3) was observed, in agreement with the nonstereospecificity of pancreatic lipase (6-8). Surprisingly, the amount of fed lipid recovered in the chyle was much smaller than for triacylglycerol. Previous studies indicated that glyceryl ethers (32-34) and their dioleate (33) were well absorbed, but then only small amounts (5-35 mg) were fed. Several reasons for the lower absorptive capacity of alkyldiacylglycerol are possible. 1-Ethers may be absorbed less efficiently than 2-ethers (34). In addition, the mixture of *rac*-1-tetradecylglycerol, free oleate, and bile in the intestinal lumen may not create the same optimal micellar phase as that with 2-monoacylglycerol (2). A contributing factor may also be a retardation of lipolysis when tetradecyldioleoylglycerol is the substrate. Most of the lipid radioactivity recovered from the intestinal contents and faeces was in free fatty acids, but significant amounts were also in the diacylglycerol fraction, including alkylacylglycerol. Likewise, most of the tetradecylglycerol in the lumen was not acylated, indicating that most luminal lipid had been completely hydrolyzed. Further studies with synthetic triacylglycerols and their analogues are necessary to distinguish between these possibilities.

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Effect of Pentadecan-2-one on Lipid Metabolism in HeLa Cells

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ABSTRACT

HeLa cells exposed to trace amounts of pentadecan-2-one showed changes in metabolism of 1-^{14}C-palmitate. These changes consisted of an increased incorporation of radioactivity into the triglycerides and free fatty acids and a decreased ^{14}C incorporation into the ether moiety of alk-1-enyl acyl phosphoglycerides. Chemical analysis of the several lipid fractions showed a threefold increase in triglyceride content but no change in the amount of alk-1-enyl acyl or diacyl phosphoglycerides in the treated cells. Pentadecan-2-one added to the culture medium apparently gains entrance to the cell since both pentadecan-2-one and pentadecan-2-ol were detected in the ketone-treated cells and their culture medium.

INTRODUCTION

Previous studies demonstrate that it is possible to alter lipid metabolism in cultured cells by providing structural analogs to a particular lipid class. Phospholipids are structurally modified by supplementing the culture medium with analogs of the acyl or base portions (1-6); these modifications sometimes result in the growth inhibition (5,6). Cholesterol biosynthesis is specifically affected by short term exposure to sterol analogs that inhibit hydroxymethylglutaryl CoA reductase EC 1.1.1.88. Prolonged exposure to these compounds causes inhibition of growth and DNA synthesis, an effect that is reversed by adding mevalonic acid to the culture medium (7,8).

Previous reports from this laboratory indicated that the long chain methyl ketone, pentadecan-2-one, was a competitive inhibitor of the fatty aldehyde reductase of cardiac muscle (9) and, when added to the culture medium, inhibited growth of a number of cell lines (10). However, this growth inhibition was relieved when hexadecan-1-ol was simultaneously added to the culture medium (10). The results presented in this paper consider the effects of pentadecan-2-one on some aspects of fatty acid metabolism.

METHODS

Cell Culture

Stock cultures of HeLa cells were maintained as monolayers in 32 oz prescription bottles containing Earle's culture medium supplemented with calf serum (10%), sodium bicarbonate (0.085%), and the antibiotics, penicillin (100 units/ml), streptomycin (100 μcg/ml), and fungizone (0.25 μcg/ml). At the end of each growth interval, the cells were harvested by draining the medium from the stock culture; the monolayers were rinsed with 0.5 ml of trypsin, drained, and then incubated again with 0.5 ml of trypsin at 37 C for 15 min. The detached cells were suspended in a known volume of Earle's medium and an aliquot removed for cell counting in a hemocytometer. The cells were then diluted with Earle's culture medium to an appropriate concentration (concentrations are given in tables) and dispensed into individual prescription bottles. When 1-^{14}C-palmitate was used, it was bound to albunim (11) and added to the cells as part of the Earle's medium to assure that each bottle had the same concentration of acid. After dispensing the cells into bottles, the serum supplement was added. When pentadecan-2-one was included in the culture medium, it was first emulsified in calf serum by sonication and the concentration of ketone in this stock serum determined by gas liquid chromatography (GLC) (10). An aliquot of the stock serum that contained a sufficient amount of pentadecan-2-one to give a 20-30% decrease in viability was added to the culture. The amount of ketone added depended upon the number of cells present. In Table I, for instance, 14.4 μg per ml of pentadecan-2-one gave a 38% inhibition when the concentration of cells plated was 10 x 10^4 per ml. However, in another experiment, the cell concentration was raised to 20 x 10^4 cells per ml, and 36 μg per ml of pentadecan-2-one was required to achieve the same level of inhibition. In both instances, the amount of ketone added per 10^4 cells was quite constant: 1.4 and 1.8 μg.

The cells were grown for one generation (22 hr) with 1-^{14}C-palmitate in the presence and absence of pentadecan-2-one. At the end of this time period, the culture medium was drained from the monolayer and saved. Cells were detached by trypsinization, diluted to a known volume with culture medium, and counted in a

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hemocytometer. Aliquots for counting were stained with trypan blue so the viability could be assessed.

After counting, the cells were harvested from the culture medium by centrifugation at 4 C, washed three times with cold isotonic saline, and the washings added to the culture medium. Both cells and culture medium were stored at -60 C until analyzed.

Lipid Extraction

Lipids were extracted from both the cells and lyophilized culture media (12). At the time of extraction, nonadecan-1-ol was added as an internal standard, the extract reduced to dryness under vacuum at 30-35 C, and the residue blanketed with nitrogen.

Quantitative and Qualitative Assays

The lipid residue was dissolved in 50-100 μl of heptane and resolved by thin layer chromatography (TLC), into the following components (13): (a) Phospholipids (origin); (b) free cholesterol; (c) free long chain alcohols and 1,2 diglycerides; (d) free fatty acids; (e) long chain methyl ketones; (f) neutral lipid esters (triglycerides, cholesteryl esters, and esterified long chain alcohols). Identification of the individual lipid components was achieved by comparing their chromatographic mobility to that of standards applied to a separate region of the same plate. Neutral lipids were eluted from the silica gel by extraction of the gel with three 3 ml volumes of $CHCl_3$:CH_3OH (1:1) in a 12 ml centrifuge tube, and the solvent was removed under vacuum. Elution of fatty acids employed the above procedure with the addition of 1%, v/v, glacial acetic acid to the extraction solvent.

The fraction containing the neutral lipid esters was saponified and the nonsaponifiable lipids extracted with diethyl ether (14). Long chain alcohols and cholesterol present in the nonsaponifiable lipid fraction were purified by TLC and converted to acetoxy alkanes prior to GLC (15). Fatty acids were isolated from the aqueous phase after acidification and derivatized to methyl esters for GLC analysis (16). The triglyceride content was calculated by subtracting the μmoles of cholesterol in this fraction from the μmoles of fatty acid in the aqueous phase and dividing the result by three. Based on the long chain alcohol content of this fraction, the amount of fatty acid present as waxes was assessed.

The lipids, of the free long chain alcohol fraction were also saponified and the nonsaponifiable lipids extracted (14). The long chain alcohols were isolated by TLC, converted

TABLE I

Effect of Increasing Concentrations of Pentadecan-2-one on the Incorporation of 1-C14-Palmitate into Various Lipid Classes[a]

ug Ketone/ml	% Growth inhibition	Total phosphoglycerides	Free fatty acids	Neutral lipid esters[b]	Alkyl and alkenyl acyl phosphoglycerides[b]
		(dpm x $10^4/10^7$ cells)			
0	0	120 ± 8	8.0 ± 0.4	10.3 ± 0.9	2.4 ± 0.8
3.6	11	90 ± 15	8.2 ± 0.8	16.9 ± 3.7	1.6 ± 0.8
7.2	30	110 ± 9	10.8 ± 0.3	26.8 ± 2.8	1.0 ± 0.4
14.4	38	120 ± 14	20.2 ± 1.0	64.4 ± 4.2	0.4 ± 0.1
N		4	4	6	4

[a]Hela cells were suspended at 1.1 x 10^5 cells/ml in Earles' medium containing 1-^{14}C-palmitate (8 μCi/μmole) at a concentration of 2.2 μM. Nine ml aliquots of this suspension were dispensed into 8 oz prescription bottles, and the proper proportion of calf serum and pentadecan-2-one treated calf serum was added to give a final volume of 10 ml. The final pentadecan-2-one concentration achieved is listed in the table. Cultures were incubated, cells harvested, and lipid fractions counted as described in the Methods section. In this experiment, the final concentration of cells was 1.5 x 10^5 cells/ml in control cultures. N = number of cultures analyzed. Values are expressed as the mean ± standard error of the mean.

[b]The composition of these lipid classes are defined in the Methods section.

to acetoxy alkanes (15), and analyzed by GLC.

Phospholipids were eluted from the silica gel using $CHCl_3$:CH_3OH:H_2O (45:45:10), reduced to dryness under vacuum, and the residue dissolved in a known volume of benzene. From this volume, a small aliquot, 1 to 2%, was removed for counting and the remainder reduced with Vitride. The reduction products, glycerol ethers and long chain alcohols, were resolved by TLC (17). 1-0-Alkyl and 1-0-alk-1-enyl glycerols were radioassayed together or subjected to methanolysis to yield dimethyl acetals of the aldehydes formed from the alk-1-enyl ethers (16). The dimethyl acetals were separated from the 1-0-alkyl glycerols by TLC and radioassayed (17). Long chain alcohols derived from the esterified fatty acids were converted to acetoxy alkanes prior to GLC. The molar concentration of the phospholipids in this study is considered to be half the molar concentration of the acetoxy alkanes derived from the phospholipids following Vitride reduction.

Radioactivity Measurements

Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer, Model 3320. The results obtained were corrected for aliquots taken for other procedures or losses due to the separation techniques.

Gas Liquid Chromatography

All lipids were quantitatively and qualitatively analyzed by GLC. The instrument employed was a Packard Becker Model 417 gas chromatograph equipped with a flame ionization detector. Silianized borosilicate glass columns, 1/8 in. OD x 6 ft, were packed with 10% SP-2340 or 3% OV-101 on 100/120 mesh Supelcoport. Peak areas were measured with an Autolab Systems I integrator. Using this system, a sensitivity of 0.100 nmoles was achieved with a reporducibility of 0.005 nmoles. Each sample was dissolved in 50-250 μl of heptane prior to analysis. Duplicate 2 μl aliquots were removed for GLC. From the remaining volume, aliquots were taken for radioassay.

Light and Electron Microscopy

For light microscopy, cells grown on histological slides from both control and experimental groups were gently rinsed with isotonic, 37 C, phosphate-buffered saline, and then fixed with 70% ethanol. the slides were stained with Papanicolau's stain (18).

For electron microscopy, the cultures were fixed in 1% osmium tetroxide in phosphate buffer for 1 hr. After rinsing in phosphate buffer, the samples were dehydrated in a graded series of ethanol and embedded in Epon-Araldite by the propylene oxide release method. Sections were stained with uranyl acetate and lead citrate, and examined with a Phillips EM 300 microscope.

Chemicals

The chemicals utilized in this study were all reagent grade. With the exception of diethyl ether, all solvents were redistilled before use. Lipid standard and prepacked gas chromatographic columns were obtained from Supelco Inc., Bellefonte, PA. Silica Gel G was obtained from Applied Science, Inc., State College, PA.

RESULTS

Effect of Pentadecan-2-one on the Utilization of 1-^{14}C-Palmitate by HeLa Cells

Incorporation of 1-^{14}C-palmitate into several lipid classes was distinctly altered in cells exposed to pentadecan-2-one as compared to their untreated controls (Table I). The response observed was dose dependent and varied with the lipid class considered. An example of this effect is that ketone treatment had no affect on the incorporation of palmitic acid into the acyl moiety of the phospholipids but greatly decreased its incorporation into the ether chain of alk-1-enyl acyl phosphoglycerides. In contrast to this response, the amount of radiolabel detected in the free fatty acids and esterified neutral lipids increased in the ketone-treated cells as the concentration of the ketone changed in the culture medium.

Metabolism of Long Chain Alcohols

In the previous experiment, the number of cells utilized were too few for accurate analysis of the amount of radioactivity incorporated into the long chain alcohols. In a second experiment, more cells were employed, and only one concentration of pentadecan-2-one (36 μg per ml) was studied (Table II). In this instance, radioactivity was detected in both the free and esterified alcohols of the cells and culture medium. The radioactivity in the esterified long chain alcohols was distributed equally between the cells and culture medium. In contrast to the esterified alcohols, distribution of the radiolabeled free long chain alcohols was disproportionate in that over 95% of the total radioactivity found in this species was detected in the culture medium. The quantitative distribution of the free and esterified alcohols was also disproportionate since at the end of the incubation interval the free alcohol content of the culture medium had increased while the esterified alcohol content had decreased. The decrease in

TABLE II

Effect of Pentadecan-2-one on the Content and Incorporation of 1-^{14}C-Palmitate into the Long Chain Alcohols of Hela Cells and the Culture Medium[a]

Treatment	Free alcohols			Esterified alcohols		
	Cells	Medium		Cells	Medium	
	dpm	dpm	nMoles	dpm	dpm	nMoles
None	216	22,700	(+) 30	90	51	(–) 5
Pentadecan-2-one	980	157,600	(+) 29	2,605	2,314	(–) 14

[a]Hela cells were suspended at 2.8 x 10^5 cells/ml in Earles' medium containing 1-^{14}C-palmitate (8 μCi/μmole) at a concentration of 2.2 μM. Eighteen ml aliquots of this suspension were dispensed into 32 oz prescription bottles, and 2 ml aliquots either of calf serum or calf serum containing 360 μg pentadecan-2-one/ml were added to each bottle. Cultures were incubated and cells were harvested as described in Methods. At the end of 22 hr incubation, the average number of cells was 4.7 x 10^5 cells/ml in control cultures and 3.3 x 10^5 cells/ml in pentadecan-2-one treated cultures. In each experiment, 4-8 culture bottles were utilized for each analysis. The results presented in the table are the average of three separate experiments.

TABLE III

Amounts and Specific Activities of Phosphoglyceride Subclasses in HeLa Cells Grown in the Presence and Absence of Pentadecan-2-one

	Sample	Diacyl		Alk-1-enyl acyl ether	
		μmole Ester 10^7 cells	Specific activity	μmoles 10^7 Cells	Specific activity
	1	.340	90 x 10^4	.012	110 x 10^4
Control	2	.460	100 x 10^4	.014	70 x 10^4
	3	.440	150 x 10^4	.021	140 x 10^4
	4	.640	110 x 10^4	---	---
	1	.420	100 x 10^4	.014	20 x 10^4
Pentadecanone	2	.740	120 x 10^4	.023	10 x 10^4
treated	3	.440	130 x 10^4	.014	10 x 10^4
	4	.420	180 x 10^4	---	---

[a]Cells were cultured under the conditions described in Table I. In this instance, the specific activity is expressed as dpm/μmole ester group or dpm/μmole hexadecyl dimethyl acetal.

the esterified alcohol content of the medium was much greater in the ketone treated than in the control cells.

Qualitative analysis of the esterified long chain alcohols from the culture medium indicated that only primary alcohols were present. These alcohols varied in chain length from 13 to 18 carbon atoms. The major species identified were octadecan-1-ol, 53.4 to 74.9%; hexadecan-1-ol, 11.2-21.5%; and tetradecan-1-ol, 4.5-10.5%, the proportion of these alcohols varying with the serum sample analyzed. The concentration of free or esterified alcohols in the cells is not reported here since it was too low for accurate analysis.

Metabolism of Phospholipids

As noted in Table I, exposure of HeLa cells to increasing concentrations of pentadecan-2-one had little effect on the incorporation of palmitate into the acyl moiety of the phospholipids but greatly decreased incorporation of the substrate into the ether chain of the alk-1-enyl acyl phosphoglycerides. This result was verified in separate experiments where the acyl and alk-1-enyl moieties of the phospholipids of both control and ketone-treated cells were quantitated and counted (Table III). In this instance, no difference was noted in the concentration of the diacyl phosphoglycerides or in the specific activity of their esterified fatty acids from either group of cells. The alk-1-enyl acyl phosphoglyceride content was also the same in both groups of cells. However, the incorporation of 1-^{14}C-palmitate into the alk-1-enyl moiety was considerably less in the ketone treated as compared to control cultures. In this experiment, the amount of the 1-0-alkyl ethers was too low for adequate quantitation, however, radioactivity was detected in this species. Qualitative analysis of the acyl moieties of the phospholipids derived from the two groups of cells indicated a similar composition. Palmitic,

stearic, oleic, linoleic, and arachidonic acid accounted for at least 93% of the total acyl moieties present. A similar analysis of the dimethyl acetals derived from the alk-1-enyl acyl phosphoglycerides indicated that dimethyl acetals corresponding to hexadecanal, octadecanal, and octadecenal represented 83% or more of the total present.

However, the qualitative distribution of the dimethyl acetals was different in the two groups of cells. The molar ratio of the saturated 18 and 16 carbon moieties averaged 1.0 ± 0.2 (SD, n=6) in the control cells and 1.4 ± 0.2 in the ketone-treated cells. These averages were significantly different at $p<0.01$.

Metabolism of the Neutral Lipids Esters

A progressive increase in the extent of incorporation of 1-^{14}C-palmitate into the neutral lipid ester fraction was previously demonstrated *in* cells exposed to increasing concentrations of pentadecan-2-one (Table I). In this instance, it was impossible to discern whether this change represented an increased synthesis or turnover of one or more of the esterified neutral lipids. To evaluate this possibility, the neutral lipid ester fraction (triglycerides, cholesteryl esters, and esterified long chain alcohols) was isolated by TLC of a total lipid extract. This mixture of lipids was saponified and the released fatty acids, cholesterol, and long chain alcohols separated by TLC and quantitated by GLC. In both the control and ketone-treated cultures, the cellular esterified long chain alcohols were present in amounts too low for quantitation. Thus, this lipid class was not considered to contribute significantly to the fatty acid content of this fraction. In the control cells, fatty acids and cholesterol were detected in equimolar amounts, 8.5 nmoles/10^6 cells. Thus, cholesteryl esters are considered the major esterified lipid of this neutral lipid fraction. In the ketone-treated cells, the concentration of cholesterol ester was essentially the same as in the control cells, while the concentration of fatty acids increased approximately fivefold. This increase in fatty acid concentration is considered to represent a large increase in triglyceride content.

Light and electron micrographs of the control and ketone-treated cells were also prepared (Fig. 1 and 2). From the light micrograph, Figure 1, it is apparent that the control cultures consisted of a well-spread monolayer of polygonal-shaped cells with round to ovoid nuclei and pale homogenous cytoplasm. Cells exposed to pentadecan-2-one, 36 μg per ml, for 24 hr show some dispersion of attached cells. The most striking change was the appearance of large globular vacuoles in the cytoplasm frequently displacing nuclei to the periphery. Over 60% of the cells contained these vacuoles. The electron micrographs, Figure 2, of control cells showed abundant free polyribosomes, sparse rough endoplasmic reticulum, and regular shaped mitochondria. A small number of lipid droplets and a few osmiophilic dense bodies were observed. In contrast, cells treated with pentadecan-2-one (36 μg per ml) showed a marked increase of lipid droplets occupying large portions of the cytoplasm.

Metabolism of Pentadecan-2-one

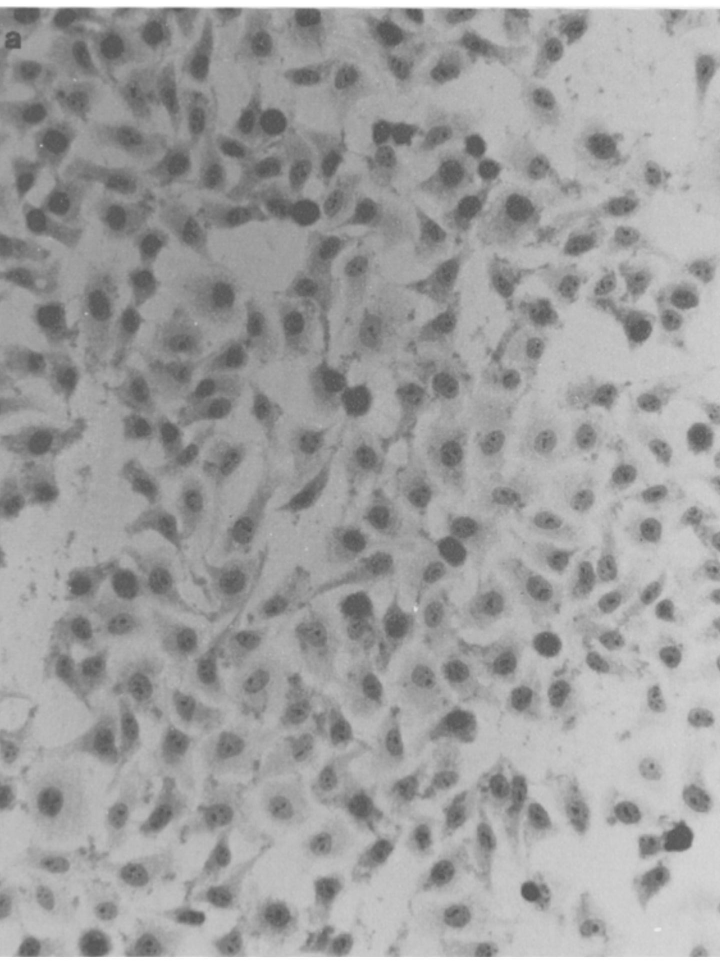
The results of a typical experiment in which HeLa cells were grown in the presenc of pentadecan-2-one is presented in Table IV. Neither the methyl ketone nor the corresponding alcohol are normal lipid components of these cells or the culture medium. Both pentadecan-2-one and pentadecan-2-ol were detected in the cells. The secondary alcohol was also found in the culture medium. These results are considered to indicate that the ketone was absorbed by the cells and reduced to the corresponding alcohol. Since over 98% of the total secondary alcohols present in this system were noted in the culture medium, it is considered that, like the primary alcohols, secondary alcohols are released by the cells.

Results from a number of experiments indicate that about half of the administered ketone can be recovered as such or as the free secondary alcohol at the end of a 22 hr incubation interval. Only very small amounts of esterified secondary alcohol were observed. Thus, it is apparent that the methyl ketone is readily catabolized by these cells.

DISCUSSION

The growth inhibitory effect of pentadecan-2-one is a recent observation (10), and at present there are no biochemical studies considering the effects this ketone has on cellular metabolism. We recognize that pentadecan-2-one may have more than one site of action, but we chose to study lipid metabolism initially since previous reports indicate that lipid metabolism in mammals is affected by pentadecan-2-one (9). The results of this study show that pentadecan-2-one dramatically affects the metabolism of two lipid classes: the 1-0-alk-1-enyl, acyl phosphoglycerides and the triacyglycerides.

The metabolism of the 1-0-alk-1-enyl, acyl, phosphoglycerides was altered in such a way that the ketone-treated cells contained their normal complement of plasmalogen, but, as im-



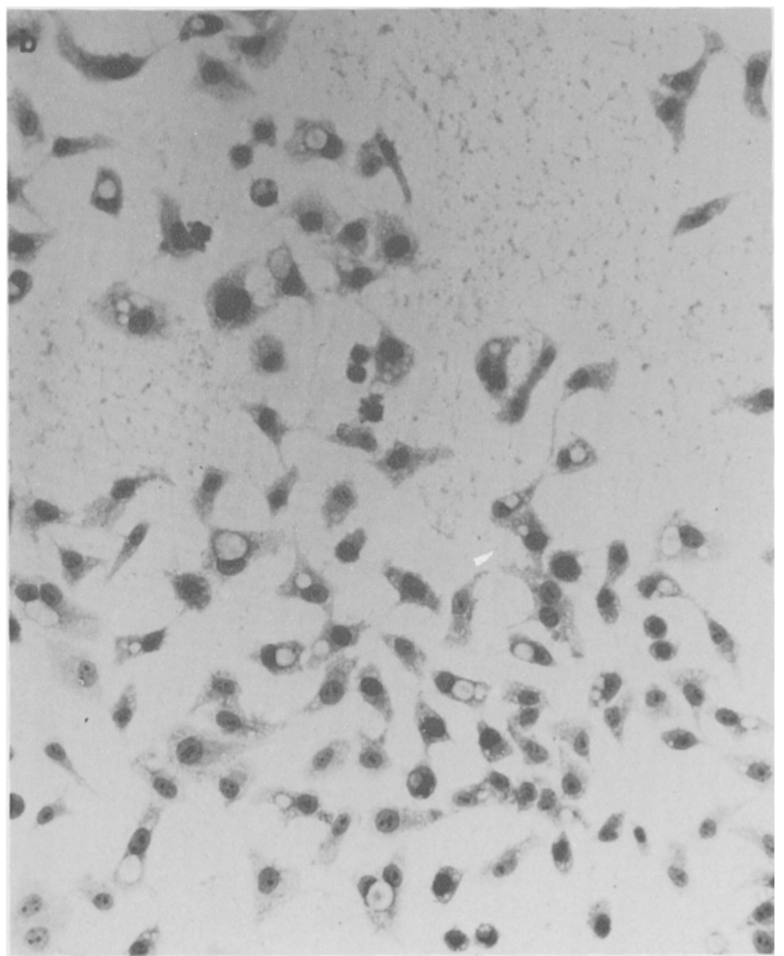


FIG. 1. (a) Light micrographs of HeLa cells in control cultures. (b) Light micrographs of pentadecan-2-one treated HeLa cells. Note the large globules present.

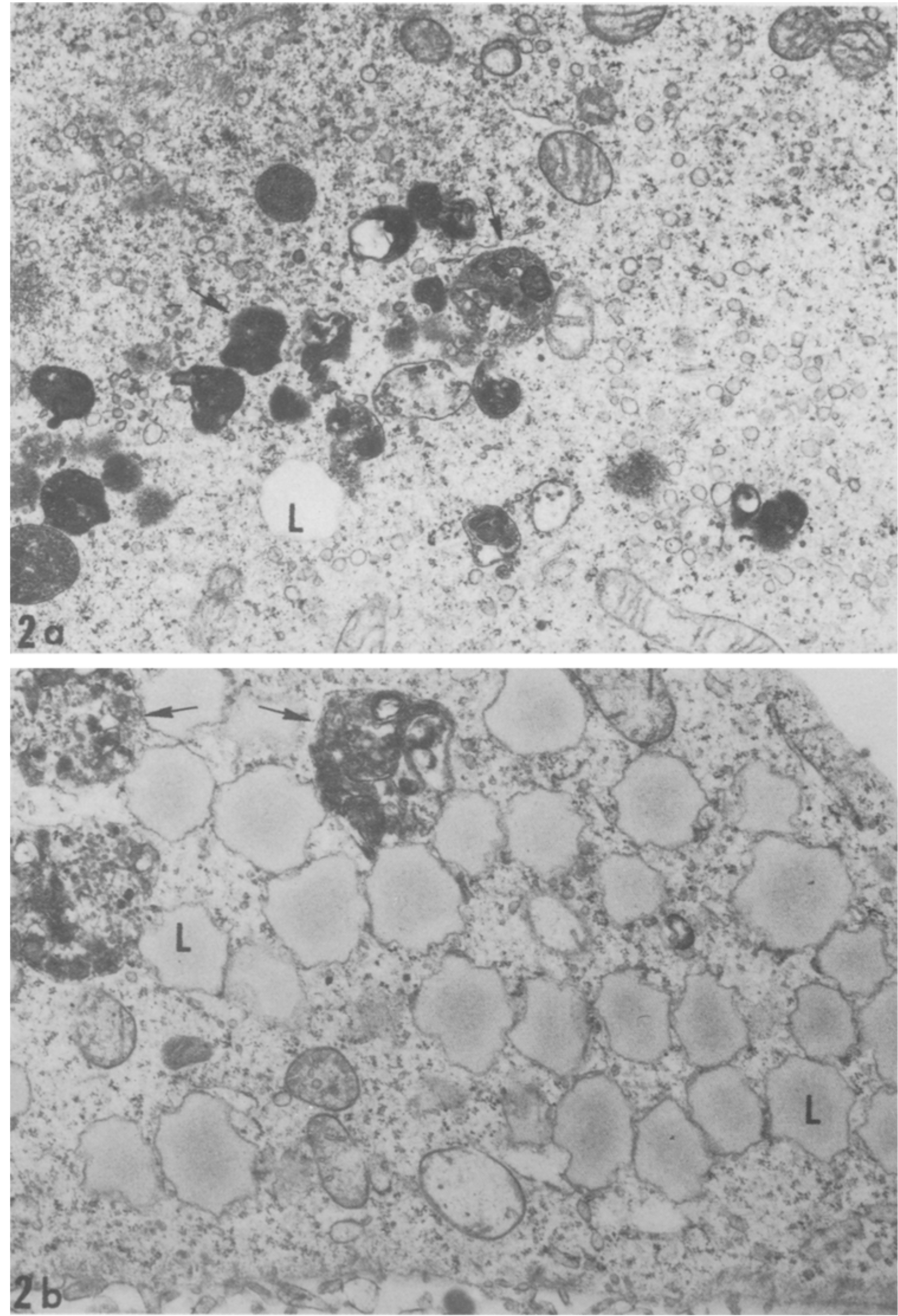


FIG. 2. (a) Electron micrographs of HeLa cells in control cultures. There are a small number of lipid droplets (L) and a few osmiophilic dense bodies (arrows). Magnification x 17,000. (b) Electron micrographs of pentadecan-2-one treated HeLa cells (24 hr). Note the marked increase of lipid droplets (L). Arrows indicate dense bodies. Magnification x 17,000.

TABLE IV

Distribution of Pentadecan-2-one and Pentadecan-2-ol between Cells and Culture Medium Following a 22 hr Incubation Interval

Component	Cell concentration	nMoles/10^6 Cells	nMoles/ml Medium
Pentadecan-2-one	30 x 10^4 cells/ml	17.5	50.0
	3 x 10^4 cells/ml	37.6	53.6
Pentadecan-2-ol	30 x 10^4 cells/ml	0.2	10.4
	3 x 10^4 cells/ml	6.2	20.8

[a]The initial concentration of pentadecan-2-one is the culture medium was 160 nanomoles/ml. The culture medium itself did not contain pentadecan-2-one or pentadecan-2-ol. In each instance, the volume of culture medium was 20 ml.

plied by the decrease in the specific activity of the alk-1-enyl ether moiety, the amount of radiolabeled fatty acid incorporated into the ether moiety was less in the ketone-treated cells as compared to the controls.

That exposing cells to increasing concentrations of pentadecan-2-one increases the synthesis of neutral lipid esters, presumably triglycerides, is supported by the fivefold increase in fatty acids in this fraction from the ketone-treated cells along with a marked increase in radioactivity and the appearance of "lipid droplets" noted on electron microscopy. While these cells show an accumulation of neutral lipid esters and are growth inhibited, they are considered to be viable since they exclude trypan blue stain and remain attached to the culture flask.

The increase in radioactivity in the cellular pool of free fatty acids can be interpreted to mean that palmitate has become enriched in this pool. An enrichment of palmitate could have occurred as a result of a depletion of the endogenous free fatty acids in the ketone-treated cells and then replacement of this pool by free fatty acids of the culture medium which contained a higher proportion of added radiolabeled palmitate. It is not unreasonable to postulate a depletion of the previously existing cellular free fatty acids upon exposure to ketone in view of the large increase of esterified neutral lipid in the ketone-treated cells.

Since neither a long chain methyl ketone or a secondary alcohol occur naturally in these cells, the observation that, following exposure of HeLa cells to pentadecan-2-one, both pentedecan-2-ol and pentadecan-2-one were found in the cell indicates that the methyl ketone has gained entrance to the cell and is reduced to the corresponding alcohol.

At present, the mechanicm by which pentadecan-2-one acts to inhibit cell growth is not apparent. However, it is apparent that exposure of HeLa cells to the long chain methyl ketone alters lipid metabolism in the treated cells.

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The Terpenyl Pyrophosphates of Wild Type and Tetraterpene Mutants of *Neurospora crassa*

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ABSTRACT

Mutations to two albino loci of *Neurospora* (*al-2* and *al-3*) are known to block tetraterpene synthesis prior to phytoene but after farnesyl pyrophosphate. Analysis of the terpenyl pyrophosphates labeled with [$2^{14}C$] mevalonic acid revealed the presence of radioactive prephytoene, pyrophosphate in both albino mutants and in the corresponding wild type strain. The *al-2* and *al-3* mutations are thus associated with lesions between prephytoene pyrophosphate and phytoene.

INTRODUCTION

Early reports elucidating the sequence of biosynthetic lesions in the carotenogenic pathway of some *Neurospora* mutants (1-3) identified three discrete blocks, viz. (a) *al-2* alleles are unable to synthesize phytoene, (b) *al-1* alleles are unable to dehydrogenate phytoene, and (c) *ylo-1* mutants cannot synthesize neurosporaxanthin (4′-apo-β,ψ-caroten-4′-oic acid). A later report (4) identified yet another albino strain (*al-3*) that was also unable to synthesize phytoene. Both *al-2* and *al-3* strains produced sterols so the mutations must have affected loci controlling reactions in he sequence between farnesyl pyrophosphate and phytoene.

We have previously reported gene linkage studies (5), polar lipid analyses (6), and analyses of carotenoid (3) and steol intermediates (7) of some of these *Neurospora* strains. With the discovery of prephytoene pyrophosphate (8), and the development of analytical techniques for the isolation and identification of the terpenyl pyrophosphates from other systems, it was reasonable to try to elucidate further the specific reactions affected by the *al-2* and *al-3* mutations.

MATERIALS AND METHODS

Strains Used

A wild type (74-OR-23-1A) *Neurospora* strain was obtained from Fungal Genetics Stock Centre in Arcata, CA. The *al-3* (RP100) strain was obtained from R.L. Phillips of the University of Minnesota, St. Paul, MN. The *al-2* strain (RES-4) and *al-1* strain (RES-6) were obtained in an earlier study (9).

Culture Conditions

About 20 g mycelial fresh weight from a 3-day aerated 1500 ml culture was used to innoculate 4.5 liters of standard Westergaard & Mitchell Neurospora medium (10) with glucose as the carbon source. After 3 days aerobic incubation at 22 C and ambient illumination, the cells were washed and incubated in a starvation medium containing the required salts (10) and 5 μCi (170 μg) of [$2\text{-}^{14}C$] mevalonic acid but no carbon source for 6 hr. The biomass was harvested by filtration on a double layer of cheesecloth, washed twice with distilled water, pressed, then frozen in liquid nitrogen and lyophilized.

Extraction of Lipids

The freeze-dried cells were ground to a fine powder with a glass pestle and mortar. The ground material (10 g) was wetted with 10 ml of water and then extracted with 100 ml of 1-butanol with magnetic stirring for 1 hr at room temperature. The mixture was filtered by suction, and the residue was extracted twice more with 100 ml portions of 1-butanol with stirring for 5 min. The combined butanol extracts were washed twice with an equal volume of butanol-saturated water in order to remove any water-soluble nonlipid material (11). The washed butanol phase was mixed with benzene and evaporated to dryness in a rotary evaporator. The residue was suspended in a minimum (< 2 ml) amount of chloroform, diluted with 30 volumes of ice cold acetone, and centrifuged. The acetone supernatant, which contained all the neutral lipids, was decanted, and the precipitate containing terpenyl pyrophosphates was dissolved in chloroform-methanol (2:1, v/v) and then fractionated by preparative thin layer chromatography (TLC). The extraction procedure used precluded the presence of pyrophosphates of C_5 and C_{10} prenols.

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Thin Layer Chromatography

The prenyl pyrophosphates were separated by preparative TLC on Silica Gel H (0.5 mm thick layer) buffered with 0,1 M ammonium phosphate buffer, pH 6.8 (12) with chloroform-methanol-water (60:40:9, v/v) as developing solvent.

Terpenols obtained after hydrolysis of the isolated terpenyl pyrophosphates (see below) were separated on Silica Gel G in benzene-ethyl acetate (80:20, v/v). They were also separated by reverse-phase TLC (13) on Silica Gel G impregnated with paraffin oil and developed with methanol-water (88:12, v/v).

The spots were detected on unimpregnated silica gel plates either with iodine vapor, with the phosphate spray reagent or by charring after spraying with sulfuric acid-ethanol (1:1, v/v) (14); with paraffin oil impregnated plates, only iodine detection was used.

Hydrolysis of Terpenyl Pyrophosphates

Three different methods were used to hydrolyze the terpenyl pyrophosphates: (a) Hydrolysis with alkaline phosphatase (0.5 mg) in potassium phosphate buffer (250 μl, 1 M, pH 8.0) at 37 C for 4-5 hr (12,15); the liberated alcohols were extracted with hexane. (b) Reduction with lithium aluminium hydride ($LiAlH_4$) in dry diethyl ether (12,13,16,17). The reaction products were worked up as described elsewhere (13), and the liberated alcohols were separated by TLC. (c) Hydrolysis with 0.1 ml of concentrated hydrochloric acid in 2.0 ml of ethanol at 37 C for 20 min. After incubation, 3 ml of water was added, and the acid-liberated terpenols were extracted with 3-ml portions of petroleum ether (13).

Determination of Radioactivity

The samples were assayed for radioactivity with a Beckman liquid scintillation spectrometer in a cocktail consisting of 0.03% POPOP and 0.5% PPO in toluene. However, for radioactive spots on TLC plates, small sections (0.5 cm) of silica gel were scraped directly into counting vials, and counting was carried out using the cocktail mixture supplemented with Beckman Bio-solv solubilizer.

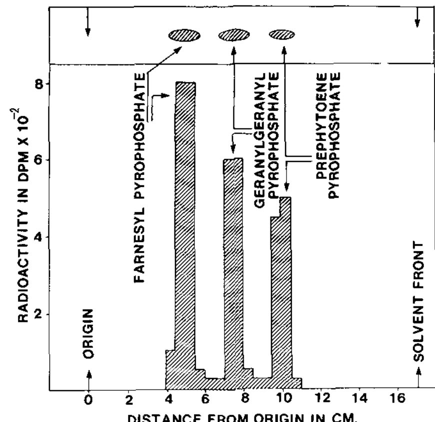


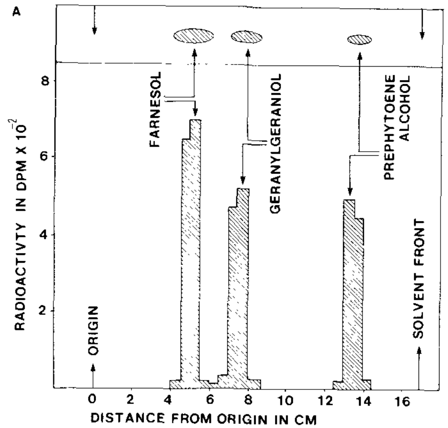
FIG. 1. Thin layer chromatography of ^{14}C-labeled prenyl pyrophosphates from Albino *al-2* mutant on Silica Gel H (buffered with 0.1 M ammonium phosphate buffer, pH 6.8) in chloroform-methanol-water (60:40:9, v/v).

RESULTS AND DISCUSSION

The terpenyl pyrophosphate fraction obtained by butanol extraction of *Neurospora crassa* incubated with ^{14}C-mevalonate was first chromatographed on a buffered Silica Gel H plate. Three radioactive bands, with R_f values are characteristic of farnesyl, geranylgeranyl, and prephytoene pyrophosphate, respectively, as indicated by the mobilities of authentic standards (Fig. 1).

To identify the prenyl pyrophosphates further, the above compounds were eluted from the silica gel with a mixture of 2% ammonium hydroxide in methanol-ethyl acetate (1:1) and hydrolyzed with alkaline phosphate as described above. The liberated ^{14}C-terpenols showed three radioactive spots on the plates with R_f values of 0.30, 0.45, and 0.80 corresponding to the mobilities of authentic farnesol, geranylgeraniol, and prephytoene alcohols, respectively (Fig. 2A). The identity of these terpenols was confirmed by reverse-phase chromatography (13), which showed the presence of three radioactive bands, with R_f values of 0.15, 0.60, and 0.70, identical with the mobilities of marker prephytoene alcohol, geranylgeraniol, and farnesol, respectively (Fig. 2B). Though allylic phosphate esters are well known for their susceptibility to chemical methods of hydrolysis (12,15,18), the alkaline phosphates procedure has been reported by Goodman et al. (15) and Sofer et al. (12) to produce the parent alcohol without any rearrangements. The results reported here are consistent with those of the above-mentioned authors (12,15), since no radioactivity was detected in any isomeric alcohols such as nerolidol or geranyllinalool (Fig. 2A,B).

However, when individual [^{14}C]terpenyl pyrophosphates were treated with $LiAlH_4$, 60-70% of the ^{14}C was accounted for by the parent alcohol, 6-8% by the rearranged isomeric alcohols, 15-20% by polar material, and the rest by hydrocarbon material (Table I). Acid



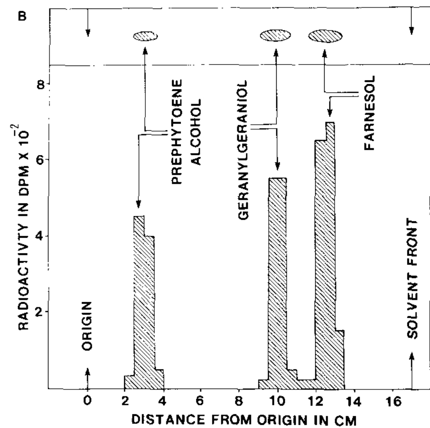


FIG. 2. Thin layer chromatography of the terpenols obtained by hydrolysis of prenyl pyrophosphates from Albino *al-2* mutant by alkaline phosphatase. A, terpenols were separated on Silica Gel G in benzene-ethyl acetate (80:20, v/v). B, terpenols were separated on Silica Gel G impregnated with paraffin oil in solvent system methanol-water (88:12, v/v) saturated with paraffin oil.

TABLE I

Distribution of ^{14}C among Products of Acid Hydrolysis and of $LiAlH_4$ Reduction of Terpenyl Pyrophosphate[a]

	^{14}C-distribution, % of total ^{14}C					
	$LiAlH_4$ reduction			Acid hydrolysis		
Product	F-PP	GG-PP	PP-PP	F-PP	GG-PP	PP-PP
Hydrocarbons (solvent front)	9	10	10	15	10	5
Prephytoene alcohol	—	—	70	—	—	65
Geranyllinool	—	7	—	—	20	—
Nirolidol	8	—	—	41	—	—
Geranylgeraniol	—	58	—	—	60	—
Unidentified	—	—	—	—	—	15
Farnesol	63	—	—	28	—	—
Polar material (origin)	15	13	14	7	4	10
Recovery	95	88	94	91	94	95

[a]The products were separated on Silica Gel G in benzene-ethyl acetate (80:20, v/v), and the radioactivity was determined by scraping off 0.5 cm sections of silica gel into counting vials (see details in methods section). Abreviations: F-PP, farnesol pyrophosphate; GG-PP, geranylgeraniol pyrophosphate; PP-PP, prephytoene pyrophosphate.

hydrolysis yielded similar products, but the proportions of rearranged alcohols were much higher than with the $LiAlH_4$ reduction procedure (Table I).

When *N. crassa* and its albino carotenoid mutants were incubated in the presence of [^{14}C]mevalonate for 6 hr, about 6-8% of the [^{14}C]mevalonate was incorporated into total lipids, of which about 1% appeared in the terpenyl pyrophosphate fraction (Table II). The ^{14}C was distributed among farnesyl, geranylgeranyl, and prephytoene pyrophosphates with the same general patterns for the various mutants (Table II). However, the proportion of ^{14}C in prephytoene pyrophosphate was highest in *al-2* and *al-3* and lowest in wild-type and *al-1* strains.

The significant incorporation of ^{14}C in geranylgeranyl and prephytoene pyrophosphates in strains *al-2* and *al-3* indicates that both strains have unblocked pathways up to prephytoene pyrophosphate. Previous reports have indicated that neither strain produces phytoene, although *al-3* tends to be a somewhat

TABLE II

Incorporation of [^{14}C] Mevalonate into Terpenyl Pyrophosphates of *N. Crassa* Mutants[a]

Mutants	^{14}C-Incorporation, %		^{14}C-distribution,[d] %		
	Total lipids[b]	Terpenyl-PP[c]	FPP	GGPP	PPPP
Wild-type *N. Crassa*	5.9	0.9	56	28	16
Albino *al-1*	8.1	0.9	44	31	25
Albino *al-2*	8.4	1.1	36	29	35
Albino *al-3*	8.0	0.9	41	28	31

[a]After incubation with 5 μCi [^{14}C] mevalonate at 22 C for 6 hr.
[b]As % of [^{14}C] mevalonate per culture.
[c]As % of [^{14}C] in total lipids.
[d]As % of ^{14}C in C_{15}, C_{20} and C_{40} terpenyl pyrophosphates.

"leaky" mutant (3). The *al-2, al-3* lesion(s) must, therefore, be between prephytoene pyrophosphate and phytoene. Because these two mutants show positive genetic complementation (4), it cannot be resolved at this time whether both loci are associated with the same function or reaction (as with the heteromultimers tryptophan synthetase or hemoglobin) or whether each locus controls a descrete reaction. The latter alternative might involve the synthesis and dehydrogenation of lycopersene.

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COMMUNICATIONS

Reduction in Medium Chain Acids and Monoenoic Acids in Livers and Plasma of Rats Fed Eicosa-5,8,11,14-Tetraynoic Acid

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ABSTRACT

Male Sprague-Dawley rats were fed for 8 weeks a corn oil (CO) diet or a hydrogenated coconut oil (HCNO) diet. These diets were fed in the absence or presence of eicosa-5,8,11,14-tetraynoic acid (TYA). The inclusion of TYA in the HCNO diet reduced the levels of 12:0 and 14:0 in the total fatty acids of livers and plasma. With either diet, the presence of TYA caused an alteration in the fatty acid composition of these tissues so as to reduce the values of the ratios: 16:1/16:0, 18:1/18:0, and 20:4/18:2. These results suggest that dietary TYA can influence the hepatic metabolism of medium chain fatty acids and that it may inhibit the desaturase enzyme involved in the synthesis of not only 20:4 but also of monoenoic fatty acids.

INTRODUCTION

Eicosa-5,8,11,14-tetraynoic acid (TYA), the acetylenic analog of arachidonic acid, is an inhibitor of the biosynthesis of cholesterol (1) and prostaglandins (2). Dietary TYA increases the tissue levels of 18:2 and decreases the levels of 20:4 (3-5) as well as 22:4 and 22:5 fatty acids (3). Thus, this compound may be an inhibitor of the desaturases involved in polyenoic fatty acid synthesis. The results presented in the present communication show that the administration of TYA also influences the hepatic and plasma levels of medium chain fatty acids and depresses the relative levels of 16:1 to 16:0 and of 18:1 to 18:0.

MATERIALS AND METHODS

One-month-old Sprague-Dawley male rats (100 g) were obtained from Hilltop Animal Supplier, Chatsworth, CA. They were divided into four groups of four each. The first group was fed for 8 weeks a high carbohydrate, 10% mazola corn oil diet (CO). The second group was fed the CO diet containing 0.033% TYA. The third group was fed a high carbohydrate, 10% hydrogenated coconut oil (HCNO) diet. Cobee 92 (HCNO) was a generous gift from PVO International, Inc., Richmond, CA. The fourth group was fed the HCNO diet containing 0.05% TYA. The composition of the diets was given previously (6).

Rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (1 ml; 50 mg) and exsanguinated using heparin-washed syringes and needles. Their livers were quickly excised, blotted, washed with ice cold saline, and either used for analysis or kept frozen. Liver slices (100 mg) were hydrolyzed, and fatty acid methyl esters were prepared as described by Grogan et al. (7). In the case of rats which were fed the CO diet, fatty acid compositions were determined using the slices of frozen livers. We have found that the fatty acid compositions determined with slices of fresh liver did not differ from those determined later with frozen livers. Whole blood was centrifuged at 1500 rpm at 22 C in a Dynac centrifuge for 7 min, and plasma was removed. Plasma was hydrolyzed, and fatty acid methyl esters were prepared (7). Analysis of methyl esters was carried out by gas liquid chromatography (150-180 C; 10 C/min) in a Varian aerograph model 2740 using a flame ionization detector and stainless-steel column (6 ft x 1/8 in.) packed with 5% diethylene glycol succinate on H/P Chromosorb G. The fatty acid methyl esters were identified by comparing retention times of known standards obtained from Applied Science Laboratories, State College, PA, and Supelco, Bellefonte, PA. Areas of peaks corresponding to various fatty acid methyl esters were calculated by triangulation, and the percent distribution of individual fatty acids was determined.

RESULTS AND DISCUSSION

When rats were fed a CO diet, their livers did not contain 12:0 fatty acid and contained only a low level of 14:0 (Table I). As would be expected, on feeding the HCNO diet, the levels of

TABLE I

Fatty Acid Composition of Livers of Rats Fed Different Diets[a]

Fatty acid	CO diet[b]		HCNO diet[b]	
	-TYA	+TYA	-TYA	+TYA
12:0	0	0	1.0 ± 0.1[c]	0.2 ± 0.03
14:0	1.3 ± 0.15[c]	0.3 ± 0.03	3.5 ± 0.1[c]	0.5 ± 0.04
14:1	0	0	0.4 ± 0.03	0
16:0	16.3 ± 0.5	16.8 ± 0.6	18.0 ± 0.5	17.9 ± 0.7
16:1	5.8 ± 0.2[c]	1.5 ± 0.1	11.5 ± 0.4[c]	8.3 ± 0.3
18:0	15.2 ± 0.3[c]	21.7 ± 0.4	16.4 ± 0.6[c]	21.4 ± 0.7
18:1	21.4 ± 0.3[c]	12.5 ± 0.3	30.6 ± 0.8	28.9 ± 0.4
18:2	17.1 ± 0.4[c]	26.9 ± 0.5	2.1 ± 0.1[c]	4.4 ± 0.1
18:3	0.4 ± 0.02	0.6 ± 0.1	0	0
20:3	0.3 ± 0.02[c]	1.1 ± 0.1	6.3 ± 0.2	7.5 ± 0.3
22:0	0.4 ± 0.02	0.5 ± 0.04	0.5 ± 0.1[c]	1.9 ± 0.1
20:4	15.8 ± 0.4	14.2 ± 0.3	6.4 ± 0.2	6.7 ± 0.2
20:5	1.9 ± 0.1	2.5 ± 0.2	1.0 ± 0.2	0.7 ± 0.1
22:4	2.2 ± 0.1[c]	0.6 ± 0.05	1.0 ± 0.1	0.7 ± 0.1
22:6	1.5 ± 0.1	0.7 ± 0.04	1.1 ± 0.2	1.0 ± 0.1
16:1/16:0	0.36 ± 0.02[c]	0.09 ± 0.005	0.65 ± 0.03[c]	0.47 ± 0.03
18:1/18:0	1.42 ± 0.04[c]	0.58 ± 0.02	1.90 ± 0.1[c]	1.37 ± 0.05
20:4/18:2	0.93 ± 0.03[c]	0.53 ± 0.01	3.07 ± 0.1[c]	1.54 ± 0.05

[a]Percent of total fatty acids is given as Mean ± SE of analysis with four rats in each diet group. Duplicate determinations were carried out with separate pieces of liver from each rat.

[b]The corn oil (CO) diet contained the fatty acids: 16:0, 10.7%; 18:0, 1.8%; 18:1, 24.8%; 18:2, 61.9%; and 18:3, 1.0%. The hydrogenated coconut oil (HCNO) diet contained the fatty acids: 6:0, 1.0%; 8:0, 11.9%; 10:0, 11.9%; 12:0, 18.6%; 14:0, 20.9%; 16:0, 16.7%; 18:0, 18.6%; and 18:1, 0.4%.

[c]P value vs. + TYA diet group <0.001.

these acids were increased. However, when TYA was present in the HCNO diet, 12:0 and 14:0 levels in the total fatty acids of the livers were decreased (Table I). The decrease may not be due to an increased mobilization of these acids from the tissue since such a depression in the levels of 12:0 and 14:0 by dietary TYA was also observed in plasma fatty acids (Table II). The reduction of medium chain acid levels in the liver may be due to the increased oxidation or the elongation for the synthesis of fatty acids of longer chain length. A relatively higher level of 22:0 in the livers and plasma of rats fed TYA suggests that the synthesis of long chain fatty acids may have been stimulated.

The diets used in this study did not contain 16:1. Hence, 16:1 found in the livers must have been produced within the tissue by the action of desaturase on 16:0. The hepatic level of the desaturase, which is also involved in the synthesis of 18:1, is enhanced when animals are fed a diet which does not contain 18:1 or 18:2 (8). This would explain the relatively higher ratios of 16:1/16:0 in the livers and plasma of rats fed the HCNO diet as compared to the CO diet (Tables I and II). A significant reduction in 16:1/16:0 observed in the liver and plasma fatty acids when TYA was fed with either diet suggests that dietary TYA may inhibit hepatic desaturase activity.

In spite of an increase in the liver desaturase activity by feeding the HCNO diet (8), the presence of even trace amounts of TYA in the diet reduced the ratio of 18:1/18:0 in the fatty acids of livers and plasma (Tables I and II). The extent of the decrease in this ratio by feeding TYA was greater with the CO diet than the HCNO diet. This may be due to the presence of 18:1 and 18:2 in the CO diet which depress the levels of hepatic desaturase (8). When added to the CO diet, TYA may exhibit an additive effect in the inhibition of the desaturase activity.

As observed previously (3-5), the ratio of 20:4/18:2 in the liver fatty acids was decreased by dietary TYA (Table I). However, although the 18:2 level was increased, unlike the previous findings (3-5), the 20:4 level was not substantially reduced by TYA (Table I). The reason for this discrepancy is not known. It would appear that the liver maintained the level of 20:4 in its fatty acids in an efficient manner under the dietary conditions of the present study. In plasma, a decrease of 20:4 and an

TABLE II

Fatty Acid Composition of Plasma of Rats Fed Different Diets[a]

Fatty aicd	CO diet		HCNO diet	
	-TYA	+TYA	-TYA	+TYA
12:0	0	0	7.2 ± 0.8[c]	3.1 ± 0.3
14:0	0.8 ± 0.1[b]	0.5 ± 0.1	6.4 ± 0.4[c]	2.8 ± 0.2
16:0	18.5 ± 0.7	18.3 ± 0.7	18.9 ± 0.7	20.8 ± 0.3
16:1	4.6 ± 0.2[c]	1.8 ± 0.1	11.7 ± 0.7[b]	9.3 ± 0.5
18:0	10.1 ± 0.3[c]	12.6 ± 0.6	11.7 ± 0.6[d]	14.8 ± 0.2
18:1	24.2 ± 0.8[c]	18.2 ± 0.5	26.3 ± 1.0	26.3 ± 0.5
18:2	25.0 ± 1.3[c]	35.3 ± 0.7	2.3 ± 0.3[c]	6.0 ± 0.3
20:3	0	0.7 ± 0.1	8.1 ± 0.6	9.6 ± 0.5
22:0	0	0	0.4 ± 0.03	1.3 ± 0.1
20:4	15.9 ± 0.6[c]	10.8 ± 0.6	6.8 ± 0.6	5.3 ± 0.4
16:1/16:0	0.25 ± 0.01[c]	0.1 ± 0.01	0.62 ± 0.03[d]	0.45 ± 0.02
18:1/18:0	2.41 ± 0.09[c]	1.49 ± 0.11	2.26 ± 0.11[d]	1.78 ± 0.04
20:4/18:2	0.65 ± 0.04[c]	0.31 ± 0.02	3.22 ± 0.67[d]	0.90 ± 0.09

[a]Percent of total fatty acids is given as Mean ± SE of analysis with plasma from four rats in each diet group. Duplicate determinations were carried out with each plasma sample.

[b]P value vs. + TYA group < 0.05.

[c]P value vs. + TYA group < 0.001.

[d]P value vs. + TYA group < 0.01.

increase of 18:2 levels were observed when rats were fed a CO diet containing TYA (Table II). The decrease of 20:4 and the increase of 18:2 levels have suggested that TYA may inhibit the synthesis of 20:4 from 18:2 (3-5). The proportion of 22:4 in liver fatty acids was also found to be decreased significantly when rats were fed the CO diet containing TYA. This observation is similar to that made earlier by Coniglio et al. (3) who had suggested that dietary TYA may be inhibitory towards the synthesis of 22:4 from 20:4.

When rats were fed the HCNO diet, liver and plasma had a relatively high level of 20:3 which is known to be produced from 18:1. Dietary TYA did not alter the proportion of 20:3 in the hepatic fatty acids. It would, therefore, appear that this compound at the levels fed does not inhibit the synthesis of 20:3 acid. It is logical to expect that 20:3 in livers of rats fed HCNO diet is the $\omega 9$ isomer, while with the CO diet, it is the $\omega 6$ isomer. Inclusion of TYA in the CO diet significantly increased the level of 20:3 in liver and plasma fatty acids (Tables I and II). This may be due to either its increased synthesis from 18:2 or its depressed conversion to 20:4.

The results on the dietary effect of TYA on the fatty acid composition of rat livers given in this study are different from those published previously with either rats (3) or mice (4,5) which did not show changes in the levels of medium chain or monoenoic fatty acids. The present study is different from the earlier ones in that there are differences in the composition of diets, duration of the dietary regimen, mode of administration of TYA, and in the sex and species of the animals used. However, our results demonstrate clearly that the dietary TYA can influence the medium chain and monoenoic acid levels of livers and plasma.

Although the administration of TYA has a variety of effects on lipid metabolism, it is not yet known whether this compound is absorbed by the intestine and is incorporated into tissue lipids. It is also not known how dietary TYA or its metabolite can influence the medium chain and monoenoic fatty acid composition of livers. Whether the reduced level of 12:0 and 14:0 in the livers of rats fed TYA is due to their utilization for fatty acid synthesis can only be ascertained by quantitating this process. Although the depressed ratios of 16:1/16:0 and 18:1/18:0 are suggestive of the inhibitory effect of TYA on desaturase, a conclusive demonstration of this effect requires the analysis of the desaturase activity in the livers of rats fed TYA.

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ERRATUM

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The Role of Dietary Protein in Hypercholesterolemia and Atherosclerosis

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ABSTRACT

Rabbits become hypercholesterolemic and develop atherosclerotic lesions when fed a cholesterol-free, semipurified diet. This provides a useful experimental model in which a moderate hypercholesterolemia can be maintained for long periods of time. The elevation of plasma cholesterol and development of atherosclerosis are dependent on the kind of protein in the diet and can be prevented by replacing casein with isolated soy protein. Feeding trials with enzymatic digests or mixtures of amino acids indicate that this difference is at least partly due to the differing amino acid composition of the two proteins. Rabbits on the soy protein diet showed higher rates of oxidation and turnover of cholesterol than those on the casein diet. Dietary protein has generally been considered to be of little significance in the etiology of atherosclerosis in humans, but evidence is accumulating which indicates that it can have a significant influence on human plasma cholesterol levels.

When Ignatowski (1,2) discovered that atherosclerosis could be produced in rabbits by feeding animal products such as meat, milk, and eggs, he thought that the lesions were due to injurious effects of animal protein on the arterial wall. This idea was largely abandoned after Anitschkow and Chalatow (3,4) showed that atherosclerotic lesions could be obtained in rabbits by feeding cholesterol, although some subsequent reports provided evidence that dietary protein could play a role in the development of atherosclerosis. Newburgh and Squier (5) observed atherosclerotic lesions in rabbits on a high casein diet. Some years later, Newburgh and Clarkson (6,7) fed a diet rich in powdered beef to rabbits and thought that beef protein was responsible for the observed hypercholesterolemia and atherosclerosis. Subsequent experiments by Meeker and Kesten (8,9) showed clearly that rabbits fed a high protein, cholesterol-free diet, with casein as the source of protein, become hypercholesterolemic and

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TABLE I

Effects of Cholesterol-Free, Semisynthetic Diets Containing Proteins from Different Sources on Weight Gain and Plasma Cholesterol Levels of Rabbits[a]

Source of dietary protein	Number of animals	Weight gain (g/day)	Plasma cholesterol (mg/dl)
Extracted whole egg	4	-1 ± 0.7	235 ± 89
Skim milk	6	18 ± 3	230 ± 40
Lactalbumin	5	9 ± 2	215 ± 69
Casein	6	14 ± 1	204 ± 44
Fish meal	6	14 ± 3	166 ± 32
Beef steak	5	20 ± 2	160 ± 60
Pork tenderloin	6	25 ± 1	110 ± 17
Raw egg white	6	9 ± 2	105 ± 28
Detoxified rapeseed flour	6	6 ± 2	91 ± 11
Wheat gluten	6	3 ± 1	80 ± 21
Peanuts	6	15 ± 2	80 ± 10
Cottonseed	6	17 ± 4	76 ± 14
Sesame seed	6	18 ± 2	70 ± 5
Soy protein isolate	6	14 ± 2	67 ± 9
Sunflower seed	6	15 ± 2	53 ± 12
Peas	6	7 ± 2	41 ± 11
Faba beans	6	8 ± 2	30 ± 4

[a]Most of the fat had been removed from the proteins used for these experiments, and the small residual amounts of fats and sterols were not considered to have any significant effect on the level of plasma cholesterol.

develop atherosclerosis, whereas these effects were not observed when soybean flour replaced the casein in the diet. The soybean flour also appeared to have an inhibitory effect on atherosclerosis produced by addition of cholesterol to the diet.

These studies on dietary protein made no lasting impression, and after the discovery in the early 1950s that feeding polyunsaturated fat caused a lowering of serum cholesterol levels in humans, most of the emphasis in atherosclerosis research was concentrated on effects of dietary fat (10,11). Thus, when two independent research groups reported some years later that hypercholesterolemia and atherosclerosis could be produced in rabbits by feeding cholesterol-free, semipurified diets (12-14), the results were interpreted as being due to an essential fatty acid deficiency. There was some justification for this view since the effects were largely prevented by including substantial amounts of polyunsaturated fat in the diet. However, the idea that nonlipid components of the diet might play an important role in experimental atherosclerosis slowly began to gain ground.

In 1964, Kritchevsky (15) pointed out that atheromata could be produced in rabbits by semipurified diets containing saturated fats, whereas no lesions were obtained when the same fats were added to a stock commercial laboratory chow. In subsequent experiments, Kritchevsky and Tepper (16) provided evidence that the small amount of polyunsaturated fat normally present in commercial feed was not responsible for this difference. The influence of various nonlipid components of the diet has been studied by these and other workers. It was reported by Howard et al. (17) that hypercholesterolemia and atherosclerosis could be reduced in rabbits by replacing the casein in the diet by whole soy flour or by hexane-extracted soybean meal, but this reduction was not obtained with purified soy protein.

Some years ago, we began a series of feeding trials designed to identify the dietary component responsible for the hypercholesterolemic response in rabbits fed cholesterol-free, semipurified diets. In these studies, the different components of the semipurified diet were varied systematically, and it was found that the hypercholesterolemia was related to the use of casein as the source of protein. Other proteins derived from animal sources, such as egg protein, lactalbumin, fish protein, or beef protein, also gave a hypercholesterolemic response, but when the casein was replaced on an isonitrogenous basis by protein preparations from plant sources, the plasma cholesterol remained low and was generally in the range seen in rabbits fed commercial diet (18-20).

The degree of hypercholesterolemia was also influenced by the carbohydrate and fat components of the diet, but dietary protein appeared to be primarily responsible for the rise of plasma cholesterol. In our experiments, a hypercholesterolemic response has so far only been obtained with cholesterol-free, semipurified diets when the dietary protein is de-

rived from an animal source (Table I). It should be pointed out, however, that some of the animal proteins, such as pork protein and egg white protein, gave levels of plasma cholesterol which were not significantly different from those obtained with some plant proteins (19). Furthermore, the work of Hermus (21) has shown that a protein mixture consisting of 6 parts casein, 4 parts gelatin, 6 parts fish protein concentrate, and 4 parts soybean protein gives a much lower cholesterol level than casein, when fed as the source of protein in a cholesterol-free, semipurified diet. It is, therefore, unwise to make a clear distinction between animal and plant proteins on the basis of their ability to produce an elevation of plasma cholesterol levels.

The exact nature of the dietary components responsible for the hypercholesterolemic effect and the mechanisms involved are of considerable interest. The response does not seem to be related to growth rate, since some of the diets containing plant protein preparations gave at least as good growth as those containing animal proteins (Table I). The preparations contained little or no fat which could influence the response, but in many cases the protein constituted no more than 60-65% of the total, and even the purest preparations, such as casein and soy protein isolate, contained as much as 5-10% of nonprotein material.

Feeding trials have been carried out with protein hydrolysates and with mixtures of amino acids in an attempt to determine whether the hypercholesterolemic response is related to the amino acid composition of the dietary protein (22,23). An enzymatic digest of casein or a mixture of amino acids corresponding to the amino acid composition of casein gave the same degree of hypercholesterolemia as the intact protein, when fed in a cholesterol-free, semipurified diet. These results give no indication that nonprotein material associated with the casein preparation has anything to do with the hypercholesterolemic response. An enzymatic digest of soy protein gave a low level of plasma cholesterol similar to that obtained with the intact protein, but a mixture of amino acids corresponding to the amino acid composition of soy protein produced a moderate hypercholesterolemia. Attempts to reverse the observed effects of casein and soy protein isolate by adding amino acids to casein (in a ratio of approximately 1:1) to give an overall amino acid composition equivalent to soy protein isolate, or by adding amino acids to soy protein isolate to give a mixture with the overall composition of casein, were unsuccessful (22). It appeared that the intact protein had an overriding effect in each case.

From these results, it is not possible to say whether the amino acid composition of the dietary proteins is entirely responsible for the observed difference in plasma cholesterol levels. A soy protein concentrate, containing about 65% protein, and soy protein isolate, containing better than 90% protein, gave similar results, but it is always possible that the residual nonprotein material in this preparation is at least partly responsible for maintaining the low cholesterol level. The possibility that the amino acid composition of the dietary protein is the major factor influencing the plasma cholesterol level is not ruled out, however, by the feeding trials with mixtures of amino acids. Feeding an intact protein probably does not provide amino acids for metabolism at the same rate or in the same proportions as feeding the corresponding mixture of amino acids. One of our current goals is to find a combination of amino acids which will maintain a normal level of plasma cholesterol and at the same time give a growth response at least equivalent to that obtained with soy protein isolate.

Identification of the specific dietary components influencing plasma cholesterol levels in rabbits on cholesterol-free diets will no doubt provide clues to the mechanisms involved. Meanwhile, other data are accumulating which may help to elucidate the mechanism of action. The hypercholesterolemia has been shown to be due mainly to an increase in the level of plasma low density lipoproteins (16,21,23). Hermus (21) studied the turnover of isotopic cholesterol in rabbits on semipurified diets or rabbit chow and found that the specific activity decay curve of serum cholesterol was in agreement with a two-pool system. Pool A was larger, and the mean rate constant for removal of cholesterol from pool A was lower in rabbits fed semipurified diets compared to chow-fed rabbits, indicating that those fed chow were better able to dispose of cholesterol than those on semipurified diets. Studies in our laboratory have given results in general agreement with these conclusions. Rabbits on commercial feed oxidized cholesterol more rapidly than those on casein-containing, semipurified diet (Carroll, Suria, Hamilton, and Huff, unpublished experiments). Further experiments showed that the rates of oxidation and turnover of cholesterol were increased by replacing the casein in the semipurified diets by soy protein isolate (24).

The higher rate of oxidation of cholesterol by rabbits on commercial feed might result from increased loss of bile acids, with consequent reduction of end-product inhibition of cholesterol oxidation. Greater loss of bile acids

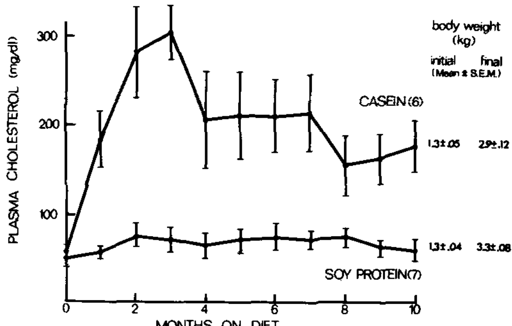

FIG. 1. Plasma cholesterol levels of male New Zealand White rabbits fed semipurified diets containing either casein or isolated soy protein. The numbers of animals per group are shown in parentheses. The vertical bars indicate the Standard Errors of the Mean. (Reproduced with permission from Springer-Verlag from "Atherosclerosis IV" (23).

could be due to increased excretion in the feces because of sequestration by indigestible material in the intestine or conversion by intestinal bacteria to products that are less readily absorbed. However, in our experiments, neither addition of fibrous materials to the cholesterol-free, semipurified diet nor prevention of coprophagy appeared to have much influence on plasma cholesterol levels (20).

Most of our feeding trials have been short term, lasting only about a month, which is sufficient time for rabbits to develop a marked hypercholesterolemia on the casein semipurified diet. However, a few longer term experiments have also been carried out to compare effects of the casein and soy protein semisynthetic diets on development of atherosclerosis (23). Rabbits on the casein diet maintained their hypercholesterolemia over a period of 10 months (Fig. 1), and sudanophilic lesions were observed in all animals maintained on the diet for 6 months or more. Little if any significant elevation of plasma cholesterol was seen in rabbits on the soy protein diet (Fig 1), and the aortas of these animals showed no sudanophilia after periods of as long as 11 months on the diet. Rabbits on this diet showed a slightly better growth performance than those on the casein diet (Fig. 1).

Cholesterol-free, semipurified diets have also been fed to a number of other animal species, and their effects on blood cholesterol levels were reviewed previously (19). Dogs (25,26), monkeys (27), and baboons (28-30) tended to show a hypercholesterolemic response, whereas pigs did not (31). The possibility that the protein component of these diets may influence the level of plasma cholesterol in these species has not, to my knowledge, been investigated.

As long ago as 1957, Yudkin (32) and Yerushalmy and Hilleboe (33) called attention to the strong positive correlation between the incidence of coronary heart disease and the amount of animal protein ingested in different countries. There is also general agreement that severe deficiency of dietary protein is associated with a reduction of serum cholesterol (34,35). This can be restored to more normal levels by supplementing the diet with proteins or amino acid mixtures (35-38). There is also recent evidence that diets high in protein have a tendency to elevate the level of serum cholesterol (39). It is less clear, however, whether at normal levels of intake the type of protein ingested has any influence on serum cholesterol levels. Anderson et al. (40) observed no differences between subjects on two different diets in which half the daily protein intake consisted of either egg white or wheat gluten, but other studies have provided evidence that a change from animal protein to plant protein in the diet is associated with a decrease in the level of serum cholesterol (41-44). Some of the observed effects may be due to associated changes in other components of the diet such as fat and cholesterol, but Sirtori et al. (44) found that addition of cholesterol to their soybean protein diet did not modify its effect.

The studies of Sirtori et al. were carried out with hypercholesterolemic subjects. We have been investigating the effect on plasma cholesterol levels of substituting soybean protein for animal protein in the diet of normocholesterolemic human subjects (Carroll, Giovannetti, Huff, Moase, Roberts, and Wolfe, unpublished experiments). The fat and sterol composition of the diet was maintained as constant as possible, and the soybean protein diet still gave a significantly lower level of plasma cholesterol.

Olson et al. (41) reported in 1958 that a marked decrease in serum cholesterol occurred in human subjects transferred from a diet containing 100 g of animal protein to an isocaloric, isofatty diet containing 25 g of vegetable protein derived from cereals, rice, and legumes. More recently, Olson and his associates (45,46) have experimented with amino acid formula diets containing three times the tentative daily requirement of the eight essential amino acids, plus a source of nonessential nitrogen to keep the diets isonitrogenous. The use of glutamic acid as a source of nonessential nitrogen gave a lowering of serum cholesterol which was not observed with other diets in which glycine plus ammonium acetate was used. There was a fall in S_f0-12 β-lipoproteins, which was associated with a decreased rate of entry of cholesterol

into the plasma pool (45). The feeding of glutamate was also observed to produce a lowering of serum cholesterol in gerbils, thus providing an experimental model with which to study this effect (47,48). Coles and Macdonald (49) observed lower cholesterol and higher triglyceride values in the serum of human subjects in which the dietary protein was replaced by an amino acid mixture. An attempt in our laboratory to reproduce this effect in rabbits by feeding a similar mixture of amino acids in a cholesterol-free semipurified diet, was unsuccessful (Huff and Carroll, unpublished experiments).

Several of the studies referred to above provide evidence that replacement of animal protein in the diet by plant protein is accompanied by a decrease in the level of blood cholesterol. Recently reported studies on a group of vegetarians living in the United States likewise indicate that they have lower plasma cholesterol levels than the population as a whole (50). These findings provide an indication that the level of plasma cholesterol in humans may be influenced by the kind of dietary protein as well as the amount in the diet.

There is good evidence that hypercholesterolemia can be reduced in human subjects by decreasing the fat content of the diet or by substituting polyunsaturated fat for saturated fat in the diet, but there is need for alternative methods to achieve even greater reduction of cholesterol levels, particularly for individuals at high risk from cardiovascular disease. More consideration should be given to the possibility that this can be achieved by varying nonlipid components of the diet such as protein.

ACKNOWLEDGMENTS

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Fiber, Hypercholesteremia, and Atherosclerosis

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ABSTRACT

Epidemiological data suggest that populations subsisting on high fiber diets are free of a number of the diseases of Western civilization, among them coronary heart disease. Studies in animals and man show that each type of fiber exerts it own specific influence. Thus, in man bran has no effect on serum lipids, but pectin lowers cholesterol levels. In animals fed atherogenic diets, alfalfa and pectin exert some measure of protection, but cellulose does not. To fully understand the mode of action of dietary fiber, it is necessary to ascertain the mechanism(s) of action of each chemical component of that fiber.

Trowell (1,2) and Burkitt et al. (3) suggest that the absence of coronary disease in certain populations, such as Africans, is due to the fiber content of their diets. Their publications have reawakened interest in the effects of fiver on lipid metabolism and atherosclerosis.

Fiber is a generic term for substances that are generally regarded as indigestible or nonnutritive. Although even this definition is open to debate, fiber is generally understood to be plant residues. The major components of plant fiber are cellulose, hemicellulose, pectin, and lignin; among the minor components are gums and cutins. With new methodology available (4,5), it is possible to determine more precisely the composition of fibrous materials. The experiments being carried out at the present time use both heterogeneous and purified plant materials.

In man, experiments with dietary fiber are limited to the sampling of serum, analysis of excreta, and monitoring of weight. In experimental animals, it is possible, of course, to assess tissue cholesterol and atherosclerosis.

Walker and Arvidson (6) suggested, in 1954, that the South African Bantu were free of heart disease because of their large daily intake of fiber. Antonis and Bersohn (7) fed four diets differing in fat and fiber content to white and black prisoners in South Africa. The fat in the diets comprised either 15 or 40% of calories, and the fiber content was either 4 or 15 grams.

TABLE I

Influence of Fat and Fiber on Fecal Steroid Excretion in Man[a]

Subjects	Fat[b] (cal %)	Fiber (g)	Steroid (g/week)	
			Acidic	Neutral
22 White	40	15	3.2	3.4
		4	3.2	2.8
21 Bantu		15	3.6	4.1
		4	3.7	2.2
18 White	15	15	3.2	3.8
		4	2.2	2.1
13 Bantu		15	2.8	3.8
		4	2.2	1.9

[a]After Antonis and Bersohn (7).

[b]On high fat diet, high fiber regimen fed 29 weeks, low fiber 22 weeks. On low fat diet, high fiber regimen fed 17 weeks, low fiber 15 weeks.

The high fiber diets resulted in increased excretion of fecal steroids. The white subjects fed the high fat-high fiber diet excreted 48% more neutral steroids in the feces than when they were fed the high fat-low fiber diet. In the Bantu, there was a 90% increased excretion of neutral steroids when the high fat diet was also high in fiber. Acidic steroid excretion was not affected in either group. On the low fat diet, however, an increase in the fiber content from 4 to 15% resulted in increased excretion of acidic (43-24%) as well as neutral steroids (81-96%) (Table I) for both whites and blacks.

Cellulose, at the level of 15 g/day, was found not to affect serum cholesterol levels in man (8). When children (10-12 years old) were fed 4 g of cholesterol and 100 g of cellulose daily, their cholesterol levels fell (9). One can only wonder at the other effects produced by the feeding of such massive doses of sterol and fiber to children.

Bran has received wide publicity as the ideal fiber. Its effects on serum lipid levels, however, are uniformly nil. Truswell and Kay (10) reviewed the literature and cited ten studies in which an average of 37 g of bran (14-100 g) was fed for periods ranging from 3 to 19 weeks. There was one report of lowered cholesterol (7%) and one of reduction of triglycerides (18%).

Walters et al. (11) reported that the feeding of 39 g/day of bran did not affect steriod excretion. Jenkins (12) and Cummings (13) and their co-workers fed 30 and 45 g of bran, respectively, and found increased excretion of steriods.

Keys et al. (8) found 15 g/day of pectin to be hypocholesteremic as did Palmer and Dixon

TABLE II

Influence of Special Diets on Atherosclerosis in Rabbits[a]

Group	No.	Fiber	Fat (%)[b]	Serum cholesterol[c] (mg/dl)	Avg. Atheromata	
					Arch	Thoracic
1	11	Cellulose	HCNO (14)	207 ± 36AB	1.2	0.5
2	12	Cellulose	HCNO (12) Stock (2)	249 ± 41CD	1.1	0.7
3	7	Stock	HCNO (14)	64 ±9ACE	0.5	0.3
4	14	Stock	HCNO (12) Stock (2)	35 ± 2BDE	0.3	0.2

[a]Diets 1 and 2 contain 40% dextrose and 25% casein. Fed 180 days. In diet 3, fiber is residue remaining after removal of stock diet fat by solvent extraction. In diet 4, stock diet was remilled with 12% added HCNO.

[b]HCNO = hydrogenated coconut oil; stock = fat present in stock diet, iodine value, 115.

[c]Values bearing same letter are significantly different.

(14) who fed 6-10 g of this substance. Durrington et al. (15) reported a hypocholesteremic effect when 12 g of pectin were fed, and Jenkins and his co-workers (16) found 36 g of pectin to lower cholesterol levels. Fahrenbach et al. (17), on the other hand, found 6-12 g doses of pectin to be without effect. Kay and Truswell (18) fed 15 g of pectin to nine men for 3 weeks. Triglyceride levels were unaffected, but cholesterol levels fell by 15%. Transit time did not change, but the dry weight of feces rose by 30%, fecal neutral steroids by 16%, and fecal bile acids by 40%. All values returned to pretreatment levels 3 weeks after cessation of pectin administration.

Guar gum has been shown to lower serum cholesterol levels (16,17). The extent of lowering increases with higher doses. A hydrophilic colloid has been shown to lower serum cholesterol levels and increase excretion of bile acids (19). Bagasse, on the other hand, did not affect serum lipids although it reduced fecal neutral steroid excretion and greatly enhanced bile acid excretion (11).

Kiehm et al. (20) have fed diabetic men a 2200 caloric diet which contained 75% of the calories as carbohydrate (72% polysaccharides) and 14.2 g crude fiber. Serum cholesterol, triglyceride, and glucose levels fell by 24, 15, and 26%, respectively. Grande (21) reviewed data from twelve studies in which sucrose was replaced isocalorically by starches (fruit, bread, legumes, etc.). In every case, there was a reduction in serum cholesterol values. It would be interesting to recalculate the composition data from these diets in order to determine what the increases in dietary fiber actually were.

In rats fed cholesterol, cellulose may actually exert an untoward effect, increasing liver as well as serum cholesterol levels (22-24). Pectin, however, significantly reduces liver cholesterol in these animals (22-28). Pectin also reduces cholesterol levels in rabbits whether they be fed cholesterol-containing (29) or cholesterol-free diets (30).

Vegetable gums such as guar gum (24,26) or carageenan (24,26,31) reduce cholesterol levels in rats or chickens. Agar (24) or alginic acid (23), on the other hand, increase liver cholesterol levels.

Grains such as barley, corn, wheat, or oats reduce plasma cholesterol levels in rabbits (30). Bran has no effect on the plasma lipids of cynomolgus monkeys (32) or rats (25).

Portman and Murphy (32) showed that substitution of a semipurified diet for stock ration in rats caused a marked drop in fecal steroid excretion. Pectin will increase bile acid excretion in rats fed cholesterol (33), and alfalfa will increase fecal steroid excretion in rats fed cholesterol-free diets (34).

Fiber will reduce the severity of atherosclerosis in animals fed atherogenic diets. Cookson et al. (35) found that a 9:1 mix of alfalfa:calf meal prevented cholesteremia and completely inhibited atherosclerosis in rabbits fed 600 mg of cholesterol daily. Fisher et al. (36) fed chickens atherogenic diets containing either 3% cellulose or 3% pectin. The severity of aortic atherosclerosis (0-3 scale) was 1.71 on cellulose and 1.36 on pectin ($p < 0.05$).

Lambert et al. (37) and Malmros and Wigand (38) reported in 1958 and 1959, respectively, that semipurified, cholesterol-free diets containing saturated fat were atherogenic for rabbits. A review of the literature (39) shows that saturated fat (up to 35%) was not atherogenic when added to stock diet but was atherogenic as part of a semipurified diet. We speculated (39) that the fiber present in the stock diet was the factor that rendered the saturated fat nonatherogenic. Experiments with extracted stock

TABLE III

Interaction of Fiber and Protein in Atherogenic Diets for Rabbits[a]

	Casein			Soy protein		
	Cellulose	Wheat straw	Alfalfa	Cellulose	Wheat straw	Alfalfa
Survival	8/14	12/14	10/14	5/14	13/14	13/14
Wt gain (g)	-99	-329	-50	-76	186	335
Serum, mg/dl						
Cholesterol	402 ± 40	375 ± 42	193 ± 34	248 ± 44	254 ± 35	159 ± 20
Triglycerides	164 ± 45	94 ± 19	60 ± 8	41 ± 8	66 ± 9	62 ± 17
Liver, mg/g						
Cholesterol	8.1 ± 0.8	8.4 ± 0.5	7.3 ± 0.7	9.3 ± 1.1	8.5 ± 0.5	7.7 ± 0.3
Triglycerides	4.3 ± 0.4	3.7 ± 0.3	4.2 ± 0.5	4.5 ± 0.5	3.9 ± 0.2	4.2 ± 0.2
Aorta						
Atheroma[b]						
Arch	1.81 ± 0.28	1.17 ± 0.22	0.70 ± 0.11	1.50 ± 0.39	1.04 ± 0.28	0.88 ± 0.22
Thoracic	1.19 ± 0.23	0.88 ± 0.18	0.55 ± 0.20	1.00 ± 0.52	0.77 ± 0.24	0.58 ± 0.17
Cholesterol						
mg/g	2.39	1.28	1.00	2.30	1.19	0.92
Free/Ester	1.81	3.92	5.25	2.38	3.25	5.57

[a]Diets contain 40% sucrose, 25% protein, 15% fiber, 14% hydrogenated coconut oil, 5% salt mix, 1% vitamin mix. Fed for 10 months.

[b]Graded (0-4) after Sudan IV staining.

diet (40,41) proved that this was indeed the case (Table II). Moore (40) fed rabbits a purified diet containing 20% butter and 19% fiber. When the fiber was cellulose or cellophane, the diet was more cholesteremic and atherogenic than when it contained wheat straw or cellophane-peat (14:5). Howard et al. (41) found that the ration obtained by mixing an atherogenic diet with an equal weight of stock diet reduced serum cholesterol levels and atherosclerosis in rabbits.

One mechanism by which fiber might affect cholesteremia and atherogenesis involves the binding and excretion of bile salts (42). This action would reduce cholesterol absorption and increase its turnover. Indirect evidence supporting this hypothesis is available from studies in primates (43,44) and rabbits (45). In vitro studies have shown that several types of fiber are capable of binding bile acids and bile salts. The extent of binding is characteristic for each type of fiber and each steroid (46,47).

The influence of dietary fiber on other variables in the atherogenic diet has been tested. Soy protein has been shown to be less atherogenic for rabbits than casein (48,49). Rabbits were fed semipurified, cholesterol-free diets containing casein or soy protein (25%). When the fiber was cellulose, soy protein was less cholesteremic and atherogenic than casein. Substitution of wheat straw for cellulose did not affect serum cholesterol levels but reduced atherogenicity of both diets. When alfalfa was the fiber, the soy protein diet was still less cholesteremic than that containing casein, but the difference was not as marked as when cellulose or wheat straw was present. The severity of atherosclerosis, however, was the same for both groups when alfalfa was used as fiber (Table III) (50).

Fiber is neither panacea nor miracle ingredient. It is a natural component of the diet which, under certain conditions, may affect lipid metabolism. To properly understand the role of fiber in lipid metabolism, we must ascertain the mechanism(s) of action of the individual components of fiber and how they interact. A diet very high in fiber may pose problems such as inhibition of absorption of essential trace minerals. This side of fiber activity should not be overlooked.

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Dietary Factors in Arteriosclerosis: Sucrose

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ABSTRACT

Epidemiological studies show that coronary heart disease is more common in wealthier countries than in poorer. Such studies cannot, however, isolate which of the dietary or nondietary characteristics of affluence help to cause the disease; they provide only clues that need to be subjected to experimental study. Experiments should be designed on the basis of their ability to produce the multiple abnormalties associated with coronary heat disease (CHD) and not only hypercholesterolemia. They should also explain the association of CHD with obesity, diabetes mellitus, cigarette smoking, and physical inactivity. These considerations suggest that the underlying abnormality that produces CHD is a disturbed hormonal balance. Experiments have shown that a high consumption of sucrose produces not only the wide range of abnormalities seen in CHD but also an increased blood concentration of insulin and cortisol. Since a low intake of sucrose confers many other health benefits, it is a more logical dietary recommendation than that of substituting polyunsaturated fat for saturated fat.

The earliest suggestion about the role of diet in coronary heart disease (CHD) was derived largely from epidemiology. Different populations with widely different prevalences of CHD were compared and were found to have differences in fat consumption that roughly paralleled the prevalence of the disease. Similarly, as one population became more and more affluent, there was an increase in both fat intake and coronary disease. These relationships are not only with fat intake but with any measure of affluence. Twenty years ago the best correlation with the increased prevalence of CHD in Britain, even better than that with any dietary constituent, was found to be with the increase in the number of people having radio and television (1). Any one of the characteristics of affluence, dietary and nondietary, could, on the basis of population comparisons, be indicted as producing CHD.

A second difficulty arises because CHD is caused not by just one factor but by several, a statement with which virtually all observers now agree. One cannot then expect to isolate any one factor by comparing populations that differ in several ways and in varying degree. A population in which many people smoke cigarettes but who are extremely active might have no more CHD than a population in which the people do not smoke but are very sedentary.

This last statement assumes that there really is evidence that both cigarette smoking and sedentariness increase the likelihood of people developing the disease. The evidence comes from the next stage in epidemiology, which moves from a study of populations to a study of individuals. This shows that, within one population, the man who does not smoke is less likely to develop the disease than the man who does smoke, who in turn is the more likely to develop CHD the more he smokes.

As regards diet, the search in individuals for a relationship between fat intake and heart disease has been unsuccessful (2). Two separate studies have shown that men with a first known coronary attack had been taking more sugar than had control subjects (3,4). The inability of other workers to confirm this observation may be due to faults in the design of their studies (5).

LIMITATIONS OF EPIDEMIOLOGY

Whatever the reason for the negative results with fat intake, and of some of the negative results with sucrose intake, epidemiological studies should be taken only as clues that determine the direction for experimental investigation. This is clearly necessary for epidemiological studies that yield contradictory, confusing, or unconvincing results, as do all the studies that have sought to identify dietary components in the etiology of CHD.

There is one epidemiological fact, however, that presents the most promising clue, but which has been entirely or largely ignored as a basis for experiments designed to discover the causes of CHD. Women before the menopause have a relative immunity to CHD, which is largely lost after the menopause, which strongly suggests the possibility that the basis for the development of the disease is an abnormality in hormone activity. This seems to be much more plausible than the commonly held view that CHD is based on a simple disturbance of some biochemical transformation, such as the synthesis of cholesterol from saturated fat.

In addition to the differences in prevalence of CHD between men and women, other features support the idea that experimental studies

should be based on the hypothesis that the disease is determined by a disturbed hormone balance. Firstly, CHD is commonly associated with an abnormally high concentration of insulin and cortisol in the blood. A very recent study has shown that the degree of coronary atherosclerosis, as revealed by angiography, is related to the concentration of cortisol in these patients (6). Secondly, in addition to the increased concentration of these hormones, and the increased blood cholesterol concentration that occurs commonly though not universally, there are increased blood concentrations of triglycerides, uric acid, and blood glucose, a diminished glucose tolerance, an increased platelet adhesiveness, and an abnormal platelet behavior in electrophoresis. It is difficult to explain this wide range of apparently unrelated abnormalities other than by a disturbance in hormone activity.

RELEVANT EXPERIMENTS

Ideally, an experimental search for a cause of coronary heart disease would be to see whether the suspected factor actually produces the disease. But none of the ordinary laboratory animals can be made to develop CHD as seen in man, and it is of course out of the question that one should attempt to do so in man. One is left then with short or long term experiments in animals, and with short term experiments in man, in which to attempt to reproduce some of the characteristics of CHD such as the changes in the blood described above. Experiments should be designed with three objectives in mind:

1. to see whether the suspected agent produces not simply an increase in the concentration of cholesterol but as many as possible of the abnormalities that are associated with CHD;
2. to discover whether the agent produces features that are also produced by other, and recognized, etiological agents – for example, cigarette smoking and physical inactivity; and
3. to see whether the results help to explain the clinical link between CHD and other conditions such as obesity and diabetes mellitus.

It is along these lines that the hypothesis that a high intake of sucrose is one of the causes of CHD has been tested. Since the increase in sucrose intake that has taken place in Western countries, especially in the past century or two, has been accompanied by a fall in the starch intake of about the same magnitude (7), these experiments have compared diets that are identical in all respects except in the nature of the carbohydrate, permitting comparison of the effects of sucrose with those of starch.

EFFECTS OF DIETARY SUCROSE

In experiments of this kind, carried out with several species including rats, spiny mice, pigs, and man, diets with sucrose produced an increase in the concentrations of plasma cholesterol, triglyceride, and uric acid; a diminution in glucose tolerance; an increased platelet adhesiveness; and a change in eletrophoretic behavior of the platelets (8). There is also an increase in the plasma concentration of insulin and of cortisol. All these features are found in patients with coronary disease.

Cigarette smoking also causes an increase in plasma insulin, whereas physical activity causes a fall, thus bringing sucrose into line with these two agents as suggested causes. Both obesity and maturity onset diabetes are fequently associated with an increase in the concentration of plasma insulin. There is also a tissue insensitivity to the action of insulin, which is another effect of dietary sucrose (9). Moreover, the diminished glucose tolerance, on which the diagnosis of diabetes rests, is a feature both of obesity and of coronary heart disease.

On quite different grounds, we suggest a more serious approach by clinicians and health educators in general to the promotion of a reduction of sucrose intake as an important health measure. In comparison to the current widespread and drastic dietary recommendations that saturated fat be reduced and that polyunsaturated fat be increased, we recommend simply a reduction of sucrose. The relative merits of these recommendations can be assessed by seeking answers to the following three questions:

1. Will it reduce the risk of developing coronary heart disease?
2. Will it have any other benefits?
3. Does it present any possible hazard?

In our view, there is a stronger case for implicating sucrose in causing CHD than for implicating fat. For example, there is no way of linking diabetes mellitus and obesity with CHD through the known effects of substituting polyunsaturated fatty acids (PUFA) for saturated fatty acids. But for the purposes of this discussion, it will suffice to say that there is equal though incomplete evidence for the fat hypothesis and the sucrose hypothesis.

BENEFITS AND HAZARDS OF DIETARY CHANGE

What are other benefits that would accrue

either by changing the composition and the amount of dietary fat, or by reducing the amount of sucrose? Little more can be expected from a change in fat composition. The total reduction of fat may be thought to cure or prevent obesity, but it will do so only if it is not unconsciosly accompanied by an increase in carbohydrate. On the other hand, a reduction of sucrose in known to result very often in a reduction of obesity, without the need for any other measures. It will certainly reduce the chances of dental caries, and there is evidence that for many people it provides immediate relief from chronic dyspepsia (10).

In principle, it is very doubtful that one can in any way profoundly modify the diet of any species, including *Homo sapiens*, without introducing some hazard. The consumption of large quantities of PUFA has been made possible only by the very recent development of sophisticated techniques of cultivating oilseeds, and extracting and refining vegetable oils. Before such techniques were available, these oils made only a small contribution to our diets, as they still do in the poorer countries. We cannot ignore the evidence that the large amounts widely recommended nowadays as a preventive of CHD can produce undesirable effects, such as increasing the risk of gallstones and possibly of carcinomatous changes in the skin (11,12). On the other hand, the reduction of the high amounts of sugar that we now consume is not known to be accompanied by any hazard. For all these reasons then, it seems foolish to continue to advocate the considerable change in our eating habits that is involved in changing our fat consumption, while making little or no attempt to reduce sugar consumption.

That CHD is basically a hormonal disease is indicated by the following quotations:

> "Clearly any statement regarding the etiology of coronary heart disease will have to explain the sex ratio .... This strongly suggests an endocrine influence." (13).
>
> ".... The administration of the hormone of the pancreas [insulin] effected a significant counteraction of the ability of estogens to protect the coronary vessels against cholesterol-induced atherogenesis [in cockerels] .... The results of this experiment pose one other problem: Is the diabetic woman's loss of immunity to coronary atherosclerosis a resultant, to any degree, of the effects of exogenous insulin?" (14).

These flashes of inspiration apparently not only failed to illuminate the minds of other research workers, but they rapidly became extinguished in the minds of these perspicacious workers themselves.

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Cholesterol and Repair Processes in Arteriosclerosis

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ABSTRACT

The high cholesterol content of the atheroma and the correlation between elevation of the serum cholesterol and myocardial infractions gave rise to the lipid theory of arteriosclerosis, which assumes that cholesterol induces arteriosclerotic lesions and that its reduction counteracts their development. However, many facts contradict this theory. Therefore, a new hypothesis has been based on the high cholesterol content of old pathological lesions of granulomatous nature and the similarity of atheromata to granulomas. In the latter, a complicated tissue containing a high percentage of cholesterol is deposited in response to the injurious agent, which becomes walled off by this tissue. Thus, cholesterol forms part of a protective mechanism, a hypothesis compatible with the known facts about the relationship of cholesterol to arteriosclerosis.

The number of investigations concerned with lipid changes related to arteriosclerosis has reached unmanageable proportions. Some facts link cholesterol with arteriosclerotic changes (1).

1. The atheroma contains a large amount of cholesterol – a fact emphasized by many investigators.
2. It has been shown over and over again that, on a statistical basis, people with high serum cholesterol levels have a significantly higher incidence of myocardial infarctions.
3. Patients with diseases associated with elevated serum cholesterol levels (e.g., nephrosis) have more advanced arteriosclerotic lesions than comparable normal persons.
4. It has been shown many times that feeding of large amounts of cholesterol to various animals, especially in conjunction with fats of animal origin, is associated with the appearance of cholesterol deposits in the aortas of some species.

These facts lend themselves to several interpretations, but the most widely accepted hypothesis is the lipid theory of arteriosclerosis, which postulated that elevated serum cholesterol is causally related to the disease and that reduction of serum cholesterol is associated with improvement.

However, it behooves us to consider other hypotheses for the development of arteriosclerotic lesions in view of many facts which do not fit in with the lipid theory. Among them are the following:

The earliest lesions are seen in infants and may even occur in utero (2). It is important that these early plaques do not contain more cholesterol than the surrounding tissue (3). Furthermore, it is difficult to explain the spotty distribution of the lesions if one assumes that we are dealing with a generalized disturbance of lipid metabolism. It seems more likely that such a change in metabolism would have to involve the whole organ. One could also cite the old, and never contradicted, reports that feeding of cholesterol increases resistance to infections (4,5). Exercise may increase serum cholesterol but, at the same time, is beneficial in preventing heart disease (6). In contrast to the lipid theory, myocardial infarction frequently precedes rather than follows coronary thrombosis (7). Also, mother's milk contains a considerable amount of cholesterol. Finally, attempts to influence the rate of myocardial infarctions by serum cholesterol lowering agents have not only been entirely negative but have entailed dangers for the patient (8,9), and a critical review of the many endeavors to influence the course of the disease by feeding diets containing large amounts of polyunsaturated oils to humans indicated no improvement (8.10).

Most of the body's cholesterol is endogenous, being produced mainly in liver and intestines. The usual American diet supplies 300-1000 mg of cholesterol/day, of which only about 150-300 are absorbed; this is true even with higher dietary levels. There has been a great deal of controversy about the influence of dietary cholesterol on plasma cholesterol levels, which is understandable from the point of view of the lipid theory. By now, it seems to be well established that there exists a homeostatic relationship between dietary and endogenous cholesterol (11,12) which tends to maintain a constant level of cholesterol in the plasma. The fact that dietary cholesterol does not influence arteriosclerosis was emphasized by Halden and Prokop about twenty years ago (6). Oliver (13) has summarized present thought in these words: "The evidence incriminating dietary cholesterol as a cause of coronary heart disease in developed countries is virtually nonexistent."

Under normal circumstances, tissue cholesterol forms an integral part of cell membranes; it forms part of the various lipoproteins in plasma and is a precursor of bile acids. About 2% is used in the synthesis of adrenal and sex hormones.

In a wide variety of pathological conditions, cholesterol forms a large part of the lesion. This is true in scars, tubercles, gummata, old fibroids, thrombi, cholesteatomata, where it forms a complicated tissue in combination with calcium, fibrin, collagen, and other substances.

The gradual accumulation of cholesterol in pathological lesions has been particularly well described for lesions of granulomatous nature. According to Hadfield (14), such lesions are a convenient term for the invasion of dying or ill-nourished tissue by the tissue ground substance (mesenchyma). The cellular reaction of the mesenchyma to the initiating agent is often rather specific. The center of such a lesion is composed of a tissue containing cholesterol, calcium, collagen, fibrin, etc., all of which serve to isolate the initiating agent of the disease (for instance, the tubercle bacillus, the spirochaete of syphilis, etc.). Cholesterol forms a large part of tissue which walls off the initiating agent; moreover, one cannot ignore the previously mentioned possibility that, in some way, it aids resistance to disease. The initial cellular response to an agent may be rather specific, but the advanced lesion may resemble many others histologically (15), and the final granuloma can be viewed as a compromise between ongoing repair processes and the continued assault of the initiating agent.

The developing atheroma has many of the features of a specific granulomatous lesion. The early changes of the mesenchyma have been particularly well studied by Hauss (16). The intima is invaded by modified smooth muscle cells (17, 18), and these early lesions do not contain any more lipid than the surrounding tissue (3). Gradually, the characteristic plaque develops made up of the modified smooth muscle cells, collagen, debris from dying cells, and varying amounts of lipid including crystals of cholesterol. In view of the similarity of many granulomas, it is inviting to speculate that also in the granuloma constituting the atheroma, the cholesterol is present as a consequence of the lesion and not as its cause. Similar ideas go back to Virchow (19) and were clearly mentioned by Leary (20).

Let us now try to look at some of the facts about cholesterol in light of the idea that cholesterol is part of an attempted tissue repair. Its accumulation in the arteriosclerotic lesion may be viewed as a protective mechanism. The undoubted correlation of high serum cholesterol levels with a higher incidence of myocardial infarction does not permit any conclusion as to which is the primary event, the lesion or the elevated serum cholesterol, and whether there is a causal relationship at all. An elevated serum cholesterol level may be a response to the increased need of cholesterol for the formation of the granuloma.

Animal experiments do not contradict the "repair hypothesis." No freely living animals, including the nonhuman primates, develop a disease which, in its natural history, resembles human arteriosclerosis. There are no myocardial infarctions and no strokes. Changes occurring after the feeding of cholesterol have been interpreted by others as a storage disease, perhaps comparable to hypercholesterolemia in man (21).

Preoccupation with the lipid theory has retarded progress in the search for the initiating agent in arteriosclerosis. At present, it may perhaps be profitable to pay more attention to speculations that the initating factor is an infectious agent (8,22).

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Hyperlipidemia and Premature Arteriosclerosis

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ABSTRACT

In the last decade, understanding of the relationship between plasma lipoprotein concentrations and arteriosclerosis has advanced considerably. Prospective and case-control epidemiologic studies in the general population have established a direct correlation between low density lipoprotein and an inverse correlation between high density lipoprotein concentrations and the risk of coronary disease. Detailed studies of patients and families with genetic hypercholesterolemia, hypertriglyceridemia, and combined hypercholesterolemia hypertriglyceridemia have identified subpopulations at particular risk. Skin fibroblast lines from patients with genetic hyperlipidemias have been used to provide important new information on the regulation by plasma lipoproteins of cellular cholesterol metabolism. We are entering a phase of investigation where epidemiological and biochemical data supplement each other in such a way that the old hypothesis linking plasma lipids to atherosclerosis has new life.

In recent years, new approaches to the study of people with elevated plasma cholesterol and/or triglyceride concentrations (hyperlipidemia) have provided important information linking serum lipids to arteriosclerosis. Hyperlipidemia is usually defined arbitrarily as being present when the plasma cholesterol and/or triglyceride is above the 95th percentile value for the population to which the individual belongs. The prevalence of the three major types of genetic hyperlipidemia (monogenic familial hypercholesterolemia, familial hypertriglyceridemia, and familial combined hyperlipidemia) has been estimated at 0.6 to 3.0% in the general population (1,2). Thus, the bulk of coronary artery disease in the general population is not a function of familial hyperlipidemia. The importance of the genetic disorders is that they provide a unique opportunity to understand mechanisms responsible for the hyperlipidemia and the relationship of hyperlipidemia to arteriosclerosis.

Prospective epidemiological data based on cohorts of healthy, free-living people in Framingham, Massachusetts (3), and in Stockholm, Sweden (4) suggest that there is a gradient of risk of developing heart disease from low to high concentrations of cholesterol and triglyceride. Thus, there are no threshold concentrations below which an individual is "safe" from arteriosclerosis. In free-living populations, factors in addition to plasma cholesterol and triglyceride concentrations such as diabetes, hypertension, and cigarette smoking also influence risk. These variables make it difficult to establish a "cause" of arteriosclerosis. Arteriosclerosis is a multifactorial process in which a set of genetic factors, only one of which may be hyperlipidemia, have interacted with a set of environmental factors, only one of which may be dietary cholesterol and/or saturated fatty acid content.

Earlier epidemiological links between serum cholesterol concentration, dietary composition, and heart disease were established in studies of different cultures and countries where the risk of developing coronary disease varied greatly. Statistically significant correlations were found, for example, between rates of coronary disease, serum cholesterol concentrations, and the intake of dietary cholesterol and saturated fatty acids in Finland with a high prevalence of heart disease and in Japan with a low prevalence (5,6). Correlations between dietary intake of cholesterol and saturated fats, and serum cholesterol concentration in populations within the United States, in Framingham, Massachusetts (7) and Tecumseh, Michigan (8), have been more difficult to establish. Dietary intervention studies in patients free of heart disease (9-11) and those known to have coronary heart disease (12,13) indicate that serum cholesterol can be reduced by changing the composition of the diet, and suggest, but do not establish conclusively, that incidence of coronary heart disease may be decreased. This is the area in which Dr. Connor and Dr. Mann have their fundamental differences.

Cholesterol and triglyceride, being insoluble in water, depend on a system on lipoproteins (14) for their transport in human beings from tissue to tissue. Chylomicrons transport dietary fat from the intestine to peripheral tissues. Very low density lipoproteins (VLDL) are synthesized mainly in and released from the liver, are rich in triglyceride relative to cholesterol, and serve to transport lipid from the liver to the periphery; VLDL are also produced by the intestine. Low density lipoproteins (LDL) are thought to arise in extrahepatic tissues as a metabolic product of VLDL and are rich in cholesterol relative to triglyceride. Although the precise function of

the LDL is unclear, LDL have been shown to regulate cholesterol synthesis and metabolism in a variety of extrahepatic cells. High density lipoproteins (HDL) are synthesized mainly in and secreted by the liver, and function as a cofactor in the esterification of free cholesterol by means of the lecithin-cholesterol acyltransferase reaction. HDL have been postulated to serve as the means by which cholesterol moves from peripheral tissues to the liver, where it is secreted into bile as cholesterol or as bile acids (15).

The pioneering work of Gofman indicated that the lipoprotein concentrations themselves might influence an individual's risk for developing arteriosclerotic heart disease. In case control studies, Gofman showed that the beta or LDL fraction appeared to be atherogenic, while the alpha function containing the HDL appeared to have some protective action (16). The Framingham prospective study established cholesterol and LDL as risk factors (3). More recent case control studies from Hawaii (17) and Framingham (18), as well as the prospective study of a cohort from Framingham (19) are consistent with the hypothesis that plasma LDL concentrations are positively correlated with risk for premature coronary disease, while HDL concentrations vary inversely as risk. The role of serum triglyceride and VLDL in promoting arteriosclerosis is less clear (4,19).

Gofman's original data suggested that younger males seemed to be particularly prone to the atherogeneic potential of LDL (16). In the study of myocardial infarction survivors in Seattle by Goldstein and his colleagues (20), 60% of the male survivors less than 40 years old were hyperlipidemic, as were 60% of the female survivors less than 40 years old. Extensive genetic analysis indicated that in the overall group of survivors less than 60 years old, monogenic disorders of lipoprotein metabolism were present in 20% (1). Goldstein et al. (1) and Rose et al. (21) have both called attention to the entity of combined elevations of plasma cholesterol and triglyceride concentrations as being genetically distinct from elevations of cholesterol or triglycerides; familial combined hyperlipidemia appears to be associated with a significantly increased risk of developing arteriosclerotic disease.

Kindred studies have provided useful information on the risk of developing the vascular complications of arteriosclerosis in the genetically determined hyperlipidemias. The data of Slack (22) and Stone et al. (23) establish a high risk of early coronary death in familial hypercholesterolemia, with male heterozygotes having a 50% risk of first heart attack and a 25% risk of death by the age of 50 years. Risks are clearly higher in men heterozygous for hypercholesterolemia than with men of the same age in the general population. Slack (22) estimates a 50% risk of coronary death in 10 years in a 50 year heterozygote man, contrasting with a 5.8% risk in the same interval in a man from the general population with a total serum cholesterol of 300 mg/dl. The risk of myocardial infarction in kindreds with familial combined hyperlipidemia and with familial hypertriglyceridemia has been studied by Brunzel et al. (24). In the familial combined disorder, the frequency of myocardial infarction among adult living relatives was 17.5% (10 of 57) with 5 (of 57) relatives having myocardial infarctions before the age of 40 and 5 between 50 and 60. The frequency of myocardial infarction in living hyperlipidemic relatives with hypertriglyceridemia was 4.7%, a frequency comparable to normolipidemic relatives (4.5%) or among spouse controls (5.2%). In contrast to the risks inherited with familial hypercholesterolemia and familial combined hyperlipidemia, kindreds with either low LDL or high HDL concentrations have been shown by Glueck and his colleagues to have increased longevity (25).

Familial homozygous hypercholesterolemia carries a nearly universal risk for death from premature arteriosclerosis. Affected children have cholesterol concentrations ranging from 600-1000 mg/dl, have extensive xanthomatosis, and die in the first or second decade from typical coronary arteriosclerosis (26). Skin fibroblast lines from such children have been used ingeniously by Brown and Goldstein (27) to demonstrate the relationship between LDL cholesterol and the intracellular regulation of cholesterol metabolism. Cells from homozygous hypercholesterolemic children lack surface (binding) receptors for LDL. Without surface receptors, the cells are unable to initiate the sequence of LDL uptake and degradation, then free cholesterol-induced suppression of endogenous cholesterol synthesis and activation of cholesterol-ester formation. In these cells, endogenous cholesterol synthesis continues at a high level in spite of the presence of increased cholesterol in LDL in the media bathing the cells. Heterozygous hypercholesterolemic cells have abnormalities of LDL binding intermediate between those of homozygote cells and controls (28). Such a mechanism of cholesterol regulation has been shown as well for human lymphocytes (29) and lymphoid cell lines (30), as well as for endothelial cells (31). Recent data from several laboratories indicate that HDL may interfere with both binding and uptake of

LDL by endothelial cells (31) and by smooth muscle cells (32). Thus, the epidemiological data from large populations relating cholesterol, LDL, and HDL in several ways to arteriosclerotic heart disease is beginning to have its correlates in terms of cholesterol flux and metabolism at a cellular level.

These studies of lipoprotein and cellular cholesterol metabolism have been supplemented by ones in nonhuman primates dealing with arterial injury and endothelial cell and smooth muscle response. Nonhuman primate data suggest that hyperlipidemia per se may result in an injury to arterial endothelial lining and thus initiate the process of platelet-endothelial cell interaction with the release of growth factors for smooth muscle cells and penetration of the arterial wall by lipoproteins (33,34). The hyperlipidemia-endothelial damage hypothesis is supported by dietary studies in nonhuman primates demonstrating induction of arteriosclerosis with cholesterol and saturated fat feedings and regression with time after a return to the animals' normal diet (35).

The studies in humans and in nonhuman primates outlined provide convincing evidence that hyperlipidemia and arteriosclerosis are linked. Data in the last ten years generated by a variety of approaches provide new life to the old hypothesis that serum lipids play a major role in influencing the arteriosclerotic process.

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The Development of Coronary Thrombosis Following Myocardial Infarction

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ABSTRACT

One hundred twenty-one patients who died from myocardial infarction were studied. Occlusive coronary artery thrombosis was present in 32%. In 68% of these, the infarct developed first. Factors inducing thrombus formation include fall in systemic blood pressure, alteration of blood flow, shock, and size of infarct. Factors inducing infarction, apart from hypoxia, may be stress, altered cell membrane permeability, and increased catecholamines.

In 1919, Herrick (1) reported three cases of thrombotic occlusion of atherosclerotic coronary arteries resulting in myocardial infarction. Since that time, most physicians believed that thrombotic occlusion of these vessels was the cause of the infarct which frequently led to the patients death. Prior to this, however, the older literature abounds with reports of myomalacia cordis in which the coronary arteries were narrowed and converted into rigid tubes but were not occluded. Such cases were first reported by Jenner (2) 200 years ago and were emphasized with graphic descriptions by Osler (3) nearly 100 years ago. These findings, however, appear to have been disregarded or forgotten by both pathologists and physicians in the first half of this century. Over the past 50 years, however, the cited frequency of coronary thrombosis as a cause of acute myocardial infarction has ranged from 21 to 100% (4,5).

In recent years, however, accumulation of evidence strongly suggests that the relationship between coronary atherosclerosis, infarction, and thrombosis may not be as direct as has been believed for the past 50 years. This evidence is epidemiological, clinicopathological, and experimental. Epidemiological evidence is based on retrospective studies in patients who were autopsied between 1910 to 1915 and between 1945 to 1950. In this series, there was more coronary atherosclerosis of an advanced type in the period 1910 to to 1915 than in 1945 to 1950, yet the incidence of infarction was greater in the 1945 to 1950 period.

The extreme variability of the incidence of occlusive thrombi found by various workers in myocardial infarction was clarified by Spain and Bradess (6,7) in two separate studies, a decade apart, who showed a correlation between the frequency of thrombi and the duration of survival from the onset of infarction. They found that, with a survival of 1 hr, the incidence was 16%; with a survival of 1-8 hr, the incidence was 36%; and with a survival more than 24 hr, incidence had increased to 54%. The suggestion that thrombosis occurs after the infarction has received confirmation from the work of Erhardt et al. (8) who administered I^{125} labeled fibrinogen to patients with myocardial infarction. They found that in a large proportion of these patients who died, the thrombus discovered in the coronary artery at autopsy was radioactive thus proving that the thrombosis had occurred after the infarction.

Further evidence suggesting that infarction may occur without thrombotic occlusion is seen in the results of long term anticoagulant studies. In terms of survival, a postinfarction population does not respond to anticoagulants homogeneously. One group is associated with thrombotic occlusion, and anticoagulants prolong the survival time and lessen the mortality. Another group exists, however, who will develop further infarcts inspite of anticoagulant therapy and without thrombotic occlusion. Finally, Selye (9) has shown experimentally how stress, combined with challenging agents, may induce widespread myocardial necrosis where the coronary arteries are perfectly normal.

This accumulating evidence suggests, therefore, that in many patients the thrombosis may result from the infarction and not visa versa as was originally believed.

The advent of coronary care units has lessened the mortality from myocardial infarction and has also provided us with carefully documented material for accurate clinicopathologic correlation in these cases. A study was undertaken to determine (a) the incidence of thrombosis in myocardial infarction, (b) the relationship of the age of the thrombus to the age of the infarct, and (c) the factors which may predispose to thrombosis if infarction has occurred earlier. The latter is of importance as the emphasis today is to prevent the infarct extending and provide healing. Should throm-

bosis occur after initial infarction, then obviously the infarct will extend.

One hundred twenty-one patients were studied who had died in a coronary care unit. The incidence of thrombi was 35.6% (44 cases). No thrombi were detected in any coronary artery in 64.4% (77 cases). The thrombi and infarct were examined microscopically, and the age of each was correlated according to known criteria (10,11). This comparison revealed that of the 44 cases in which occlusive thrombia were detected, the infarct and the thrombus were the same age in 14 cases (32%), while the infarct was older than the thrombus in 30 cases (68%).

The infarcts were classified into massive (a full thickness infarct), laminar (in the center of the wall), and endocardial. It was found that of the 34 cases of massive infarction, thrombi were present in 28 cases (82%), but of these the infarct was older than the thrombus in 22 instances (64%). In the laminar group, there were 39 cases in which thrombi were detected in 10 (25%), and of these the infarct was older than the thrombus in 4. The endocardial infarction group comprised 48 cases of which thrombi were detected in only 6 cases (12.5%), and of these the infarct was older than the thrombus in 4 instances. Apart from time of survival and size and type of infarction, the other factors which seemed to determine the incidence of thrombus formation were cardiac enlargement, severe degree of coronary artery narrowing, cardiogenic shock, and, in some cases, a definite precipitating factor such as blood loss (spontaneous or postoperatively) which initiated thrombosis.

SUMMARY

The presence of occlusive coronary artery thrombosis resulting in myocardial infarction is about 32%. In 68%, the infarct would appear to have developed first. The factors which induce thrombus formation in this latter group may be: fall in systemic blood pressure after the infarction, alteration in hemodynamic blood flow in the area of infarction, endothelial damage, and release of thromboplastic substances from the necrotic myocardium. In addition, cardiogenic shock, survival time, enlargement of the heart, and size of infarct are also contributory factors in the induction of postinfarction thrombosis.

The causes of the initial myocardial cell damage are hypothelial, but a combination of factors such as stress, altered cell membrane permeability with local changes in potassium, calcium, magnesium, and sodium ions, and increased catecholamines may render the myocardium increasingly sensitive to hypoxia, the latter induced by coronary artery atherosclerosis and possibly cigarette smoking (12). In some instances, this would result in progressive necrosis of the myocardium followed by thrombosis with extension of the now infarcted area.

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Neural Factors in Experimental Degenerative Arteriopathy

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ABSTRACT

Intermittent electrical stimulation of the lateral hypothalamus of rats performed for 30 min to 6 hr, results in hyperlipidemia and endothelial cell damage of the aorta and coronary arteries. Hyperlipidemia is related to transient biliary obstruction elicited by hypothalamic stimulation and is characterized by elevation of the cholesterol, phospholipid, and triglyceride fractions. Endothelial cell damage is observed ultrastructurally as plasma membrane degeneration with detachment and the formation of large spaces ("vacuoles"). Thus, neural factors may be implicated in inducing conditions associated with early atherogenesis. Stimulation carried out for longer time intervals would be expected to produce more advanced lesions. However, the role of neural transmission per se (i.e., without hyperlipidemia) in producing arteriopathy is not clearly defined from these experiments. In rats, the lesser splanchnic nerve forms the major innervation of the abdominal aorta. In animals fed normal diets, chronic intermittent stimulation of this nerve (up to 3 weeks) resulted in advanced arteriosclerotic changes with intimal fibrosis and calcification. On histologic examination, lipid deposits appeared to be absent from these lesions. Animals stimulated for shorter periods of time exhibited earlier changes associated with atherogenesis, such as endothelial damage, elastic reduplication, and adherent microthrombi. Thus, direct neural transmission, especially if excessive, plays a role in producing arteriopathy. Hyperlipidemia, if persistent, could modify these lesions so that they would accumulate plasma lipids. Experiments to test this hypothesis are currently in progress.

In recent years, behavioral responses to psychosocial conditions have been strongly correlated with the development of human ischemic heart disease (IHD) (1,2). The latter implies the underlying existence of advanced coronary atherosclerosis, although in a few cases nonorganic, vasospastic coronary changes may occur.

Based on extensive studies, epidemiologists have gone so far as to imply that "neural factors" may play an even larger role in atherogenesis than do the recognized "risk factors" of hyperlipidemia, hypertension, diabetes mellitus, and cigarette smoking (3). Furthermore, it is implied that these neural factors may act independently of the accepted risk factors, although the potential for an interaction is not excluded.

The clinical evidence concerning neuropsychologic mechanisms in IHD implies that they may be etiologic agents in the development of atherosclerosis (4). In support of such a possibility is a substantial amount of data in experimental animals, to which our own laboratory has contributed over the past 15 years. The work was performed in young male and female Sprague-Dawley rats. The findings may be summarized as follows:

EXPERIMENTAL PROCEDURES

I. Experiments on the Peripheral Nervous System (PNS)

It was found that animals which were subjected for 3 weeks to intermittent electrical stimulation of autonomic nerves innervating the aorta, demonstrated morphologic changes of the vessel ranging from slight injury to advanced fibrocalcific arteriosclerosis (5). These

TABLE I

Plasma Lipid Changes in Hypothalamically Stimulated Rats[a]

Group	Triglyceride	Total cholesterol	Free cholesterol	Phospholipid
Control	-9.8 ± 24.7[b]	-11.9 ± 9.4	-16.7 ± 13.0	-17.2 ± 13.3
Stimulated	61.9 ± 37.7[c]	41.3 ± 24.6[c]	315.3 ± 224.5[c]	104.5 ± 76.4[c]

[a]Control and stimulated animals consisted of groups of 24 animals each. A lipid of 1 ml of heavy cream (40% butter fat) was administered by intubation. Blood samples were obtained before and 1 hr after hypothalamic stimulation and were analyzed for plasma lipid concentrations. Increments for each animal were averaged in control and experimental groups for all of the lipids concerned and were compared statistically by Student's t test for differences in means. [Reproduced from Gutstein et al. (7) with the permission of the publisher].

[b]Mean values ± standard errors for increments after stimulation; units in percent change.

[c]$p < 0.05$.

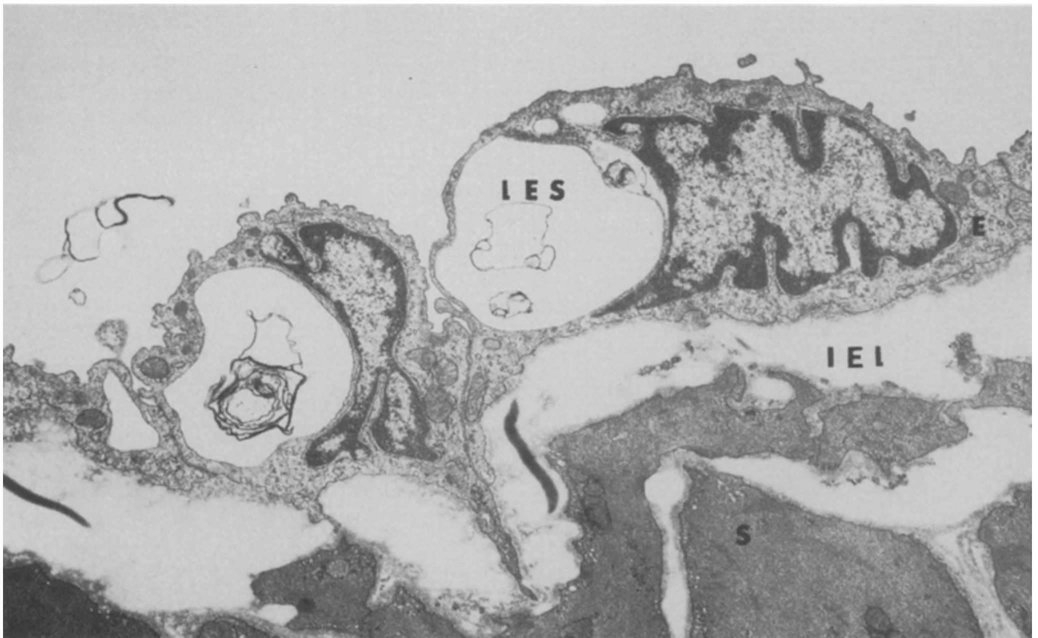

FIG. 1. Endothelial changes in coronary artery of rats intermittently stimulated for 6 hr in LHA. L = lumen; E = endothelial cell; LES = large endothelial space containing membrane fragments; IEL = internal elastic lamina; S = smooth muscle cell. (X 18,000.)

animals had not been placed on special dietary regimes.

II. Experiments on the Central Nervous System (CNS)

These were of two types, those in which plasma lipid changes were predominantly studied and those in which morphologic observations were made. In all experiments, electrical stimulation was delivered intermittently to the lateral hypothalamic area (LHA) for varying periods of time which ranged from 30 min to 6 hr (6).

Plasma lipid concentrations: Baseline blood samples were obtained. Animals were given an intestinal lipid load by intubation (1 ml heavy cream containing 40% butter fat). Analysis of poststimulation blood samples compared with nonstimulated controls revealed elevation of plasma triglyceride, cholesterol, and phospholipid concentrations in the stimulated group. Free cholesterol was more markedly increased than the esterified fraction. Cholesterol/phospholipid (c/p) ratios were decreased (Table I) (7).

Subsequent experiments disclosed the mechanism by which hyperlipidemia was induced in hypothalamically stimulated animals (8). It was discovered that functional biliary obstruction occurred during stimulation. Hence, the hyperlipidemia was similar to that observed in obstructive biliary disease in experimental animals or in man and was consistent with the changes in free cholesterol and c/p ratio noted above.

The role of the intestinal lipid load appeared to be one of inducing choleresis, resulting in a greater effective biliary obstruction at the time of stimulation. Omission of the intestinal lipid load in stimulated animals failed to produce plasma lipid increases.

Additional experimentation disclosed that the plasma lipid responses of stimulated animals could be prevented by pretreating them with pentaerythritol tetranitrate, an agent which dilates not only vascular smooth muscle but biliary smooth muscle as well (9). Another interesting finding which emerged in further experiments was the relationship between the magnitude of the plasma lipid response in stimulated animals and the types of lipid introduced into the intestinal lumen (10). There was an inverse relationship between the magnitude of increase and the carbon double bond number in fatty acids administered in pure form. Thus, highest responses occurred with fully saturated fats (18:0 = +18%), no changes with polyunsaturated ones (18:2 = 0.2%), and intermediate responses were observed with monounsaturated fatty acids (18:1 = +7%). A possible explanation for this relationship is that

the degree of choleresis induced by the administered lipid is an inverse function of the double bond number. This would result in greater effective obstruction in the case of saturated fats in the intestinal lumen than when mono- or polyunsaturated ones are administered.

Endothelial cell changes: Animals subjected to intermittent stimulation of the LHA over a 6 hr period disclosed pronounced endothelial cell changes in their major coronary arteries by electron microscopic examination (11). These were independent of plasma lipid responses or of lipid feeding. They consisted of plasma membrane degeneration, intracellular edema, separation of endothelial cells from the underlying basement membrane accompanied by widening of the subendothelial space, and the formation of other types of large endothelial spaces, both intra- and extracellular (Fig. 1). Some of these alterations could also be found in control animals but with significantly less frequency and severity.

INTERPRETATION OF RESULTS AND DISCUSSION

The morphological observations reported demonstrate that increased neurotransmission, either at the CNS level or regionally at the PNS level, produces arteriopathy in experimental animals. Indeed, if sustained, fibrocalcific arteriosclerosis similar to advanced human lesions may be seen in the rat. Similarly, CNS-elicited neurotransmission induces hyperlipidemia in animals challenged with an intestinal lipid load. It may be anticipated that sustained stimulation in such animals will result in lesions resembling those of man even more closely. Experiments are in progress to test this hypothesis.

Neurogenically induced arteriopathy thus appears to consist of two pathogenetic mechanisms. In one, arterial wall injury is produced. Endothelial cell injury implies that the lining may alter its permeability resulting in accelerated influx of plasma components. The arterial wall will eventually respond to this stimulus with intimal fibrosis and calcification. The second mechanism is that of hyperlipidemia resulting from an interaction of CNS stimulation with intestinal lipid. As described, this hyperlipidemia is mediated via transient, functional biliary obstruction. Lesions may be further modified by accumulating lipid. Such a sequence of events can be demonstrated by mechanically inducing a microscopic injury (12). Fibroelastic plaques then develop at the site of injury. If, in addition, hyperlipidemia is present, lesions accumulate lipid and resemble human fatty streaks.

The investigations described above provide experimental evidence in animals for the role of neural factors in degenerative arteriopathy and atherogenesis. Thus, they support the increasing awareness of the role played by the nervous system in the development of human atherosclerosis. Additional data in animals concerning both experimentally induced and spontaneously appearing atherosclerosis can be found in the literature (1,2,4). It appears then, that the time may have arrived for including neuropsychological mechanisms among the important factors which place the individual at high risk for the development of this major arteriopathy.

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Antioxidants in Neoplastic Cells: I. Changes in the Antioxidative Capacity of Mouse Neuroblastoma Cells Measured by a Single-Phase Assay

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ABSTRACT

Cultured mouse neuroblastoma cells exhibit a striking increase in antioxidative capacity during the transition from logarithmically dividing cells to nondividing, neurite-bearing cells. Two physically separable phenomena are involved: (a) the membrane pellet of neurite-bearing cells is highly resistant to lipid peroxidation, and (b) the postmicrosomal supernatant of these cells inhibits peroxidation in rat liver mitochondria and other biological membranes. A precise, single-phase assay has been developed for assessing antioxidant levels in lipid extracts. By means of this assay, the increase in membrane resistance to lipid peroxidation has been correlated with a threefold increase in the antioxidant activity of the neuroblastoma neutral lipid fraction. This finding implies that generation of a neutral lipid antioxidant (or antioxidants) is involved in the profound increase in antioxidative capacity which occurs in differentiating neuroblastoma cells.

INTRODUCTION

The ease with which the homogenates of most mammalian tissues can be made to undergo lipid peroxidation has been observed in many laboratories (1); in contrast, a number of investigators have reported that neoplastic cells and some other rapidly proliferating cells and tissues are highly resistant to peroxidation (2-10). Over the past three decades, this resistance has been reported in homogenates of solid tumors (2,3,5), ascites tumors (4,10), bone marrow (11), regenerating rat liver (12), and normal and neoplastic cells in tissue culture (5,9). Many of these reports have also shown that the addition of homogenates of peroxidation-resistant cells strongly inhibits lipid peroxidation in biological membranes that ordinarily undergo this reaction. Growth and cell division appear to be common denominators for cells exhibiting these phenomena (1).

The mechanism or mechanisms underlying these observations are unknown. Although a number of explanations could be proposed, the presence of a lipid-soluble antioxidant or antioxidants in cells resistant to peroxidation presented itself as a readily testable hypothesis. This approach was suggested by impressive evidence that α-tocopherol (13) and other antioxidants (14) administered in vivo can prevent lipid peroxidation in biological membranes in vitro and in vivo.

The following report details solutions to two major problems which we encountered in eliciting evidence for such an antioxidant: (a) developing a reliable, single-phase assay system for the antioxidative capacity of lipid extracts; and (b) accounting for the verity that an antioxidant which is active in highly structured biological membranes may not retain its potency in solution, while an antioxidant which is active in solution may not have been active in the membranes from which it was extracted. Means had to be found to correlate the inhibitory potency of lipid extracts with the resistance to peroxidation exhibited by the membranes from which these extracts were derived. Employment of an experimental system in which the extent of resistance to peroxidation could be varied would be a valuable approach to this problem. In such a system, an increase in membrane resistance should be paralleled in lipid extracts by an increase in the concentration of the antioxidant or antioxidants responsible for the resistance phenomenon.

The present work began with our observation of a dramatic increase in resistance to peroxidation during differentiation of mouse neuroblastoma cells (C1300) in tissue culture. A variety of treatments can induce logarithmically growing neuroblastoma cells to undergo a morphological and biochemical differentiation to nondividing cells with neuron-like properties (15). The most striking morphological change is the appearance of long, axon-like extensions (neurites) (16). In this paper, the terms *log cells* and *neurite cells* will designate, respectively, the logarithmically growing and the neurite-bearing forms of neuroblastoma cells. The transition

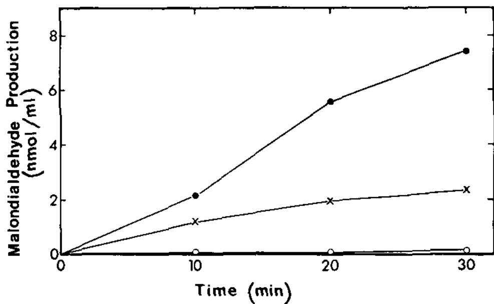

FIG. 1. Response of log and neurite neuroblastoma cells to peroxidizing conditions. •——•, rat liver mitochondria, 0.2 mg/ml protein; o——o, neurite cells, 0.4 mg/ml; x——x, log cells, 0.4 mg/ml. Mitochondria or once frozen-thawed neuroblastoma cells incubated with shaking at 37 C in 0.1 M KCl, 0.05 M potassium phosphate, pH 7.0, 1.0 μM $FeSO_4$, and 0.2 mM ascorbic acid.

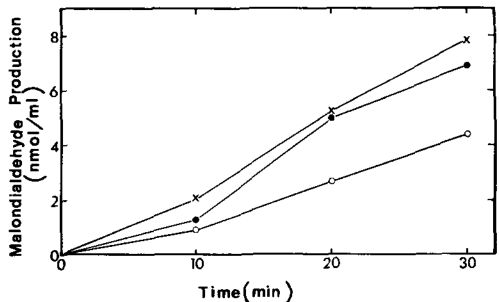

FIG. 2. Inhibition of lipid peroxidation in rat liver mitochondria by once frozen-thawed neuroblastoma cells. •——•, mitochondria alone, 0.2 mg/ml protein; o——o, mitochondria, 0.2 mg/ml, and neurite cells, 0.4 mg/ml; x——x, mitochondria, 0.2 mg/ml, and log cells, 0.4 mg/ml. Conditions are the same as in Figure 1.

from log cell to neurite cell bears a striking resemblance to neuronal differentiation, and the term differentiation is often applied to this process in the current literature. A discussion of the relationships between such modulations and more comprehensive views of differentiation is found in Foulds' monograph (17).

In this communication, we describe the antioxidative phenomena occurring in neuroblastoma cells and relate an increase in the antioxidative potency of the neutral lipid fraction from these cells to the increase in resistance to peroxidation which occurs during differentiation. These studies have provided the basis for pursuing the isolation, characterization, and assay of an antioxidant molecule from differentiated neuroblastoma cells (18).

MATERIALS AND METHODS

Culture and Analysis of Neuroblastoma Cells

Mouse neuroblastoma cells (C1300, clone N-18) were obtained from Dr. Marshall Nirenberg. Cells were grown without antibiotics in Eagle's medium as modified by Dulbecco (Gibco, Grand Island, NY) in 10% CO_2-90% air with 10% fetal bovine serum (Microbiological Associates, Bethesda, MD). The cells were subcultured every 6 days using one-fiftieth of the cells from one of the original flasks to inoculate each new flask. All cell culture was carried out in 75 cm^2 flasks (Falcon Co., Oxnard, CA). The cells were fed every 2 days with 15 ml of medium containing 10% fetal bovine serum except in those cases where 15 ml of medium without serum was added to induce neurite formation.

Cells were harvested as follows. The medium was decanted, and each flask was washed gently with 5 ml of Blume's modified D_1 solution (19). Both medium and wash solutions were discarded. The cells were removed from each flask by serial treatments with 5-ml portions of modified D_1 solution, versene solution (20), versene solution, and modified D_1 solution, in that order. The flasks were incubated 15 min at 37 C with the second 5-ml portion of versene solution. The cell pellet obtained by centrifugation at 400 x *g* for 10 min was resuspended in 40 ml of modified D_1 solution. A sample of the isolated cells was counted in a hemacytometer, and cell viability was determined by the dye-exclusion test using trypan blue (21). Acetylcholinesterase was assayed by the method of Schubert and co-workers (22). One unit of activity is defined as 1.0 nmol of product produced per minute. The cells used in these studies were found to be free of mycoplasma by the method of McGarrity (23).

Neurite cells were produced from log cells by incubation in culture medium prepared without fetal bovine serum. Serumless incubations lasted either 1 or 2 days prior to harvest. In the case of 2-day serumless incubations, fresh medium was introduced on the first day after withdrawal of serum. The preparations used most commonly in these studies were: (a) undifferentiated 3-day cells grown in serum; and (b) differentiated 6-day cells grown in serumless medium from the fourth day after subculture.

An alternative culture method was employed in some initial experiments using once frozen-thawed cell suspension. These experiments include the one illustrated in Figures 1 and 2. In this procedure, the number of cells used to inoculate flasks incubated without serum was ca. 20 times greater than the inoculum for

flasks incubated with serum so that after a 3-day incubation the yield for both log and neurite cells was about 2.5 x 10^6 cells per flask. This equal-yield approach was replaced by the 6-day culture cycle described above after it was found that the latter procedure gave more uniform results.

Estimation of Rates of Lipid Peroxidation in Biological Membranes

Unless otherwise stated, all operations were carried out in a standard buffer: 0.10 M KCl, 0.05 M potassium phosphate, pH 7.1 (at 38 C). All biological samples were estimated as mg protein by the method of Lowry and co-workers using bovine serum albumin as a standard (24). The peroxidation test system is based on the procedure of Ottolenghi (25). The contents of each incubation tube were stored on ice prior to incubation. The reaction was started by addition of freshly dissolved $FeSO_4$ and ascorbic acid in 0.05 ml distilled water. Zero-time samples were taken, and the tubes were incubated at 38 C in a shaking water bath. Aliquots were generally taken at n min and $2n$ min after the addition of the catalysts, n varying widely with the purpose of the experiment. All samples were run in duplicate. The final concentrations used in most incubations were 2 μM $FeSO_4$, 0.4 mM ascorbic acid, 0.2-1.0 mg/ml rat liver mitochondria, and 0.4-4.0 mg/ml neuroblastoma fractions. The malondialdehyde produced during incubation was estimated by the thiobarbituric acid (TBA) procedure of Placer and co-workers (26), with the modification that a final concentration of 0.033 M EDTA was added prior to the 100 C incubation step (27).

Rat liver mitochondria were prepared by the method of Johnson and Lardy (28) and stored in standard buffer at -85 C prior to use. Mitochondrial membrane preparations were made by centrifuging once frozen-thawed mitochondria at 100,000 x g for 90 min at ca. 5 C and homogenizing the resultant pellet in standard buffer.

The procedures of May and McCay were employed to prepare rat liver microsomes and to assay enzyme-dependent, NADPH-dependent lipid peroxidation (29). The incubation mixture contained microsomes (1 mg/ml protein), 4 mM ADP, 0.012 mM $FeCl_3$, 0.3 mM NADPH, 5 mM glucose 6-phosphate, and 1 unit/ml glucose 6-phosphate dehydrogenase, all in 0.1 M Tris-HCl buffer, pH 7.5. A unit of glucose 6-phosphate dehydrogenase is the amount of enzyme which causes an absorbance change of -1.0/min at 340 nm at 25 C under the above conditions. The microsomal incubation mixture was shaken at 38 C and aliquots were assayed for malondialdehyde by the procedure given above.

Disruption of Neuroblastoma Cells

The following preparations were made at a low temperature: Once frozen-thawed suspensions of neuroblastoma cells were prepared from cell pellets stored at -85 C. These pellets were resuspended by hand, using a tube and pestle homogenizer. Hemacytometer counts of these resuspensions indicated that the majority of the cells originally present survived as grossly intact, trypan blue-staining bodies. Attempts to disrupt neuroblastoma cells by osmotic shock failed; however, two methods gave rapid and complete disruption of these cells: (a) homogenization by four, 30-sec treatments with the Brinkman Polytron (Model PT 10 ST) at setting No. 5; 20-sec cooling intervals separated the treatments, and (b) pressure homogenization (30) using 900 psi nitrogen in the Parr cell-disruption bomb, Model 4635.

Preparation and Assay of Neuroblastoma Lipid Extracts

Neuroblastoma lipids were extracted by the procedure of Bligh and Dyer (31). A solvent blank (using standard buffer in place of the cell suspension) was carried through the entire set of extraction and assay procedures described below as a means of detecting interfering substances in the extracting solvents. The phospholipid and neutral lipid fractions were separated by acetone precipitation (32), stored in benzene at -85 C, and used directly in the assay described below.

The following method was developed for the assay of antioxidant activity in neuroblastoma lipid extracts. Cumene (2-phenylpropane; Eastman Organic Chemicals, Rochester, NY) was distilled in a glass apparatus and percolated through a column of neutral alumina to remove peroxides. Azobis(isobutyronitrile) (AIBN; Aldrich Chemical Co., Milwaukee, WI) was recrystallized from methanol. Each tube contained 0.20 M cumene and 2.0 mM AIBN, with or without lipid extract, in a final volume of 250 μl benzene. The tubes were sealed with a Teflon-lined cap and kept in an ice bucket prior to incubation for various times in a shaker-water bath at 60 C; tubes were promptly returned to the ice bucket at the end of the incubation, and the cumyl hydroperoxide thus produced was determined by the following modification of the ferrous thiocyanate reduction method (33). Each tube was diluted with 1.8 ml of 6.7% (w/v) NH_4SCN in methanol. Just before the peroxide determination, the

TABLE I

Growth of Mouse Neuroblastoma (N18) Cultures in the Presence and Absence of 10% Bovine Fetal Serum

Days in culture	Cell counts[a] per flask x 10^{-6}	
	Flasks cultured in 10% bovine fetal serum	Flasks cultured in the absence of serum[b]
3	1.14 ± 0.10	--
4	3.90 ± 0.55	--
5	8.84 ± 0.88	5.12 ± 0.49
6	10.20 ± 0.73	4.97 ± 0.13

[a]Mean ± standard error of the mean for three flasks.

[b]All cells were from the same passage; serum was withdrawn from the flasks indicated on the fourth day of culture. From the fourth day onwards, the medium in each flask was changed daily.

tube was allowed to warm to room temperature, whereupon 10 μl of 0.44% (*w/v*) $FeCl_2$ was added. The absorbance at 490 nm was determined at 5 min after addition of the ferrous salt. The determination was calibrated using standard solutions of ferric ion (33).

RESULTS

The Behavior of Log and Neurite Cells with Respect to Peroxidation

The decline in cell division (Table I), the formation of neurites, and the increase in specific activity of acetylcholinesterase which we have observed upon withdrawal of serum from neuroblastoma cultures were essentially the same as reported by other workers (19). In addition to these changes, withdrawal of serum induced a striking increase in the antioxidative capacity of neuroblastoma cells. When cells were frozen, thawed, resuspended, and incubated in the test system described in the Methods section, the log cells underwent lipid peroxidation, while the neurite cells were completely resistant (Fig. 1). Comparison of Figures 1 and 2 reveals that lipid peroxidation by rat liver mitochondria is inhibited by the neuroblastoma cells, and that neurite cells are more effective than log cells in effecting this inhibition. Thus, the modulation from log cells to neurite cells is accompanied both by an increased resistance to peroxidation and by an increased capacity to inhibit peroxidation in biological membranes.

When neurite cells were disrupted and centrifuged at 100,000 x *g* for 120 min, the two phenomena seen in frozen-thawed cells could be separated. The membrane-containing pellet fraction from neurite cells was completely resistant to peroxidation for at least 180 min under the conditions described in the Methods section; however, pellet fractions from log cells so treated peroxidized rapidly. The neurite cell supernatant fraction was a potent inhibitor of peroxidation in rat liver mitrochondria, but the corresponding pellet fraction transferred no inhibitory effect. Log cell supernatant fractions stimulated peroxidation in the mitochondrial test system.

Yields of inhibitory activity varied, but a neurite cell supernatant concentration of 2 mg/ml protein generally resulted in 80-100% inhibition of mitochondrial peroxidation. The inhibitory activity of supernatants decreased during incubation in the presence of mitochondria, and this decline was more rapid for supernatant preparations having low initial activity (Table II). Attempts to fractionate neuroblastoma supernatants by the usual procedures failed due to the lability of the inhibitory principle; however, the activity could be concentrated using UM10 and UM05 Amicon ultra-filtration membranes (Table II).

A variety of peroxidizing systems was inhibited by neuroblastoma supernatants. Thus, 1 mM cysteine could be substituted for 0.4 mM ascorbate, and the post-nuclear membrane fraction from mouse brain could be substituted for rat liver mitochondria. The enzyme-dependent, NADPH-dependent peroxidation system of rat liver microsomes (29) was also inhibited by neuroblastoma supernatants (Table II).

Antioxidation in Proliferating Cells

A brief survey was made of two tumors and an established cell line available from nearby laboratories. The samples used were rabbit VX2 carcinoma (34), rat adrenocortical carcinoma 494 (35), and human lymphoid cell line PGLC-33H (36). The supernatants from all three cell types were strongly inhibitory, while only a trace of inhibitory activity was seen in

TABLE II

Inhibition of Lipid Peroxidation in Rat Liver Mitochondria and Microsomes by Neuroblastoma Neurite Cell Supernatants[a]

Sample	Time (min)	Malondialdehyde production (nmol/ml)	Percent inhibition
	Incubation A		
Mitochondria	15	4.4	---
	30	13	---
	45	15	
Supernatant alone	15	0	---
	30	0.16	---
	45	0.28	---
Mitochondria plus supernatant	15	0	100
	30	0.16	99
	45	0.32	98
	Incubation B		
Mitochondria	10	6.9	---
	20	16	---
Mitochondria plus supernatant	10	5.1	26
	20	16	0
Mitochondria plus supernatant	10	4.5	35
	20	14	13
Mitochondria plus concentrated supernatant	10	2.5	64
	20	7.0	56
Mitochondria plus concentrated supernatant	10	3.2	54
	20	8.3	48
	Incubation C[b]		
Microsomes	5	4.6	---
	10	9.2	---
Microsomes plus supernatant	5	2.6	44
	10	5.3	42

[a]Mitochondria and supernatants were incubated in 0.1M KCl, 0.05M K_2HPO_4, pH 7.1, 2 μM $FeSO_4$, and 0.4 mM ascorbate at 38 C in a shaker-water bath. The supernatant used in incubation A had been freshly prepared from a neurite cell homogenate while the supernatant used in incubation B had been stored for 4 days at -85 C. Final concentrations (mg/ml protein) were: incubation A, mitochondria, 0.5 mg/ml; supernatant, 0.96 mg/ml; incubation B, mitochondrial membrane preparation, 0.58 mg/ml; supernatant, 1.3 mg/ml; supernatant concentrated on an Amicon UM-05 ultrafiltration membrane, 3.8 mg/ml.

[b]Incubation C: microsomes, 1 mg/ml protein, with or without 0.8 mg/ml neurite cell supernatant, were incubated at 38 C in a mixture containing 4 mM ADP, 0.012 mM $FeCl_3$, 0.3 mM NADPH, 5 mM glucose,6-phosphate dehydrogenase in 0.1 M Tris-HCl, pH 7.5. Data are average values for duplicate incubations.

some of the membrane fractions. The lymphocyte membranes underwent rapid peroxidation, and adrenal tumor membranes peroxidized to a small extent, but rabbit tumor membranes were completely resistant to peroxidation.

A Precise, New Assay for Antioxidant Activity in Lipid Extracts

We have developed a clear, precise, reliable assay for antioxidant activity in lipid extracts. Details of the assay procedure are presented in the Methods section. Peroxide formation in the cumene-AIBN system was linear with time in the presence or absence of lipid extract (Fig. 3); however, extrapolation of the data presented in Figure 3 to zero time indicates that the rate of reaction was slightly higher during the first 5 min than afterwards. Under our assay conditions, the decline in cumyl peroxide production was approximately linear with increasing amounts of neutral lipid extract until the reaction was about 50% inhibited (inset, Fig. 3).

The antioxidant activity of neutral lipid extracts was related to the extent of resistance to peroxidation exhibited by the membranes from which the extracts were derived. Table III presents a comparison of 3-day log and 6-day neurite cells demonstrating the striking increase in resistance to peroxidation which invariably

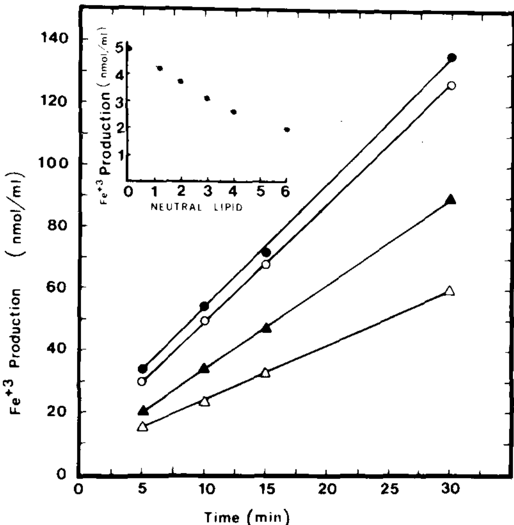

FIG. 3. Time course of cumyl hydroperoxide production at 60 C in the cumene-AIBN assay system for several concentrations of neurite cell neutral lipid extract. The amount of extract added is given in terms of the mg protein of neuroblastoma cells from which the extract was derived: ●——●, 0.0 mg; ○——○, 0.24 mg; ▲——▲, 1.2 mg; △——△, 2.4 mg. Extracts were dissolved in a final volume of 250 μl benzene containing 0.2 M cumene and 2.0 mM AIBN. Cumyl hydroperoxide was estimated in terms of Fe^{+3} production. The 6-day cells employed had been incubated without serum for 2 days prior to harvest. Inset: Inhibition of cumyl hydroperoxide production by a neuroblastoma neutral lipid extract.

accompanied serum withdrawal. The antioxidant activity of neutral lipid extracts from neurite cells was threefold higher than the activity of log cell extracts.

DISCUSSION

A basic goal in the study of neoplasia is to define on the molecular level those changes which distinguish neoplastic cells from normal cells. In contrast to normal tissues, neoplastic cells and some other rapidly dividing cells exhibit a remarkably high resistance to lipid peroxidation (1). In spite of the likelihood that an explanation of this phenomenon would enhance our understanding of neoplasia, only a few reports on the antioxidative capacity of neoplastic cells have appeared sporadically over a period of three decades. We believe that this problem has been neglected largely because there appeared to be no clear approach for relating the observed phenomena to underlying molecular events. Although we acknowledge that other factors, such as a change in membrane structure (37), may also participate in the antioxidative effect, we have undertaken to ascertain whether an antioxidant or antioxidants contribute to the resistance of neoplastic cells to lipid peroxidation, the presence of an antioxidant offering technically the simplest hypothesis to test.

In this paper, we present evidence for the involvement of neutral lipid antioxidants in the resistance of neuroblastoma membranes to lipid peroxidation. This finding resulted after implementation of two technical advances: The first of these was the development of a tissue culture system in which resistance to peroxidation could be varied by manipulation of the culture conditions. The dramatic increase in resistance to peroxidation which occurs in neuroblastoma cultures after withdrawal of serum provides a touchstone for identifying the chemical principles responsible for this resistance. Secondly, we have developed a practical assay for scaling the antioxidant activity of lipid extracts. The TBA reaction is a semiquantitative assay of low precision (Table II), and it is subject to artifacts (27,38). The unsuitability of this test for purification studies led us to develop the cumene-AIBN assay. The temperature dependence of AIBN homolysis (39) ensures reliable control of free radical production in the incubation, and the cumyl hydroperoxide generated can be determined precisely and conveniently by the ferrous thiocyanate method (33). In the presence or absence of neutral lipid extract, standard errors applicable to the values obtained by this method were uniformly below 5%. This assay has proved adequate to permit the purification and ultimate isolation of a chemical principle associated with and possibly involved in the evolution of neuroblastoma membrane resistance to peroxidation (18).

Table III indicates that the increase in resistance to peroxidation is a more dramatic phenomenon than the concomitant threefold increase in neutral lipid antioxidant activity; however, no quantitative comparison can be made between the activity of an antioxidant in solution and its activity in biological membranes. Furthermore, the increased antioxidant activity of neutral lipid extracts from neurite cells may be due to antioxidants which are (a) highly effective in biological membranes, and (b) absent or present in low concentration in extracts from log cells.

Another explanation for neurite cell resistance to peroxidation would be diminished amounts of the polyunsaturated fatty acids which are the substrate for lipid peroxide formation; however, it has been shown that the

TABLE III

Changes Occurring upon Differentiation of Mouse Neuroblastoma Cells[a]

Assay	Passage number	3-Day log cells	6-Day neurite cells
Malondialdehyde production by cell suspensions[b] (nmol/ml; TBA test)	66[c]	13	0.12
	66[d]	24	0.28
Antioxidant levels of neuroblastoma neutral lipid extracts[e] (cumene-AIBN assay; percent inhibition)	66	25.8	72.7
	67	21.5	76.3
Acetylcholinesterase (units/mg)	66	11	62
	67	7.3	44

[a]Undifferentiated neuroblastoma cells (3-day cells) and differentiated cells (6-day cells) were harvested from each of two cell passages (Nos. 66 and 67). Cells harvested on the third day of each passage were cultured in 10% fetal bovine serum, whereas cells harvested on the sixth day had been incubated without serum for 2 days. With the exception of the acetylcholinesterase assays, all data are the means of duplicate or triplicate determinations.

[b]Neuroblastoma cells, frozen-thawed once, were incubated at 4 mg/ml in 0.1M KCl, 0.05 M potassium phosphate, pH 7.1, 2 μM $FeSO_4$ and 0.2 mM ascorbate at 38 C. for the times indicated.

[c]Incubated for 30 min.

[d]Incubated for 180 min.

[e]Aliquots of neutral lipid extract derived from 2.0 mg neuroblastoma protein (ca. 5 x 10^6 cells) were dissolved in benzene and assayed by the cumene-AIBN procedure described in Methods.

fatty acid compositions of log cells and neurite cells are essentially identical (40).

In neuroblastoma cells, and in the other neoplastic or transformed cells which we have examined, resistance to peroxidation is a property of the membrane pellet, while the capacity to inhibit peroxidation in rat liver mitochondria is found in the high-speed supernatant. Although physically separable, these phenomena may be due to the same antioxidant or antioxidants. An antioxidant present in neuroblastoma membranes could act as a protective agent while being unavailable to exogenously added mitochondrial membranes, while the same antioxidant, bound to soluble proteins or other supernatant components, could migrate into mitochondrial membranes. Another possibility is that an enzyme or enzymes in the supernatant is responsible for the inhibition. In addition, an enzyme could be required for the transfer of an antioxidant from supernatant proteins into biological membranes. Although we cannot distinguish between these models at present, neutral lipid extracts from the supernatants of differentiated neuroblastoma cells exhibit high antioxidant activity in the cumene-AIBN assay.

A recent study in this laboratory indicates that glutathione peroxidase, an effective inhibitor of lipid peroxidation (41), is not involved in the antioxidative phenomena seen in neuroblastoma cells (42). In the absence of added selenium, log cells exhibit only trace glutathione peroxidase activity whereas cells differentiated by withdrawal of serum have no detectable activity (42).

Although previous workers have consistently identified high antioxidative capacity with cell division and growth (2), the increase in antioxidative activity in neuroblastoma cells occurs as cell division declines. However, an increase in cell number occurs during the first day after withdrawal of serum (Table I), and a preliminary study indicates that neuroblastoma membranes are almost entirely resistant to lipid peroxidation one day after the withdrawal of serum. Thus, we are not certain whether the capacity for cell division is a necessary condition for these antioxidative phenomena. Additional unknown factors are certainly required, since rapidly proliferating undifferentiated neuroblastoma cells peroxidize readily (Fig. 1), and other dividing cells have been reported to be extremely labile to lipid peroxidation (43,44).

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Antioxidants in Neoplastic Cells: II. Isolation and Partial Characterization of a Phenolic Antioxidant from Differentiated Mouse Neuroblastoma Cells

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ABSTRACT

The generation of an antioxidant has been shown to be associated with the dramatic increase in resistance to lipid peroxidation which occurs during the differentiation of mouse neuroblastoma cells in culture. The antioxidant has been isolated from the neuroblastoma neutral lipid fraction and partially characterized by means of low-resolution and high-resolution mass spectrometry and other lines of evidence. All presently available information suggests that this antioxidant is a highly aromatic, monosubstituted phenol having the molecular formula $C_{19}H_{14}O_2$.

INTRODUCTION

A variety of treatments has been shown to cause mouse neuroblastoma cells growing in culture to undergo a process of morphological and biochemical specialization which strongly resembles neuronal differentiation (1). During this process, the round, logarithmically growing cells flatten and extend long, axon-like processes termed neurites, while cell division is generally diminished or arrested (1).

During differentiation, neuroblastoma membranes exhibited a dramatic increase in resistance to lipid peroxidation (2). This observation suggested that the molecular mechanism underlying resistance to peroxidation might be identified by comparing the compositions of low-resistance and high-resistance cells. Following this approach, we have demonstrated a threefold enhancement in the antioxidative capacity of the neutral lipid fraction of neuroblastoma cells during differentiation (2).

In this paper, we report the isolation and partial characterization by mass spectrometry of a highly aromatic, apparently phenolic antioxidant which is generated during the differentiation of neuroblastoma cells. For convenience, we have designated this antioxidant A274.

METHODS

Mouse neuroblastoma cells (C 1300, clone N-18) were cultured in Eagle's medium as modified by Dulbecco (Gibco, Grand Island, NY) in 10% CO_2-90% air with 10% fetal bovine serum (Microbiological Associates, Bethesda, MD). Harvesting of cells and the preparation of neutral lipid extracts were carried out as previously described (2). Preparations of differentiated or undifferentiated cells were produced by two protocols reported in the preceding paper (2). In the first protocol, cells were cultured in 10% fetal bovine serum and harvested on the third day of subculture (3-day cells). In the second protocol, differentiation was induced by withdrawing fetal bovine serum from the medium on the fourth day of subculture; fresh serum-free medium was introduced on the fifth day, and the cells were harvested on the sixth day (6-day cells). When cultured in this manner, 3-day cells were undifferentiated and readily underwent peroxidation, while 6-day cells had formed neurites and were highly resistant to peroxidation (2). The test system for assessing lipid peroxide production by neuroblastoma membranes has been described (2). Protein was estimated by the method of Lowry and co-workers (3).

The assay developed in this laboratory (2) for estimating the antioxidative activity of lipid extracts was modified as follows in order to increase its sensitivity: an extract sample was tested in a final volume of 250 µl benzene containing 1.2 M isopropylbenzene (cumene, Eastman Organic Chemicals, Rochester, NY) and 0.1 mM azobis(isobutyronitrile) (AIBN, Aldrich Chemical Co., Milwaukee, WI). This mixture was incubated for 15 min in a sealed tube at 60 C in a shaking water bath. Cumyl hydroperoxide formation was determined by the ferrous thiocyanate method (4).

Extracts of neutral lipid from neuroblastoma cells were fractionated on an LH-20 Sephadex column using freshly distilled ethanol as the eluting solvent (5). A column 19 cm in height and having a bed volume of 17 ml was generally employed, but smaller columns were also used, including some made from Pasteur pipets. The Pasteur pipet columns were 7.0 cm in height and had a bed volume of 1.4 ml. Neutral lipid

samples derived from neuroblastoma (100 mg to 300 mg protein) were dissolved in 0.3 ml ethanol, placed on the large column, and eluted with ethanol; 1.0 ml fractions were collected. Since ethanol interferes with the cumene-AIBN assay, column fractions were dried in a vacuum oven at 60 C and redissolved in an appropriate volume of benzene. A water aspirator provided the vacuum. Experiments have shown that the antioxidant activity of column fractions is stable to incubation in a vacuum oven up to at least 135 C. Aliquots (10 μl-20 μl) from column fractions were assayed by the cumene-AIBN procedure and the results expressed as percent inhibition. The mid-peak tubes from the inhibitory peak (containing about three-fourths of the total activity of A274) were pooled and stored at -85 C.

Silica gel TLC medium (ITLC-SA) was purchased from Gelman Instrument Co., Ann Arbor, MI. Silicic acid, type CC-7 (Mallinckrodt Chemical Works, St. Louis, MO) was activated for 1 hr at 110 C. Silicic acid columns ca. 1 cm in height were prepared using Pasteur pipets and washed with several column volumes of ethanol prior to use.

The monoacetate of A274 was prepared by a modification of Gill's method (6). The acetic anhydride used was twice-distilled. Sylon BTZ (Supelco, Inc., Bellefonte, PA) was employed to prepare the monokis(trimethylsilyl) ether of A274; the sample of A274 was evaporated to dryness with nitrogen in a 0.3 ml reaction vial (Pierce Chemical Co., Rockford, IL), whereupon the vial was sealed, and 20 μl Sylon BTZ was injected. The contents were mixed, allowed to stand for 10 min at room temperature, and then incubated at 60 C for 30 min. The monomethyl derivative of A274 was prepared using diazomethane generated from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (Aldrich Chemical Co., Milwaukee, WI) by the micromethod of Schlenk and Gellerman (7).

Low-resolution mass spectrometry was carried out using the following Finnigan components: a 1015D electron impact mass spectrometer and a 3200 chemical ionization mass spectrometer were separately interfaced to 9500 gas chromatographs and collectively interfaced to a 6000 minicomputer data system. Gas chromatography-mass spectrometry (GC-MS) was carried out using a 3% SP-400 column (80/100 Supelcoport, 2 mm ID, length, 45 cm) (Supelco, Inc., Bellefonte, PA). The temperature program generally employed was 150-240 C at 10 C/min. Methane (18 ml/min) served as both the carrier gas and the reagent gas for chemical ionization. A direct insertion probe was also used to introduce samples into the source. These studies employed 20-μl samples in benzene, which were derived from ca. 1 mg protein. About 5 μl of a sample was used for each test.

High-resolution GC-MS was carried out with an A.E.I. MS50/DS50 system, using a 2% SE-30 column with helium (35 ml/min) as the carrier gas. Data were obtained during the course of each run by rapid, repetitive scanning at high resolution ($>$10,000 M/ΔM).

RESULTS

A 120-fold increase in the cumene/AIBN ratio afforded a 16-fold increase in the sensitivity of the assay. The kinetics of the high-sensitivity assay were similar to those reported for the original assay (2), except that the inhibitory potency of a lipid extract was no longer approximately constant with time but declined measurably during the course of the incubation. This result is reasonable since lower extract concentrations were employed, and essentially all chain-breaking antioxidants are degraded in the course of their action (8).

Chromatography of the neutral lipid fraction from differentiated, 6-day neuroblastoma cells on LH-20 Sephadex resulted in two major peaks whose antioxidant activity could be demonstrated by the cumene-AIBN assay (Fig. 1). Using the large column, we found these peaks at 14 ml (peak I, 0.82 V_{bed}) and 24 ml (peak II, 1.4 V_{bed}). An identical elution pattern was observed on the smaller columns. We believe these two peaks are due to different chemical substances. When peak I was rechromatographed, the elution volume of the activity was still 0.82 V_{bed} and no activity appeared in the region of peak II. Similarly, peak II rechromatographed at 1.4 V_{bed} and no activity appeared in the region of peak I.

In contrast to neutral lipid extracts from 6-day cells, extracts from undifferentiated, 3-day cells clearly exhibited peak I but showed only a trace of peak II when chromatographed on LH-20 Sephadex (Fig. 2). Comparison of Figures 1 and 2 indicated that peak II activity was associated with differentiation in neuroblastoma cells, whereas differentiated and undifferentiated cells yielded comparable peak I activity.

Trace contaminants found in a variety of polar solvents also inhibit the peroxidation of cumene, and these contaminants have proved difficult to remove. For example, a trace of antioxidant activity derived from the solvent remained in the column fractions after the 60 C vacuum evaporation step. One-milliliter portions of ethanol were concentrated

in vacuo as blanks and redissolved in 1.0 ml benzene; 10-μl aliquots of this solution gave background readings of 5% to 10% inhibition. In consequence, the amount of neutral lipid chromatographed must be adjusted so that analysis of the column fractions can be carried out on 10-μl to 30-μl aliquots. We have not been able to detect these contaminating trace antioxidants by mass spectrometry.

A274 was strongly absorbed (or inactivated) by silicic acid. Only traces of antioxidant activity could be recovered when neutral lipid extracts were spotted on strips of silica gel-impregnated TLC sheets and extracted with methanol overnight. A silicic acid column, ca. 1 cm, was set up as described in the Methods section, and a sample of A274 purified by LH-20 Sephadex chromatography was dissolved in ethanol and placed on the column. The column was eluted with 6 ml of ethanol. No antioxidant activity was recovered in the effluent.

Low-resolution chemical ionization mass-spectrometric studies were carried out on lipid fractions from about ten passages of neuroblastoma cells, and electron-impact spectra were occasionally examined. A 274-dalton molecule was invariably found in samples of LH-20 Sephadex peak II but has not been detected in other column fractions. Minor contaminants, the majority of which appeared to be phthalates, occurred irregularly in the spectra, but the only recognized constituent of peak II was the molecule which we have designated A274 (Antioxidant, molecular weight 274 daltons). This molecule underwent a strongly favored cleavage to produce a fragment of *m/e* 181. Indeed, the chemical ionization spectrum of A274 virtually consists of two ions, *m/e* 275 and *m/e* 181, and the electron-impact spectrum contains only *m/e* 181, the molecular ion, *m/e* 274, being extremely weak in intensity.

The formation of the monomethyl, monoacetyl, and monokis(trimethylsilyl) derivatives of A274 was confirmed by the upward shift in mass of the M+1 ion (14 daltons, 42 daltons, and 72 daltons, respectively) in the chemical ionization mass spectrum. Despite overnight exposure to diazomethane, only a partial conversion (ca. 25%) into the monomethyl ester occurred, but complete conversion into the monoacetyl and monokis(trimethylsilyl) derivatives readily occurred. The *m/e* 181 fragment was the most abundant ion in all of the spectra we have obtained for A274 and its derivatives.

The high-resolution mass spectrometric study of a typical peak II sample confirmed that a very low level of contaminants was present and revealed the same two ions observed by low-resolution mass spectrometry.

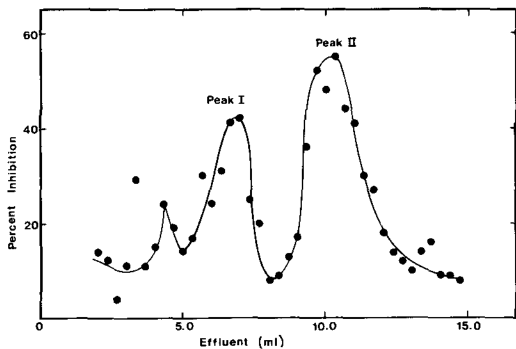

FIG. 1. Chromatography on LH-20 Sephadex of the neutral lipid fraction from 6-day (differentiated) neuroblastoma cells. A 0.10 ml neutral lipid sample derived from 24 mg neuroblastoma protein was applied to a column (0.7 cm x 20 cm, V_{bed} = 7.6 ml) equilibrated with ethanol; 0.33 ml fractions of ethanol effluent were collected, dried at 60 C in a vacuum oven, and redissolved in a volume of benzene five times the original volume of ethanol. Antioxidant levels were assessed by assaying 100-μl aliquots of each fraction using the cumene-AIBN procedure described in Methods. The 60 C incubation step was 15 min in duration. The data shown represent the means of triplicate assays.

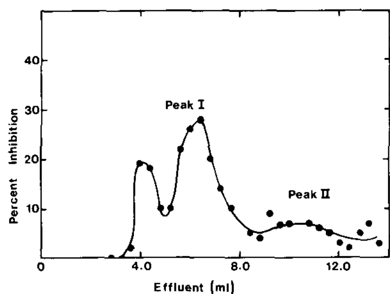

FIG. 2. Chromatography on LH-20 Sephadex of the neutral lipid fraction from 3-day (undifferentiated) neuroblastoma cells. Conditions were identical to those described in Figure 1, except that each fraction of effluent was 0.40 ml.

The exact masses of A274 (274.1000 amu) and of the fragment (181.0653 amu) best accord with the empirical formulations $C_{19}H_{14}O_2$ and $C_{13}H_9O$, respectively; the portion lost in the fragmentation process is undoubtedly C_6H_5O (from a phenol group).

DISCUSSION

A remarkably strong resistance to lipid peroxidation by the membranes of neoplastic cells and some other rapidly dividing cells and tissues has been reported by a number of investigators over a period of three decades (9).

The mechanism or mechanisms underlying these observations are unknown. The well-documented association of resistance to peroxidation with neoplastic cells led us to investigate the mechanism of this phenomenon.

The dramatic increase in peak II antioxidant activity during the differentiation of neuroblastoma cells suggests that peak II (more specifically A274, which is the only recognizable component) is involved in membrane resistance to peroxidation. We have reported that, although the membranes of differentiating neuroblastoma cells underwent a dramatic transition from rapid peroxidation to profound resistance to peroxidation, only a threefold increase in the antioxidant activity of neutral lipid extracts occurred during differentiation (2). This apparent discrepancy was probably due largely to the presence of peak I antioxidant activity in neutral lipid extracts from both undifferentiated and differentiated cells. The antioxidant component or components of peak I did not confer upon biological membranes the same magnitude of resistance to peroxidation as that observed for membranes yielding peak II, but the presence of peak I in the neutral lipid fraction produced a high background in the cumene-AIBN assay.

Mass spectrometry of A274 and its derivatives yielded findings which imply that A274 possesses a single, only moderately reactive, alcoholic group located on a portion of the molecule which weighs only 93 amu and which is selectively lost upon fragmentation. Thus, a partial structure of A274 may be proposed as illustrated:

$C_{13}H_9O$ — (benzene ring) — OH

where $C_{13}H_9O$ is (or rearranges as a cation to become) highly aromatic. Such a structure is consonant with the facts that (a) the *m/e* 181 fragment is extremely stable, (b) potent antioxidant activity is commonly associated with phenols, and (c) peak II (A274) experiences strong, selective adsorption to the aromatophilic LH-20 Sephadex-ethanol chromatographic system (demonstrated in the elution volume, which is considerably greater than the bed volume of the column), which indicates considerable π-electron delocalization (aromaticity) (10).

The mass spectrum of A274 is absent from the ca. 40,000 entries in the Cyphernetics Mass Spectral Search system (Cyphernetics Corp., Ann Arbor, MI), and a search of the formula indices of *Chemical Abstracts* failed to identify any obvious candidates having this atomic composition. This suggests that A274 may not have been previously described, despite the large number of lipid composition studies on neoplastic material (11). A274 is undoubtedly present in a concentration that is very low relative to that of the major components of the neutral lipid fraction, so an extremely sensitive and specific assay is required for its detection. Further, we observed that the peak II antioxidant activity disappeared whenever a sample was applied to a silicic acid column or a silica gel TLC strip and that the activity could not be recovered by extensive washing with ethanol or methanol. This effect could have resulted either from inactivation of A274 or from extremely tight binding of it to silicic acid. Thus, the conventional methods for purifying lipids would eliminate A274.

The proposed structure for A274 is consistent with potent antioxidant activity, but we have yet to prove that all or most of the peak II activity is accounted for by the A274 present. If A274 is the sole or major principle acting, then acetylation of A274 must remove the antioxidant activity. Aliquots of peak II were reacted with several concentrations of acetic anhydride, and the remaining inhibitory activity was determined by the cumene-AIBN assay. A qualitative decrease in inhibition correlated with increasing acetic anhydride concentration (and presumably, extent of acetylation). A quantitative analysis is being developed for A274 which will allow us to refine this experiment by comparing the observed decline in activity with the relative amounts of A274 acetate and unreacted A274.

A major question which arises from this work is whether A274 or similar compounds can generally account for the extreme resistance to peroxidation observed in many neoplastic cells (9,12). The survey required to answer this question will follow the complete characterization of A274 and the development of an inexpensive, quantitative assay for this antioxidant.

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Effects of Dietary Vitamin E, Selenium, and Polyunsaturated Fats on In Vivo Lipid Peroxidation in the Rat as Measured by Pentane Production

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ABSTRACT

Starting at 21 days of age, groups of six rats each were fed a basal Torula yeast diet supplemented with 0.4% L-methionine and varying amounts of vitamin E as dl-alpha tocopherol acetate, selenium as sodium selenite, and with either 10% stripped corn oil, stripped lard, or coconut oil. By 7 wk, pentane production by rats fed a corn oil diet deficient in both vitamin E and selenium was twice that by rats fed 0.1 or 1 mg of selenium per kg of the same basal diet. Blood glutathione peroxidase activity after 7 wk was proportional to the logarithm of dietary selenium. Groups of rats fed the vitamin E- and selenium-deficient diets with lard or coconut oil had one-half the pentane production of rats fed the vitamin E- and selenium-deficient corn oil diets. The plasma level of linoleic plus arachidonic acid was 1.8 times greater on a wt % basis in rats fed corn oil than in rats fed lard or coconut oil as the fat source. Pentane production by rats fed 40 i.u. dl-alpha tocopherol acetate per kg of the selenium-deficient corn oil diet was one-sixth of that by rats fed the same diet without vitamin E; the plasma of the rats fed the vitamin E-supplemented corn oil diet had a level of vitamin E that was about six times greater than that of the rats fed the vitamin E-deficient corn oil diet.

INTRODUCTION

Hydrocarbon gases have been related to lipid peroxidation and rancidity in vitro by a number of workers (1-5). Riely et al. (6) were the first to show that ethane, a decomposition product of ω3-fatty acid hydroperoxides, serves as an index of lipid peroxidation in vivo in mice. Hafeman and Hoekstra (7,8) reported that vitamin E and selenium protected rats against lipid peroxidation as determined by measurement of evolved ethane. These investigators (7) showed that ethane evolution was related to the ω3-fatty acid content of the diet. Rats fed a 5% cod liver oil diet evolved about six times more ethane than did rats fed a diet with tocopherol-stripped lard, which contains a lower level of ω3-fatty acids than does cod liver oil.

In this laboratory, in vivo lipid peroxidation in rats fed a vitamin E-deficient diet during a 7-wk period was followed by measurement of expired ethane and pentane (9). Pentane, which derives from ω6-fatty acid hydroperoxides, was shown to be a better index of peroxidative damage in vivo than was ethane when a 10% corn oil diet was fed. Since corn oil contains a relatively high level of linoleic acid, one would expect the rapid incorporation of this fatty acid into membranes of growing rats. The present study was designed to examine the influence of dietary ω6-fatty acids, mainly linoleic acid, on pentane production by rats. A Torula yeast-based diet deficient in both vitamin E and selenium was selected to provide conditions in vivo that would most rapidly result in peroxidation of unsaturated fatty acids. Vitamin E serves as a lipid peroxidation chain-breaker (10), while selenium is required for the function of the lipid hydroperoxide and H_2O_2 decomposing enzyme, glutathione peroxidase (11). The dietary fat sources chosen were tocopherol-stripped lard, tocopherol-stripped corn oil, and coconut oil. In addition to testing the effect of linoleic acid content of the diet on pentane production, the experiments were designed to test (a) the effect of different levels of dietary selenium on pentane production in rats fed a 10% corn oil, Torula yeast-based diet without vitamin E and (b) the effect on pentane production of feeding rats 0.1 ppm selenium together with 40 i.u. dl-alpha tocopherol acetate/kg of a 10% corn oil, Torula yeast-based diet.

MATERIALS AND METHODS

Animals and Diets

The animals used were specific pathogen-free male rats, descendants of the Sprague-Dawley strain. The rats were obtained at 21 days of age from Hilltop Lab Animals, Inc. They were housed in hanging wire cages and were kept on a 14-hr light and 10-hr dark cycle.

The basal diet was a powdered selenium- and vitamin E-deficient diet obtained from Teklad Test Diets. This basal diet contained in percentage: Torula yeast, 30; L-methionine, 0.4;

sucrose, 55.97; fat, 10; mineral mix[1], 3.5; and vitamin mix[2] exclusive of vitamin E, 0.13. The fat source was one dietary variable. The three fats used were tocopherol-stripped corn oil (CO), tocopherol-stripped lard (LD), and coconut oil (CN). The diets were supplemented with vitamin E and selenium as follows (fat source – i.u. dl-alpha tocopherol acetate, mg selenium/kg diet): CO-O E, O Se; CO-O E, 0.01 Se; CO-OE, 0.1 Se; CO-O E, 1.0 Se; CO-40 E, O Se; CO-40 E, 0.1 Se; LD-O E, O Se; and CN-O E, O Se. Selenium was added as sodium selenite. Groups of six rats each were fed the diets and deionized water ad libitum, with fresh diet being provided daily except for Saturdays. The animals were fasted overnight prior to pentane analyses. Weights were recorded at 21 days of age and on each day on which pentane was measured.

Pentane Analysis

Pentane analysis was done on either a Varian 3700 or 1520 gas chromatograph with a flame ionization detector and fitted with a six-way, nut type gas sample valve. Stainless steel columns (1/8 in. x 5 ft for the 1520 model and 1/8 in. x 10 ft for the 3700 model) were filled with 80-100 mesh activated alumina, and a 30 ml/min nitrogen carrier gas flow was used with each column. For the 1520 model, column temperature was programmed as previously described (9). The Varian 3700 was programmed at 20 C/min from 55 to 210 C. The chambers used to hold the rats during sample collection have been described previously (9). The rats were placed in the holding chambers to breathe hydrocarbon-free air (Matheson, ultra pure) for 25 min prior to collection of the samples. One-half of the breath-air stream (120 ml/min) from each rat was collected over a 10-min period to give a 600 ml sample in an activated alumina-filled gas sample loop immersed in liquid nitrogen-ethanol at -130 C. Breath samples were collected from each rat during week 3, 5, 6, and 7 of the dietary feeding program. The relative peak area of pentane on each chromatogram was calculated by triangulation, and the picomoles of the gas were calculated from a standard. One-milliliter portions of the 0.8 ppm pentane standard (Matheson Gas Products) were measured at an electrometer setting of 2 x 10^{-12} amps/mv on the 1520 model and at a setting of 4 x 10^{-12} amps/mv on the 3700 model.

Glutathione Peroxidase, Vitamin E, and Fatty Acid Analyses

After 7 wk of feeding the diets, two to four rats in each group were anesthetized with pentobarbital, and blood was removed by heart puncture via a needle and heparinized syringe. One-half ml of fresh blood was used for assay of glutathione peroxidase (12). The remaining blood was centrifuged for 25 min at ca. 2700 rpm in an IEC clinical centrifuge, and the plasma was frozen for later vitamin E and fatty acid analysis. Total vitamin E in plasma pools from each of the eight dietary groups and in samples of the CO-O E, O Se, CO-40 E, O Se, LD-O E, O Se, and CN-O E, O Se diets was measured by the fluorometric assay of Taylor et al. (13). For analysis of diet vitamin E, 1 g of diet was mixed with 5 ml of redistilled absolute ethanol, 7.5 ml of water, and 2.5 ml of 25% ascorbic acid. After heating for 5 min at 70 C, 5 ml of 10 N KOH was added for saponification. Aliquots of 3 ml were used for extraction. Aliquots of each plasma pool were extracted by the Bligh-Dyer (14) procedure, and methyl esters of the fatty acids were prepared by a modification of the procedure of van Wijngarden (15) for analysis on an automated Hewlett-Packard gas chromatograph with a model 3352B data system. Protein analysis was done by an automated Folin technique (16).

RESULTS

Pentane Production

Pentane production by rats during the third, fifth, sixth, and seventh weeks of feeding the eight diets is shown in Figure 1. Table I gives the statistical significance of differences between mean values as determined by Student's *t*-test. There were several findings of interest. At all four weeks of testing, pentane production by the CN-O E, O Se group was significantly (all $P < 0.05$ were considered significant) lower than by the CO-O E, O Se group, whereas, the production of pentane by the LD-O E, O Se group was lower only at week 6. At weeks 5, 6, and 7, there was no significant difference in pentane production by the CO-O E, O Se and CO-O E, 0.01 Se groups. The CO-O E, 0.1 Se and CO-O E, 1.0 Se groups had significantly lower pentane production than the CO-O E, O Se group, yet they were not significantly

[1]Salt mix (% composition): calcium carbonate, 20.71; calcium phosphate, diabasic, 32.29; cupric sulfate, 0.04; ferric citrate, 0.43; magnesium sulfate, 6.57; manganese sulfate, 0.44; potassium chloride, 20.86; potassium iodate, 0.002; sodium phosphate, dibasic, 18.60; and zinc carbonate, 0.06.

[2]Supplying (g/kg diet): thiamin HCl, 0.0004; riboflavin, 0.0025; pyridoxine HCl, 0.002; calcium pantothenate, 0.02; niacin, 0.1; biotin, 0.001; folic acid, 0.002; choline chloride, 1.0; vitamin B_{12} (0.1% trituration in mannitol), 0.1; dry vitamin A palmitate (500,000 u/g), 0.028; dry vitamin D_2 (500,000 u/g), 0.0064; and menadione, 0.001.

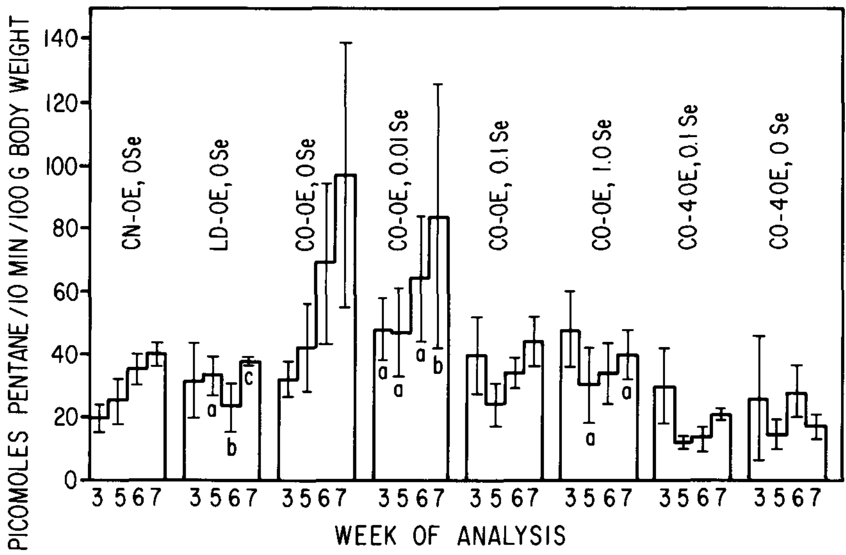

FIG. 1. Pentane production by rats fed diets variable in fat source, selenium and vitamin E. Rats were fed the diets starting at 21 days of age, and breath samples were collected during the following third, fifth, sixth, and seventh weeks. Pentane represents the amount of pentane expired during 10 min of sampling (1200 ml air-breath). The number of rats in each group was six except where indicated: a=5, b=4, and c=2. Bars represent mean ± standard deviation.

different from each other. Only at week 6 was pentane production by the CO-40 E, 0.1 Se group significantly lower than that of the CO-40 E, O Se group. The two groups of rats supplemented with 40 i.u. dl-alpha tocopherol acetate/kg of diet had significantly lower pentane production than all of the rats in the non-supplemented groups.

Glutathione Peroxidase

Table II shows glutathione peroxidase activity in the blood of rats fed the eight diets for 7 wk. The effect on glutathione peroxidase activity of a less than nutritionally required amount of selenium is shown by the absence of activity in the blood of rats fed a 10% corn oil diet with O or 0.01 mg selenium/kg of diet. Glutathione peroxidase activity in the CO-40 E, O Se group was about 16-17% of that in the CO-40 E, 0.1 Se group or the CO-O E, 0.1 Se group, while the activity in the CN-O E, O Se group was about 10% of the levels of these two groups. There was a doubling of specific activity in the blood of rats fed a ten-fold greater level of selenium, that is, in the CO-OE, 1.0 Se group.

Animal Weight Gain and Mortality

Table III shows the effect of the eight diets on final weight attained by the rats. The final weights attained were greatest by the groups of rats supplemented with 0.1 or 1.0 ppm selenium, regardless of dietary vitamin E content. By the seventh week, statistical analysis of the data by Student's t-test showed no difference in weights among the CO-O E, O Se, LD-O E, O Se, and CN-O E, O Se groups. The CO-40 E, 0.1 Se group of rats was significantly ($P < 0.01$) heavier than the CO-40 E, O Se group. The mean weight of the CO-O E, O Se group was significantly lower than that of any other corn oil-fed group except for the CO-O E, 0.01 Se group as indicated. CO-40 E, 0.1 Se, $P < 0.001$; CO-40 E, O Se, $P < 0.02$; CO-O E, 1.0 Se, $P < 0.001$; CO-O E, 0.1 Se, $P < 0.001$; and CO-O E, 0.01 Se, N.S.

Deaths resulting from dietary causes occurred mainly in the group fed lard as a fat source; one rat died during week 5, two during week 6, and one during week 7. During week 7, one rat from the CO-O E, 0.01 Se group died. Hematuria and rusty nasal secretions were noted during the sixth and seventh weeks in all groups but the vitamin E-supplemented groups.

Plasma and Diet Vitamin E

Analyses of total tocopherols were done on two different days with duplicate samples of pooled plasma from rats in the eight dietary groups and in triplicate samples of the diets. The average values obtained for the plasma samples were (mg/100 ml): CO-O E, O Se, 0.12; CO-O E, 0.01 Se, 0.22; CN-O E, O Se, 0.18;

TABLE I

Significance of Differences in Pentane Production by Rats Fed Varying Levels of Vitamin E and Selenium and Different Fat Sources[a]

Dietary groups compared[b]	Week			
	3	5	6	7
CO-O E, Se vs. CN-O E, O Se[c]	<.01[c]	<.05[c]	<.01[c]	<.01[c]
CO-O E, O Se vs. LD-O E, O Se[c]	NS	NS	<.05[c]	NS
CO-O E, O Se[d] vs. CO-O E, 0.01 Se	<.02[d]	NS	NS	NS
CO-O E, O Se[d] vs. CO-O E, 0.1 Se[c]	<.02[d]	<.02[c]	<.01[c]	<.02[c]
CO-O E, O Se[d] vs. CO-O E, 1.0 Se[c]	<.05[d]	NS	<.05[c]	<.02[c]
CO-O E, O Se vs. CO-40 E, O Se[c]	NS	<.001[c]	<.001	<.001[c]
CO-O E, O Se vs. CO-40 E, 0.1 Se[c]	NS	<.001[c]	<.001[c]	<.001[c]
CO-O E, 0.01 Se vs. CO-O E, 0.1 Se[c]	NS	<.01[c]	<.01[c]	<.05[c]
CO-O E, 1.0 Se[c] vs. CO-O E, 0.01 Se	NS	NS	<.02[c]	<.05[c]
CO-O E, 1.0 Se vs. CO-O E, 0.1 Se	NS	NS	NS	NS
CO-40 E, 0.1 Se[c] vs. CO-40 E, O Se	NS	NS	<.01[c]	NS
CN-O E, O Se[c] vs. LD-O E, O Se[d]	<.05[c]	NS	<.05[d]	NS
CO-40 E, O.1 Se[c] vs. CO-O E, 0.1 Se	NS	<.02[c]	<.001[c]	<.001[c]

[a]*P* values determined by Student's *t*-test.
[b]Diets are described in Materials and Methods.
[c,d]*P* value and dietary group with same superscript refers to group with lowest pentane production.

TABLE II

Effect of Dietary Vitamin E and Selenium on Rat Blood Glutathione Peroxidase[a]

Diet[b]	Specific activity[c]
CO-O E, O Se	0.0
CO-O E, 0.01 Se	0.0
LD-O E, O Se	0.0
CN-O E, O Se	2.4 ± 3
CO-O E, 0.1 Se	22.9 ± 3.7
CO-O E, 1.0 Se	41.3 ± 3.3
CO-40 E, O Se	4.0 ± 1.7
CO-40 E, 0.1 Se	25.0 ± 3.7

[a]Rats were fed their respective diets starting at day 21 for 7 wk prior to collection of blood.
[b]Diets are described in the text. Fat source, i.u. dl-alpha tocopherol acetate/kg and mg selenium/kg are indicated.
[c]Nanomoles NADPH oxidized/min/mg protein. The values are the mean ± SD for activity in whole blood from three rats in each dietary group except for the LD-OE, O Se group, from which blood from two rats was analyzed.

TABLE III

Effect of Vitamin E, Selenium, and Fat on Rat Weight[a]

Diet	Body wt[b]
CO-40 E, 0.1 Se	291.8 ± 21.1
CO-40 E, O Se	252.1 ± 21.6
CO-O E, 0.01 Se	212.0 ± 33.6
CO-O E, 0.1 Se	305.0 ± 34.4
CO-O E, 1.0 Se	294.5 ± 16.8
CO-O E, O Se	216.2 ± 21.5
CN-O E, O Se	234.1 ± 32.2
LD-O E, O Se	211.7 ± 27.8

[a]Weights are final weights attained by 7 wk of feeding the diets.
[b]Weight in g ± std. dev.

CO-40 E, O Se, 0.69; CO-O E, 1.0 Se, 0.16; LD-O E, O Se, 0.18; CO-40 E, 0.1 Se, 0.52; and CO-O E, 0.1 Se, 0.14. The average value for the vitamin E in plasma of the rats not supplemented with vitamin E was 0.17 mg/100 ml, and for the vitamin E-supplemented rats, the average value was 0.61 mg/100 ml. The average values of vitamin E obtained for four of the diets were (mg/kg diet); LD-O E, O Se, 2.4; CN-O E, O Se, 5.9; CO-O E, O Se, 3.9; and CO-40 E, O Se, 44.

Plasma Fatty Acid Analysis

Fatty acid analysis on pooled plasma samples from rats fed the eight diets was done primarily to compare the relative amounts of linoleic acid and arachidonic acid. The relative amounts of these two fatty acids were (linoleic acid, arachidonic acid, wt %): CO-O E, O Se, 25.9, 23.3; CO-O E, 0.01 Se, 25.1, 24.6; CO-O E, 0.1 Se, 20.9, 23.5; CO-O E, 1.0 Se, 20.5, 29.6; CO-40 E, O Se, 24.0, 28.0; CO-40 E, 0.1 Se, 23.7, 28.7; CN-O E, O Se, 10.0, 18.9; and LD-O E, O Se 12.3, 18.5.

DISCUSSION

As summarized previously (9), pentane has been shown by other workers to derive from oxidized linoleic acid (4,17) or from lipoxidase oxidized linoleic acid (2,18,19). Dumelin and Tappel (20) showed in vitro that decomposition of preformed linoleate hydroperoxide yielded 1.3 mol % pentane. As one would expect the

peroxidation of linoleic acid in vivo to be dependent upon the amount of linoleic acid provided by the diet and upon the antioxidant status of an animal, the aim of this study with rats was to investigate some of the relationships among linoleic acid, vitamin E, selenium, and pentane production. A diet doubly deficient in vitamin E and selenium was chosen in order to determine whether the effect of selenium, presumably via glutathione peroxidase activity, could be determined in the absence of vitamin E in the diet. A vitamin E-deficient diet is known to increase pentane production in rats (9). Interrelationships between vitamin E and selenium have been studied by many, but it has been difficult to obtain a quantitative approximation of the protective effect of selenium against lipid peroxidation in relationship to the protection afforded by vitamin E. Three fat sources were chosen for use in the hope of providing differing levels of linoleic acid in the diet. Corn oil, lard, and coconut oil have been reported to contain about 34%, 6%, and trace amounts of linoleic acid, respectively (21).

Selenium in the diet had a very definite effect on decreasing the production of pentane by rats fed a vitamin E-deficient 10% corn oil diet. This effect was related to the activity of blood glutathione peroxidase. The blood glutathione peroxidase activity probably can be considered a good index of selenium status in the rat. When fed a selenium-deficient diet, weanling rats lose their glutathione peroxidase activity in 4-5 weeks (22). The doubling of glutathione peroxidase activity when the diet level was increased from 0.1 to 1.0 mg/kg of diet confirms the log relationship of dietary selenium levels to the glutathione peroxidase level in plasma reported by Smith et al. (23). A nutritionally adequate level of selenium decreased the production of pentane by a factor of two. For growth in rats, the nutritional level recommended is 0.04 mg/kg of diet (24). However, increased dietary selenium above the recommended level did not further decrease pentane.

Coconut oil or lard in the vitamin E- and selenium-deficient diet resulted in a level of about one-half of the pentane produced by rats fed a diet with corn oil by 7 wk. The decreased pentane was related to the lower amount of linoleic acid in the diet and linoleic and arachidonic acid in the plasma. Plasma fatty acids can be used as a reflection of the composition of dietary fat in short range experiments (25). It was expected that the coconut oil diet would have a lower level of linoleic acid than the lard diet, but fatty acid analysis of the plasma of rats at the termination of the experiment showed no difference in the amount of linoleic and arachidonic acid in the plasma of rats fed these two diets.

The effect of feeding 40 i.u. dl-alpha tocopherol acetate/kg of diet (CO-40 E, O Se or CO-40 E, 0.1 Se) for 7 wk was to decrease pentane production to one-sixth that produced by rats fed no vitamin E (CO-O E, O Se). The lack of difference between production of pentane by the vitamin E-supplemented rats not fed selenium and those fed 0.1 mg selenium/kg of diet suggests that at this level of vitamin E supplementation no further effect of selenium on pentane production could be seen.

A comparison of pentane production by rats fed a vitamin E- and selenium-deficient Torula yeast-based diet with 10% corn oil and supplemented with 0.4% methionine was made with that produced by rats fed a casein-based, 10% corn oil and tocopherol-deficient diet (9). Pentane production by rats fed these two diets supplemented with 40 i.u. dl-alpha tocopherol acetate/kg of diet was comparable at weeks 5, 6, and 7. Rats fed the Torula yeast-based and methionine-supplemented vitamin E- and selenium-deficient diet in this study produced 16% less pentane by week 7 than did the rats fed a tocopherol-deficient, casein-based diet in the previous study (9). Since the fat source, 10% corn oil, was the same in the diets, one can speculate that L-methionine provided some protection to the rats. Hafeman and Hoekstra reported that methionine was protective as indicated by lower ethane production by methionine-fed rats treated with carbon tetrachloride than by similarly treated rats not fed methionine (7).

The results obtained in this study are in accord with present concepts of in vivo lipid peroxidation and its inhibition by vitamin E and selenium-glutathione peroxidase. Overall lipid peroxidation in bulk lipids is proportional to the content of polyunsaturated fatty acids and inversely proportional to the chain-breaking antioxidant (26), in this case, vitamin E. As an example of a study of the above, Witting (27) studied the relationship of vitamin E to polyunsaturated lipids in the diet and tissues. Results of this study and of others show that the above relationship holds in vivo. In vivo lipid peroxidation is known (28) to be initiated by reactions of the endoplasmic reticulum. Organs that are probable major sources of in vivo lipid peroxidation per unit weight would include liver and testes. Hydroperoxides produced by in vivo lipid peroxidation at a steady state concentration may be reduced by selenium-glutathione peroxidase. The amount of hydroperoxides reduced should be propor-

tional to the amount of enzyme present, although McCay et al. (29) found no hydroxy fatty acids produced in vitro when microsomal phospholipid peroxides were incubated with glutathione peroxidase. Glutathione peroxidase may function to prevent the initiation of peroxidation by H_2O_2 in vivo, since H_2O_2 is an excellent substrate for the enzyme as first reported by Mills (30). Pentane is produced from hydroperoxides by metal-catalyzed decomposition (20). The method described in this paper measures the total body production of pentane as it is expired in the breath. Although it is a minor product (20) of in vivo lipid peroxidation, pentane offers a number of advantages for measurement. Once produced it is an inert product in contrast to most of the products of lipid peroxidation. If there are other sources of pentane, they are small enough not to interfere with the primary measurement. The very low level of pentane produced by the rats fed the CO-40 E, 0.1 Se diet was considered to arise from very low level lipid peroxidation in vivo. Pentane, though soluble in fat, is transported to and volatile enough to be degassed through the lungs. The technique described makes it possible to apply noninvasive measurements to animals.

The lower weight attainment by four groups of rats (CO-O E, O Se, LD-O E, O Se, CN-O E, O Se, and CO-O E, 0.01 Se) was related to a deficiency of dietary vitamin E and selenium, but not necessarily to the fat source. The findings suggest an apparent greater influence of selenium than of vitamin E on the weight attained by the rats during seven weeks of feeding the special diets (see Table II).

Deaths resulting from dietary causes occurred mainly in the group of rats fed lard as the fat source. In a study of essential fatty acid deficiency, Kaunitz (31) compared the mortality of rats. During the first year, the mortality was about the same for rats fed coconut oil, lard, and corn oil, so the deaths were probably unrelated to essential fatty acid deficiency. Hafeman and Hoekstra (8) found deaths with hematuria and lung and liver necrosis 35-105 days after feeding a methionine-supplemented Torula yeast-based diet similar to the diet fed to the rats in this study.

Observations that pentane production is related to dietary linoleic acid, vitamin E, and selenium, via glutathione peroxidase, are of interest in themselves, but the implications of the wider applicability of the technique to areas involving lipid peroxidation is of even greater interest. Evidence for lipid peroxidation being involved in hepatic injury has been shown in rats following treatment with hydrazine (32), acute and chronic doses of ethanol (33), acute and chronic doses of carbon tetrachloride (9,34,35) and orotic acid (36), and during choline deficiency (37). The injury induced by these hepatotoxic agents, as well as lipid peroxidation in the livers is modifiable by antioxidants. Both lipid-soluble and water-soluble antioxidants modify ethanol- and carbon tetrachloride-induced injury, even though the steps in their metabolism to the toxic species is by a different route (38). In 1973, Di Luzio (38) stated that the lipid peroxidation concept had gained increased support, but that the validity of the concept was yet to be unequivocally established. Use of the breath analysis for pentane, which arises in vivo during lipid peroxidation, should prove useful to more firmly establish this process as being basic to many types of liver injury or damage caused by toxic substances.

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Effects of Dietary Antioxidants on In Vivo Lipid Peroxidation in the Rat as Measured by Pentane Production

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ABSTRACT

The hypothesis that pentane is an in vivo product of lipid peroxidation was confirmed by a study of the effects of a nonbiological antioxidant on pentane production in rats fed a diet deficient in vitamin E and supplemented with 0.01% N,N'-diphenyl-p-phenylenediamine (DPPD). Seven rats were fed a vitamin E-deficient diet starting at 3 wk of age. After 5 wk, 0.01% DPPD was added to the diets of three rats (group DPPD) while the diet of the other four rats remained unchanged (group OE). Within 2 wk of the diet change, rats in group DPPD exhaled 65% less pentane than rats of the same age in group OE. After 5 wk of being fed the DPPD-supplemented diet, rats in group DPPD were again fed the basal vitamin E-deficient diet; within 3 wk, these rats produced pentane levels similar to those of rats in group OE. The effects of vitamin E depletion and repletion on in vivo lipid peroxidation in rats were also studied. Three groups of three rats each were initially fed a vitamin E-deficient diet starting at 3 wk of age. After 8, 8, and 5 wk of being fed this diet, the three groups were fed diets supplemented with 3.3 (group 0→3.3E), 11 (group 0→11E), and 200 (group 200E) i.u. vitamin E acetate/kg diet, respectively. Another group of three rats (group 11E) was fed a diet supplemented with 11 i.u. vitamin E/kg starting at 3 wk of age for the duration of the study. There were significant decreases in pentane production by rat groups 0→3.3E, 0→11E, and 200E within 2 wk of the change to the vitamin E-supplemented diets. After about 5 wk of being fed their respective vitamin E-supplemented diets, pentane breath levels had stabilized. Breath pentane levels were inversely proportional to the log of dietary vitamin E concentration.

INTRODUCTION

The importance of vitamin E in nutrition has been attributed to its function as an in vivo inhibitor of lipid peroxide formation. Evidence that supports this function is the finding of higher levels of malonaldehyde in tissues of animals deprived of dietary vitamin E and the presence of fluorescent products from the reaction of malonaldehyde with biological amines. One indication of in vivo antioxidant deficiency is increased susceptibility to hemolysis of red blood cells. Recently, a new approach to the study of in vivo lipid peroxidation was reported (1). This method involves measurement in an animal's breath of volatile hydrocarbon scission products from peroxidizing lipids. Ethane and pentane are released from the hydroperoxides of ω3 fatty acids (the linolenic acid family) and ω6 fatty acids (the linoleic acid family), respectively. Riely et al. (1) first reported that ethane production was characteristic of mice injected with carbon tetrachloride, a known inducer of lipid peroxidation. Hafeman and Hoekstra (2,3) reported similar results with rats fed diets deficient in vitamin E and selenium and injected with carbon tetrachloride. Pentane and ethane in breath samples were correlated with in vivo lipid peroxidation in rats deprived of dietary vitamin E (4) and in rats deprived of both dietary vitamin E and selenium (5).

Various nontocopherol antioxidants effectively prevent or delay symptoms of vitamin E deficiency. Prevention of vitamin E deficiency symptoms is attributed to inhibition of lipid peroxidation chain reactions by these antioxidants. A commonly studied antioxidant in this regard is N,N'-diphenyl-p-phenylenediamine (DPPD), which delays the onset of muscle dystrophy in guinea pigs (6), reverses sterility in female rats (7), prevents resorption-gestation in rats (8), and prevents exudative diathesis in chicks (9).

In this experiment, the effects of the nonbiological antioxidant DPPD on in vivo lipid peroxidation in rats were studied by monitoring levels of pentane in the breath. The dynamics of vitamin E depletion and repletion were studied via this new method. The aim was to test more critially whether pentane in the breath of animals can be used as a measure of in vivo lipid peroxidation.

MATERIALS AND METHODS

Nineteen male Sprague-Dawley rats were obtained as weanlings, about 21 days old, from Simonsen Laboratories, Gilroy, CA. For the duration of the experiment, the rats were housed three to a cage in hanging wire mesh cages in a room kept at a constant temperature of 22-23 C. They were subjected to a daily cycle of 14 hr light and 10 hr dark.

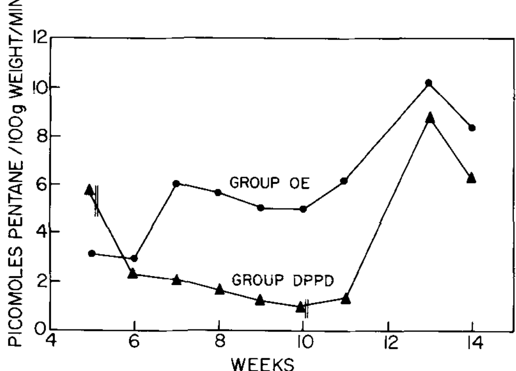

FIG. 1. Picomoles pentane/100 g wt/min produced by rats in group OE (●) and group DPPD (▲). The values plotted are the averages for the four rats in group OE and the three rats in group DPPD. The diet composition was changed at 5 wk as described in the text, and as indicated by vertical parallel lines on the figure.

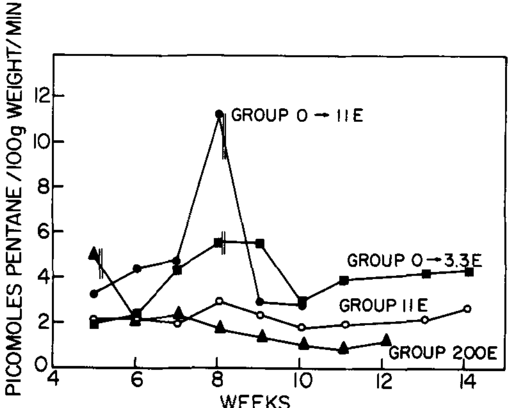

FIG. 2. Picomoles pentane/100 g wt/min produced by rats in groups 0→3.3E (■), 0→11E (●), 11E (○), and 200E (▲). The values plotted are the averages for three rats in each group. The vertical parallel lines denote the times when diet compositions were changed as described in the text.

Diets

The vitamin E-deficient diet used was described by Draper et al. (7). The powdered diet contained in percentage: dextrose, 65.9; casein, 20; vitamin E-stripped corn oil, 10; and mineral-vitamin mix, 4.

One group of four rats was fed the basal vitamin E-deficient diet (group OE) and another group of three rats was fed the basal diet supplemented with 11 i.u. dl α-tocopherol acetate/kg (group 11E) for the duration of the study. Three rats (group DPPD) were fed the vitamin E-deficient diet for 5 wk, then fed the same diet supplemented with 0.01% DPPD for 5 wk, and again fed the vitamin E-deficient diet for 4 wk. Three rats (group 200E) were fed the vitamin E-deficient diet for 5 wk and then fed the same diet supplemented with 200 i.u dl-α-tocopherol acetate/kg for the following 7 wk. Two groups of three rats each were fed the basal vitamin E-deficient diet for 8 wk. One group was then fed the diet supplemented with 3.3 i.u. dl-α-tocopherol acetate/kg (group 0→3.3E) and the other group was fed the diet with 11 i.u. dl-α-tocopherol acetate/kg (group 0→11E).

Rats were fed the powdered diets and tap water ad libitum. The diets were stored at -10 C and were provided to the rats fresh each day.

Gas Chromatography

The gas chromatographic procedure used for breath analysis was similar to that described by Dillard et al. (4). Each rat was fasted 18-24 hr, weighed, and placed in a holding chamber for 25 min prior to sampling. During this time, the rat was provided pentane-free air either prepared by the method of Dillard et al. (4) or supplied as commercial hydrocarbon-free air (Matheson).

A Varian 1520 gas chromatograph was used for the separation and quantitation of pentane. The chromatograph was equipped with a flame ionization detector and a 5 ft x 1/8 in. stainless steel column packed with 80-100 mesh activated alumina. The instrument was standardized with 1 ml aliquots of 0.8 ppm pentane in N_2 at an electrometer setting of 2×10^{-12} amps/mv. A comparison of peak areas, measured by triangulation, for the standard sample and the breath sample allowed calculation of the molar amount of pentane.

The following computation was then applied:

$$\frac{\text{(picomoles pentane) (incoming flow rate of hydrocarbon free air)}}{\text{(100 g rat body wt) (sample volume)}} =$$

total pentane produced/100 g/min. This value is independent of sample volume and the rate at which pentane-free air is supplied to the rat.

Tocopherol Content of Diets

The basal diet, assayed for total tocopherol content by the method of Taylor et al. (10), contained a residual tocopherol content equivalent to 1.5 i.u. α-tocopherol/kg. Vitamin E-supplemented diets fed to groups 11E, 0→11E, 0→3.3E, and 200E contained 15.8, 15.8, 5.3, and 190 i.u. α-tocopherol/kg, respectively.

RESULTS

Statistics

The Mann-Whitney *U* test, a nonparametric statistical method, was applied to the data to determine differences ($\alpha = 2P = 0.1$) in breath pentane evolution from rats fed different diets. Some data were analyzed by the Student's *t*-test. The variations in the amount of pentane produced, as shown by the standard deviations, were due to individual variations among the rats within each dietary group and not to the analytical procedure. Because of individual variation and the very small number of animals in each group, there was a standard deviation of about 30% in weekly pentane measurements of rats within a dietary group. However, the variation observed among rats was similar to that found previously (4).

Effects of Dietary Vitamin E on Pentane Production

After the animals were fed their respective diets for 7 wk, group OE rats exhaled approximately three times more pentane per minute than those in group 11E (Fig. 1 and 2).

Effect of Dietary DPPD on Pentane Production

Two weeks after the addition of 0.01% DPPD to the vitamin E-deficient diet, rats in group DPPD produced 65% less pentane than rats in group OE (Fig. 1). The decrease was shown to be significant by the Mann-Whitney *U* test. The sudden decrease in pentane production after the diet change was also apparent from results of a Student's *t*-test comparing groups of data points before and after the diet change. Within 3 wk of a diet change back to the vitamin E-deficient diet without DPPD, rats in group DPPD produced pentane at levels similar to those in group OE (Fig. 1).

Vitamin E Repletion

Rat groups 0→3.3E and 0→11E, initially fed the basal vitamin E-deficient diet for 8 wk, both exhibited significant decreases in pentane production after being fed vitamin E supplemented diets for 2 wk. Within 2 wk of the diet change, rats in group 0→3.3E exhaled 41% less pentane than the rats in group OE. After 3 wk, rats in group 0→3.3E produced pentane levels significantly different from those of group 11E, and their pentane levels stabilized at twice the pentane levels of rats of group 11E. After 5 wk, rats in group 0→3.3E produced pentane levels significantly different from those of groups OE and 11E (Fig. 2). After 6 wk of being fed the vitamin E-supplemented diet, rats in group 0→3.3E produced 47% less pentane per minute than those in group OE. Rats initially deprived of vitamin E and then fed a diet supplemented with 11 i.u. of vitamin E/kg of diet (group 0→11E) showed a significant pentane decrease, and within 2 wk of the diet change the pentane levels were similar to the levels found in group 11E (Fig. 2). Within 2 wk of a diet change to 200 i.u. vitamin E/kg, rats in group 200E displayed a significant reduction in the rate of pentane production to a level that was 61% less than that of group OE (Fig. 2). Six wk after the diet change the rats in group 200E exhaled 86% less pentane per minute than the rats in group OE.

DISCUSSION

Upon Fe-catalyzed decomposition, in vitro peroxidizing lipids release hydrocarbon gases. Ethane and pentane were released during decomposition of hydroperoxides derived from purified linolenic acid and linoleic acid, respectively. In vitro, the iron-catalyzed decomposition of purified linoleic acid hydroperoxide produced 1.3 mol % pentane (11). Evidence is accumulating for the in vivo formation of these hydrocarbon gases during lipid peroxidation. Dumelin et al. (12) showed that rats fed a vitamin E-deficient diet and subjected to a short period of ozone exposure exhaled significantly greater quantities of ethane and pentane than ozone-exposed rats fed vitamin E. Köster et al. (13) observed increased ethane production in rats and mice treated with carbon tetrachloride and ethanol.

Nondestructive methods for measurement of in vivo lipid peroxidation previously have been limited to hemolysis of red blood cells. Measurement of hydrocarbon scission products of lipid hydroperoxides, including ethane and pentane, in breath samples promises to provide a sensitive indication of in vivo lipid peroxidation. The observation that rats fed a diet deficient in vitamin E produce significantly greater amounts of pentane than those fed sufficient vitamin E was again made in this study. Vitamin E-stripped corn oil comprised 10% of the Draper diet; about 35% of the corn oil is linoleic acid. With this high percentage of dietary ω6 unsaturated fatty acid, the tissue lipid composition should reflect the dietary composition, and pentane should be the major hydrocarbon released from hydroperoxide decomposition in vivo. For this reason and for simplicity in the chromatographic procedure, only pentane levels were measured in breath samples.

The nontocopherol antioxidant DPPD has been shown in numerous studies to relieve or

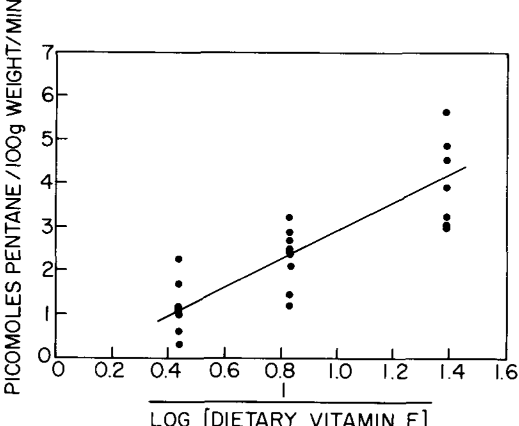

FIG. 3. Picomoles pentane/100 g wt/min vs. $\frac{1}{\log\ [\text{dietary vitamin E}]}$. Points represent pentane expired by individual rats in groups 0→3.3E, 11E, and 200E at wk 11-14, 11-14, and 9-12, respectively, after pentane levels had stabilized.

postpone symptoms of vitamin E deficiency. In this study, rats initially deprived of dietary vitamin E exhibited a significant decrease in pentane production within 2 wk of dietary supplementation with 0.01% DPPD (Fig. 1). After returning these rats to a vitamin E-deficient diet without DPPD, the rats exhaled levels of pentane similar to those exhaled by rats continuously fed the vitamin E-deficient diet. These findings strongly support the hypothesis that pentane is a product of in vivo lipid peroxidation, and that it can be used reliably as an index of such. It also was apparent that the DPPD was incorporated into the body tissues and substituted for vitamin E in a protective antioxidant role.

Previous studies indicate that DPPD is effective in reversing the physical symptoms of vitamin E deficiency. It is important to note that the ability of DPPD to substitute for vitamin E was observed, via the pentane method, before any gross physical symptoms of vitamin E deficiency appeared.

Rat groups 0→3.3E, 0→11E, and 200E produced increased amounts of pentane while being fed the vitamin E-deficient diet. Two weeks after their diets were supplemented with vitamin E, pentane production in all three groups was significantly less than that of group OE. After 5 wk of being fed the vitamin E-supplemented diets, rat groups OE, 0→3.3E, 11E, and 200E produced pentane levels significantly different from each other. Breath pentane levels increased with decreasing amounts of dietary vitamin E. The rats were fasted for 18-24 hr prior to breath sampling to assure that the pentane measured was not derived from food peroxidized in the digestive tract. Ingestion of rancid food was prevented by storing the diets at -20 C and providing it fresh each day to the rats.

The data obtained here and in vitro (11) provide an estimation of the amount of lipid hydroperoxides decomposing in vivo. Rats in groups OE and 11E produced about 10 and 2 pmol pentane/100 g body wt/min, respectively. If 1.3 mol % pentane results from the decomposition of ω-6 lipid hydroperoxides, the pentane produced in vivo came from 770 and 154 pmol ω-6 hydroperoxides present in the tissues of rats in groups OE and 11E, respectively. We assume that these amounts of hydroperoxides are part of a larger steady state pool and that some are also being removed via the glutathione peroxidases. The small amount of residual pentane in rats of group 200E probably represents formation and decomposition of linoleic acid hydroperoxides that occur before the free radical chain reaction is broken by vitamin E. The very low level of pentane production observed in group 200E rats indicates that there is no other significant source of pentane in vivo. The two lower levels of dietary vitamin E, 3.3 i.u./kg and 11 i.u./kg, were chosen to accomplish a twofold difference in the tissue vitamin E levels between rats fed these diets. Vitamin E levels in tissue vary with the logarithm of dietary vitamin E levels (14). According to our concept of the source of pentane, a doubling in tissue vitamin E should halve the amount of pentane produced by the rat. Thus, the relationship obtained is pentane $\propto \frac{1}{[\text{tissue vitamin E}]} \propto \frac{1}{\log\ [\text{dietary vitamin E}]}$. A plot of pentane vs. $\frac{1}{\log\ [\text{dietary vitamin E}]}$ using data from this study (Fig. 3) does, in fact, yield a linear relationship that is significant at the 99% confidence level. These correlations support the relationship between pentane production and in vivo lipid peroxidation.

Because the method used in this study is a relatively new one, attempts were made to optimize conditions. The actual time involved in testing each animal is rather long. Each rat must be fitted with rubber and Teflon collars and placed into a holding chamber. The rat must remain in the chamber 25 min prior to the 10 min breath sampling time to allow sufficient time for all room air to be flushed from the system and to equilibrate lung gases with

hydrocarbon-free air. The gas chromatographic program takes about 10 min, the column must then be heated to drive off remaining adsorbed compounds and cooled before the next sample can be injected. This procedure limits the number of animals that can be tested in one day. Due to these time limitations, an attempt was made to test only the minimum number of rats necessary for reasonable statistical treatment of the data. The nonparametric Mann-Whitney *U* test was used to compare weekly values of exhaled pentane from rats in different dietary groups. The use of only three rats per dietary group yields data that can be significantly compared at a confidence level no higher than 90%. The use of very small numbers of animals in this study severely limited the statistical treatment of the data. For example, groups 0→11E and 200E displayed large decreases in pentane production during the first week following their respective diet changes, while group 0→3.3E displayed a more gradual decrease (Fig. 2). With the small number of animals per group, however, the differences in pentane levels between group OE and each of the three other dietary groups were not statistically significant until 2 wk after the diet changes.

The experiments described in this paper provide examples of the potential applicability of the pentane method to the study of many factors that affect lipid peroxidation in vivo. The dynamics of vitamin E depletion and repletion have been investigated by a number of researchers. Bieri (14) determined the tissue vitamin E levels of a large number of rats fed diets with varying amounts of vitamin E. This method requires the killing of many animals and is impractical for research on more expensive animals. Vitamin E studies on primates (15,16) utilized analysis of hemoglobin and reticulocytes obtained from blood samples. The method of analysis of blood and fat samples to determine the dynamics of vitamin E depletion and repletion was employed in a study of human subjects (17). Although the method of blood sampling does not require killing the animal, it has been observed that the repeated sampling of blood may severely stress an animal and therefore introduce unknown variables into the results (18).

The use of breath pentane measurements has many advantages over other methods. The taking of breath samples does not harm the test subject, so fewer subjects are needed than in tests requiring killing to obtain data. The risks inherent in blood sampling are eliminated. The painless nature of the measurement of breath pentane may increase the availability of human subjects for studies of the effects of antioxidants and other factors on lipid peroxidation. Because the method is very sensitive, the extent of in vivo lipid peroxidation may be detected before gross physical symptoms of antioxidant deficiency become apparent in laboratory animals. In general, the measurement of breath pentane is a very sensitive and noninterfering method for determination of the effect of antioxidant activity on in vivo lipid peroxidation.

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Cerebral Prostaglandin Synthesis during the Dietary and Pathological Stresses of Essential Fatty Acid Deficiency and Experimental Allergic Encephalomyelitis[1]

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ABSTRACT

Rats of the Lewis strain were fed diets adequate or deficient in essential fatty acids (EFA). At 70-80 days of age experimental allergic encephalomyelitis (EAE) was induced using adjuvants containing either *Mycobacterium butyricum* or *Mycobacterium tuberculosis H37Ra*. When the former *Mycobacterium* was used, the incidence of EAE was greater in the EFA-deficient than in EFA-adequate controls; but when the rats challenged with *M. tuberculosis*, the incidence of the disease was the same in both dietary groups. Brain slices from EFA-deficient rats had a marginally depressed synthesis of prostaglandin F (PGF) compared to that of controls. Immunochallenge with adjuvant alone or adjuvant plus antigen tended to depress further PGF synthesis by brain slices from EFA-deficient rats and significantly depressed synthesis by slices from rats receiving adequate EFA. Whether or not rats were paralyzed had no effect on PGF synthesis when the diet was adequate in EFA, but a significant difference was seen in the EFA-deficient group. The results indicate a possible role for PGF synthesis in the degree of susceptibility of the rats to EAE under different dietary regimens.

Clausen and Moller (1) and Selivonchick and Johnston (2) showed that essential fatty acid (EFA) deficiency in the rat leads to a greater susceptibility to the autoimmune disease, experimental allergic encephalomyelitis (EAE). Since the prostaglandins (PGs) are apparently involved in the inflammatory and immune responses (3-5), Selivonchick and Johnston (2) suggested that one explanation for the greater susceptibility to EAE in EFA-deficient animals was a change in the PG synthesizing capacity.

The effect of EFA deciciency on the ability of a tissue to produce PGs is not predictable, however, Prostaglandin synthesis from EFA-deficient animals was shown to be increased over PG synthesis from EFA-adequate animals in kidney medulla homogenate (6), and in lung and small intestine homogenate (7), but decreased in intact epidiymal fat pads (8), in carrageenan-induced inflammatory exudate (9), and in ground vesicular glands (10).

In EFA deficiency, tissues are not completely depleted of arachidonic acid, and availability of the precursor for PG synthesis may not be the limiting factor. Essential fatty acid deficiency is marked by an accumulation of fatty acids of the ω9 series. It has been reported that many fatty acids that are not precursors for PGs act as inhibitors to the PG synthesizing system (11), and inhibition has been shown for 18:1ω9, 18:2ω6, and 18:3ω3 (12) and for the "deficiency triene" 20:3ω9 (13). The source of the fatty acid precursors of PGs is the 2-position of the phospholipids, and the action of phospholipase A_2 is the rate-limiting step in PG synthesis (14). It has not yet been established that the acylhydrolase is specific for arachidonate; therefore, nonspecific release of fatty acids from the 2-position of phospholipids of EFA-deficient animals could result in the presence of increased levels of inhibitors of PG synthetase. In addition to the effects of competitive inhibition, an EFA deficiency could change the lipid environment in the membranes in which the PG synthetase system resides. Changes in activity in EFA deficiency have been reported for some membrane-bound enzymes in brain (15,16). The PG synthetase system may be similarly influenced.

Although an EFA deficiency is reflected in the central nervous system by some changes in brain and spinal cord fatty acids, the effects are less marked than those seen in other tissues (17-21). The ability of the normal brain to synthesize PGs from exogenous arachidonic acid appears to be limited (22,23). Brain slices, however, have considerable capacity to produce PGs from endogenous substrates (22-25), and measurements of this endogenous synthetic capacity appears to be a valid procedure to study PG synthesis in brain (23).

The following study of the PG synthesizing capacity of brain during EFA deficiency and

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[1] Part of a dissertation submitted by Patricia G. Weston to the University of Illinois in partial fulfillment of the requirements for the Ph.D. in Nutritional Sciences.

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TABLE I

Composition of Semipurified Diets[a]

	EFA-adequate (g/100 g)	EFA-deficient
Corn oil	5	---
Hydrogenated coconut oil[c]	---	5
Casein[d]	20	20
Dextrose[e]	67	67
Cellulose[f]	4	4
Salt mix[g]	4	4

[a]Vitamins (mg/kg diet): thiamin • HCl, 30; riboflavin, 30; pyridoxine • HCl, 8; calcium pantothenate, 100; nicotinamide, 100; inositol, 220; folic acid, 2; biotin, 0.2; cyanocobalamin, 0.05; vitamin K_5, 2; retinyl acetate, 10; vitamin D_2, 4; dl-α-tocopherol, 200; choline chloride, 1,000.

[b]Fatty acid analysis: 16:0, 12.2%; 18:0, 2.9%; 18:1ω9, 23.8%; 18:2ω6, 61.1%. Linoleic acid represents 6.95% of calories.

[c]Fatty acid analysis: 8:0, 3.0%; 10:0, 6.4%; 12:0, 60.4%; 14:0, 13.4%; 16:0, 7.9%; 18:0, 7.6%; others, 1.3%.

[d]Shamrock Brand, Erie Casein Company, Erie, IL.

[e]Staleydex 333, A.E. Staley Company, Decatur, IL.

[f]Alphacel, Nutritional Biochemicals Corporation, Cleveland, OH.

[g]Jones-Foster Salt Mix, Nutritional Biochemicals Corporation, Cleveland, OH.

EAE was undertaken in order to determine if changed PG synthesis in the target organ of EAE is associated with the degree of susceptibility to the disease.

MATERIALS AND METHODS

Dietary Treatment

Male weanling rats, inbred Lewis/Mai F strain, (Microbiological Associates, Walkersville, MD) were divided into two dietary groups. Group 1 was reared on an EFA-adequate diet and group 2 on an EFA-deficient diet (Table I). The animals were housed individually in polypropylene "shoebox" cages, with Sanicel bedding (Paxton Processing Company, Inc., Paxton, IL), a diurnal light cycle of 12 hr was maintained, and food and water were available ad libitum. The rats were weighed weekly until induction of EAE.

Induction of EAE

At 70-80 days of age each dietary group was further divided into the following groups: (a) animals in which EAE was induced (EAE); (b) animals injected with adjuvant only; and (c) animals given no treatment and killed for baseline levels of PGF. EAE was induced by a single injection of 0.25 ml into each hind foot pad of (a) Freund's complete adjuvant containing *Mycobacterium butyricum* or (b) Freund's adjuvant containing *Mycobacterium tuberculosis H37Ra* (Difco Laboratories, Detroit, MI), each homogenized with an equal volume of a 0.9% saline suspension of 35 mg of homogenized fresh or frozen adult guinea pig spinal cord (Pel-Freeze Biologicals, Inc., Rogers, AR). Freund's control (FC) animals were injected with a single injection of 0.25 ml into each hind foot pad of Freund's complete adjuvant homogenized with an equal volume of 0.9% saline.

After the injections, animals were weighed daily and examined for signs of EAE. From the first incidence of EAE, two observers graded each rat, using the following scoring system: weight loss of at least 10 g, 1; urinary incontinence, 1; fecal impaction, 1; paresis, 2; paralysis, 4; paralysis plus lethargy, 5.

Preparation and Incubation of Slices

Paralyzed animals were killed in the acute phase of the disease. Those animals whose only symptom was weight loss were killed when weight loss had ceased and gain had again been resumed. Animals free of symptoms were killed not later than 4 weeks post induction. Freund's control animals were killed at the same time as a weight-matched paralyzed rat, and the animals used for baseline levels (no injections) were killed at 70-80 days of age.

The rats were decapitated and the brains and livers removed immediately. Livers were stored at -10 C until analyzed. The brain was weighed, and one slice of cerebral cortex, 0.5 mm thick, was cut from each hemisphere with a Stadie-Riggs micrometer at room temperature in a humid chamber (26). For the incubation, one-half of each slice was weighed and placed in 3 ml of Krebs-Ringer-phosphate medium, pH 7.4. The other half slice was weighed and incubated in 3 ml of the same medium, acidified to pH 1-2 with 1 N HCl. The slices were shaken at constant speed in a 37 C water bath in air for

TABLE II

Fatty Acid Composition of Total Liver Lipids and Brain Ethanolamine Glycerophosphatides (EPG) of Rats Fed Diets Adequate or Deficient in Essential Fatty Acids (EFA-A or EFA-D), Sampled at 70-80 Days of Age

Fatty acids, percent of total

Diet	Liver lipids EFA-A n=7	Liver lipids EFA-D n=6	t-test
16:0	21.1 ± 4.1	22.3 ± 2.6	N.S.
18:0	19.5 ± 3.0	20.3 ± 2.1	N.S.
18:1ω9	9.3 ± 1.6	21.2 ± 3.2	$p < 0.001$
18:2ω6	14.6 ± 2.9	3.0 ± 1.0	$p < 0.001$
20:3ω9	. . .	12.2 ± 2.1	$p < 0.001$
20:4ω6	26.0 ± 2.1	9.9 ± 2.3	$p < 0.001$
22:6ω3	3.3 ± 0.9	4.3 ± 1.1	$p = 0.08$
Others	6.2	6.8	. . .
Ratio $\frac{20:3\omega 9}{20:4\omega 6}$	. . .	1.2	. . .

Diet	Brain EPG EFA-A n=5	Brain EPG EFA-D n=4	t-test
16:0	7.8 ± 1.6	10.3 ±1.8	p=0.06
18:0	26.6 ± 2.0	25.4 ± 5.1	N.S.
18:1ω9	8.5 ± 0.4	11.7 ± 4.1	N.S.
18:2ω6	Trace	Trace	. . .
20:1ω9	1.0 ± 0.1	2.4 ± 1.3	N.S.
20:3ω9	0.8 ± 0.4	2.4 ± 1.8	N.S.
20:4ω6	15.9 ± 1.0	13.7 ± 2.2	p=0.08
22:6ω3	25.6 ± 1.2	21.2 ± 2.6	p=0.01
Others	13.8	12.9	. . .
Ratio $\frac{20:3\omega 9}{20:4\omega 6}$	0.05	0.18	. . .

30 min. At the end of the incubation, the tissue slice was removed by filtration, and 0.1 ml of incubation medium was assayed directly, in duplicate, for PGF. Because preliminary investigations had indicated that the concentration of PGF in the medium was still increasing linearly with time of incubation at 45 min, an incubation time of 30 min was chosen for the study. Preliminary work had also shown that the concentration of PGF in the medium at 0-1 min of incubation was not different from the values obtained for the incubation medium alone, therefore, a zero time control was omitted in subsequent samples. However, the levels of PGF in the acidified medium after a 30 min incubation of the tissue did differ from the values obtained for the medium alone; therefore, an acidified control was included for every brain slice. The value of PGF obtained for this acidified control presumably represents the PGF formed during the excision, slicing, and weighing procedures, and it was subtracted from the PGF value obtained from the slice incubated in the nonacidified medium. No interference in the binding of $PGF_2\alpha$ to the antibody was observed for the medium or acidified medium, before or after 30 min incubation.

Radioimmunoassay (RIA)

Radioimmunoassay for PGF was carried out according to the procedure provided with freeze-dried rabbit antiprostaglandin F-bovine serum albumin serum (Miles Laboratories, Elkhart, IN). This antiserum was specific for the F prostaglandins and showed negligible cross reaction with PGE. The RIA was carried out in a buffer consisting of 0.01 M potassium phosphate pH 7.4, 0.15 M sodium chloride, 0.1% sodium azide, and 0.1% bovine serum albumin. Standards ranging from 0.15 ng/ml to 2.50 ng/ml were prepared in the buffer from $PGF_2\alpha$. Tritiated $PGF_2\alpha$ (178 Ci/mmole) was purchased from New England Nuclear, Boston, MA. Radiopurity was checked by thin layer chromatography (TLC) using Silica Gel G (Brinkmann Instruments, Des Plaines, IL) and a solvent system of chloroform-methanol-glacial acetic acid-water, 90:9:1:0.65 by vol. The antiserum was dissolved in buffer to a concentration that would bind 40 to 50% of 10 pg of tritiated $PGF_2\alpha$ under the conditions of this assay. Standards of $PGF_2\alpha$ ranging from 15 pg to 250 pg in 0.1 ml buffer, 0.1 ml of incubation supernatant, or 0.1 ml buffer (zero control) were placed in test tubes. A solution of antibody (0.5 ml) was added to each tube, except to a blank and a total tube, to which 0.5 ml buffer was added. After an incubation of 30 min at 4 C, 0.1 ml of tritiated $PGF_2\alpha$ in buffer

TABLE III

Incidence and Severity of Experimental Allergic Encephalomyelitis in Rats Fed Diets Adequate or Deficient in Essential Fatty Acids (EFA-A or EFA-D), Challenged with Guinea Pig Spinal Cord in Freund's Adjuvant Containing *Mycobacterium butyricum* or *Mycobacterium tuberculosis H37Ra*

Age	70-80 days		70-80 days	
Adjuvant	*M. tuberculosis H37Ra*		*M. butyricum*	
Diet	EFA-A	EFA-D	EFA-A	EFA-D
Incidence	11/11 (100%)	25/25 (100%)	10/18 (55%)	17/19 (90%)
Total caudal paralysis	5/11 (46%)	13/25 (52%)	5/18 (31%)	3/19 (16%)
Score	3.82	4.48	2.54	2.05
Onset (days)	13 ± 1	12 ± 1	14 ± 3	14 ± 3
Range	(12-16)	(9-14)	(10-19)	(11-23)
Weight at induction (g)	331 ± 27	286 ± 17	278 ± 15	236 ± 26

(10 pg) was added to all tubes, and then all tubes were incubated again for 60 min at 4 C. Free PG was then separated from antibody-bound material by the addition of dextran-charcoal (0.2 ml of buffer containing 1% w/v Norit A charcoal and 0.1% w/v Dextran T-70 (Pharmacia Fine Chemicals, Uppsala, Sweden), excluding the total tube to which 0.2 ml buffer was added. This was followed by vigorous mixing, incubation at 4 C for 10 min, and finally centrifugation at 4 C for 15 min at 600 x g. An aliquot (0.5 ml) of the supernatant was added to a counting vial containing 6 ml of toluene-biosolve (Beckman Instruments, Fullerton, CA), 85:15 by volume, containing 6 g 2,5-diphenyloxazole (PPO) per liter of toluene. Samples were counted for 10 min in a Packard liquid scintillation spectrometer, model 3320 (Packard Instruments Company, Inc., Downers Grove, IL), and efficiency was monitored using a channels ratio method. The calibration curve was expressed as fraction bound = dpm/min in sample - dpm in blank / dpm in zero control - dpm in blank on the ordinate plotted against the levels of nontritiated $PGF_2\alpha$ on a logarithmic scale on the abscissa.

Lipid Analysis

Lipids were extracted from brain slices and livers using the method of Folch et al. (27). The brain lipids were separated by thin layer chromatography (TLC) on Silica Gel H with chloroform-methanol-water, 65:25:4 by vol, and the ethanolamine glycerophosphatide (EGP) spot was removed. The methyl esters of brain EPG and total liver lipids were prepared using 4% sulfuric acid in methanol. The dimethylacetals from the EPG were separated from the methyl esters by TLC on Silica Gel G with toluene. Liver lipid methyl esters were separated from cholesterol by chromatography on silicic acid. The methyl esters were analyzed by gas liquid chromatography (GLC) using a Hewlett-Packard gas chromatograph model 7610A equipped with a 180 cm x 0.4 cm glass column packed with 10% SP 2340 on 100/200 chromosorb WAW (Supelco Inc., Bellefonte, PA). Fatty acid methyl esters were identified by means of equivalent chain lengths, based on the retention times of standards (Nu-Chek-Prep, Elysian, MN).

RESULTS

At the time of induction of EAE, rats in the EFA-deficient diet showed a scaly dermatitis and had a significantly lower body weight than control animals. Deficient rats weighed 262 ± 23 g and controls, 300 ± 15 g ($p<0.001$). Food intake, however, did not differ, both groups consuming 16 g ± 2 at 70 days of age. Fatty acid analysis of total liver lipids showed significant increases in 18:1ω6 and 20:3ω9 and decreases in 18:2ω6 and 20:4ω6 in EFA-deficiency (Table II) and an elevated triene/tetraene ratio indicative of a moderate degree of EFA deficiency (28). Fatty acid analysis of EPG from cerebral cortex slices from deficient animals also showed changes typical of EFA-deficiency (Table II), although they are less pronounced than in other tissues.

An initial weight loss immediately after the injections in both EAE and Freund's control (FC) rats, was followed by slow growth again until the first clinical signs of EAE were observed, usually an abrupt weight loss. Paralysis, when it occurred, was usually observed two to three days after the first signs of weight loss. There was no incidence of disease in FC animals from any groups. The days postinduction when symptoms were first observed and the incidence and severity are shown in Table III. In agreement with previous findings (1,2,29) when the adjuvant contains *M. butyricum* and the diet is

TABLE IV

Biosynthesis of Prostaglandin F in Brain Slices of Rats Fed Diets Adequate or Deficient in Essential Fatty Acids (EFA-A or EFA-D) and Challenged with Guinea Pig Spinal Cord in Adjuvant (EAE), Challenged with Adjuvant Only (FC), or Unchallenged, at 70-80 Days of Age[a]

	Nanograms PGF/100 mg wet weight	
Diet	EFA-A	EFA-D
Unchallenged	7.16 ± 1.72 (8)[b]	5.01 ± 2.47 (10)
EAE	3.46 ± 1.77 (35)	3.94 ± 2.00 (54)
FC	4.39 ± 1.83 (15)	3.94 ± 1.50 (21)
Paralyzed	3.29 ± 1.81 (18)	4.73 ± 1.97 (24)
Nonparalyzed	3.64 ± 1.77 (17)	3.31 ± 1.82 (30)

Student's t-test for PGF levels.
Unchallenged, EFA-A vs. EFA-D p=0.05; EFA-A, unchallenged vs. EAE $p < 0.00005$; unchallenged vs. FC p=0.03; EFA-D, paralyzed vs. nonparalyzed p=0.008.
All others NS.

[a]Statistical analysis showed that the *Mycobacterium* used (*M. butyricum* or *M. tuberculosis H37Ra* had no effect on the PGF levels. Therefore the levels given are for a mixture of rats receiving either *Mycobacterium*.
[b]Mean value ± SD for number of determinations in parentheses.

deficient in EFA, the incidence of EAE is increased. However, when the adjuvant contains *M. tuberculosis H37Ra*, the greater immunochallenge leads to 100% incidence of EAE irrespective of the EFA status of the animals (29).

Preliminary results showed no PG synthesis by rat brain homogenate using exogenous precursors, confirming reports by Wolfe and coworkers (22). Brain slices from immunologically unchallenged animals on the EFA-adequate diet produced more PGF than those from rats on the EFA-deficient diet (Table IV), but this difference was only marginally significant (p = 0.05). When the diet was adequate in EFA, a challenge with adjuvant alone or antigen plus adjuvant led to a depression in the ability of brain slices to produce PGF (Table IV). This depressed synthesis was highly significant in EAE ($p < 0.001$) and significant in FC rats (p = 0.03). When the diet was deficient in EFA, the immunochallenge depressed PGF synthesis, but the differences from the control values are not significant (Table IV). Whether or not rats were paralyzed had no effect on the PGF synthesis of brain slices in the EFA-adequate groups, but a significant difference was seen in the EFA-deficient groups (Table IV).

DISCUSSION

The detection of PGF in the medium after incubation of brain slices and the continued production of PGs by the slices for at least 30 min confirm the findings of several investigators (22-25). Our failure to detect PG synthesis by brain homogenates from exogenous precursor agrees with the findings of Wolfe (23). Although Kataoka et al. (30) found that extracts of homogenized brain contained equal amounts of PGE, and PGF, all investigators using incubated brain slices have found PGF to be the major product. Leslie (25) suggested that an active prostaglandin E_2 9-ketoreductase in rat brain slices may convert PGE_2 to $PGF_2\alpha$. Because of the predominance of PGF production in brain slices, only the F series was assayed in this study. The antiserum used did not distinguish between $PGF_1\alpha$ and $PGF_2\alpha$. However, in view of the negligible amount of 20:3ω6 in brain and the demonstration by gas chromatography-mass spectrometry (GC-MS) that brain PG is predominantly $PGF_2\alpha$ (23,24), the PG detected in this study was probably largely $PGF_2\alpha$. The levels of PGs detected in the present study (7.16 ± 1.72 ng/100 mg wet tissue for unchallenged animals) are lower than some previously reported: 81.4 ± 7.1 ng/100 mg (24); 16 ng/100 mg (25). However, if corrected for the amount present in unincubated slices due to excisional damage (8.5 ng), the biosynthetic capacity of rat brain slices determined by Wolfe et al. (22) is remarkably close to that reported here, namely 6.5 to 9.5 ng/100 mg tissue. Thus, if conditions are carefully standardized, results obtained by RIA and GC-MS are comparable. It is unlikely that the PGs obtained in the present study were catabolized, since it has been shown by several inbestigators that the brain of several species, including the rat, lacks the PG degrading enzyme, 15-hydroxy-prostaglandin dehydrogenase (EC 1.1.1.141) (23,25,31,32). Removal of PGs from the brain is probably achieved by diffusion or active transport into the venous system, and degradation is effected in other tissues (23,33).

Thus, several groups of workers agree that

the endogenous synthesis of PGs by brain slices lead largely to the accumulation of PGF. This, however, does not necessarily mean that all the PG synthesis is due to neural cells, i.e., the neurons and/or the neuroglia. While glioma in culture have been shown to synthesize PGs (34), there is apparently no definitive proof that neurons do so. Much of the synthesis by the slices may, therefore, be due to extraneural elements such as the brain capillary endothelial cells and, in the case of immunochallenged animals, by lymphocytes and other invasive cells in the CNS tissue. The results of the present study must, therefore, be considered in terms of the heterogeneous source of the PGF produced.

The findings that, in brain EPG, the EFA deficiency is poorly expressed in terms of a deficit of the PG precursor 20:4ω6 is in agreement with previous findings that the effects of the deficiency are not as marked in brain as in other tissues (17-21). Lymphocyte CPG and EPG, however, do show significant changes in fatty acid composition characteristic of EFA deficiency (unpublished results). Moreover, Selivonchick and Roots (personal communication) have found that the major phosphoglycerides of rat brain capillary endothelial cells also show similar significant changes in fatty acid composition in EFA deficiency. The marginally depressed PGF synthesis by brain slices cannot, therefore, be attributed to a lack of precursor in the neural cells themselves. While a change in the kinetics and/or microenvironment of the PG synthetase cannot be ruled out, it is possible that the decreased synthesis occurred in the capillary endothelial cells.

The effects of EFA deficiency on PG synthesis in other tissues have varied. Christ and Nugteren (8) found that incubated epidiymal fat pads from EFA-deficient rats showed a decreased PG synthesis compared to controls. This fat pad, like the brain slices, is morphologically heterogeneous, consisting of adipocytes and stromovascular elements. On the other hand, PG synthesis has been found to be increased in EFA deficiency in lung and intestine (7) and in kidney medulla (6) but decreased in vesicular glands (10). A direct comparison is not possible in these cases, however, since in these studies, homogenized tissue or powder was incubated with exogenous precursor.

While a trend towards decreased PGF synthesis was noted in all cases of immunochallenge, this was consistently significant only when the diet was adequate in EFA. Again it appears unlikely that the decreased PGF production reflects a direct effect on neural PGF synthesis. More likely it reflects a change in synthesis by peripheral activated lymphocytes and, in the case of EAE, also by those cells invading the neural tissue. Additionally, the immunochallenge may affect synthesis by the capillary endothelia. The absence of a significant decrease in PGF synthesis by brain slices from EFA-deficient immunochallenged rats can be explained by the fact that in this group the PGF synthesis was already depressed by the deficiency. A further depression to levels approximately the same as those seen in the EFA-adequate group does not prove to be statistically significant.

The use of *M. butyricum*-containing adjuvant gave varying degrees of severity of EAE and thus a comparison between the PGF synthesis of brain slices from paralyzed and nonparalyzed can be made. No difference was seen in the rats on the EFA-adequate diet, whereas a significant difference was seen in the EFA-deficient rats. The only explanation that can be offered is that when the stress in more severe, i.e., both the deficient diet and the neurological stress, there is a tendency for the PG synthesis to return to the unchallenged levels.

It is concluded that, to a large extent, the changes in PGF synthesis by brain slices observed in this study reflect events in the extraneural components of the slice. Thus, decreased brain PGF synthesis in EFA-deficient rats is more logically attributable to events in the capillary endothelia (and possibly in their luminal inclusions) in which greater changes in phospholipid fatty acid composition occur. The decreased synthesis in EFA deficiency may be due to decreased availability of the 20:4ω6 precursor, changed activity of PG synthetase, or its kinetics, or some combination of all three. That the changes in PGF synthesis may not have occurred in the neural elements themselves does not mean that events in the neural tissue were not influenced by the change in PGF production. Decreased levels of PGF could be expected to lead to decreased local vasoconstriction (23) and to decreased levels of cyclic GMP. Such effects are compatible with changed permeability of local vascular elements and with increased cellular immunity (35). Studies aimed at separating the contribution of the various elements of the slices to the observed PGF synthesis are indicated. Such studies should contribute to understanding of how changed PG synthesis may be involved in the increased permeability of the brain to the invading transformed lymphocytes which are involved in the neurological manifestations of EAE.

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High-Pressure Liquid Chromatography of Autoxidized Lipids: I. Methyl Oleate and Linoleate

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ABSTRACT

Autoxidized methyl oleate and linoleate were reduced with $NaBH_4$ and fractionated with a preparative high-pressure liquid chromatography (HPLC) reverse phase column. Products characterized from reduced-oxidized oleate included monohydroxy- and dihydroxyoctadecenoates, dihydroxy- and epoxyoctadecanoates. Products characterized from reduced-oxidized linoleate included hydroxy-*cis,trans*- and *trans,trans*-octadecadienoates, monohydroxy-, dihydroxy-, trihydroxy-, epoxyhydroxy-, and epoxyoctadecenoates. Quantitation of oxidation products by HPLC was in agreement with gas chromatography of trimethylsilyl ether derivative. Epoxyoctadecanoate in oleate and epoxy- and epoxyhydroxyoctadecenoates in linoleate were the most abundant secondary oxidation products. Some mechanisms are discussed to explain formation of these secondary products.

INTRODUCTION

In previous papers, we described the application of gas chromatography-mass spectrometry (GC-MS) for the analysis of autoxidation products of methyl oleate, linoleate, and linolenate (1-3). This approach provided valuable structural information on allylic hydroperoxides and some of the secondary products which may serve as nonvolatile precursors of off-flavors. For further characterization of oxidation products and for decomposition studies, it was necessary to develop a nondestructive chromatographic system useful on a preparative scale. This paper reports a high-pressure liquid chromatography (HPLC) system which meets these requirements.

Several HPLC methods have recently been reported for the separation of isomeric 9- and 13-hydroperoxides of oxidized linoleate (4,5) and products of lipoxygenase using linoleic acid (6) and γ-linolenic acid (7) as substrates. These studies were carried out with either adsorption (4-7) or reverse phase (5) chromatographic systems. In this paper, we describe a preparative method using a reverse phase HPLC system suitable for both spectral and GC-MS studies of the structure and the origin of oxidation products from methyl oleate and linoleate.

EXPERIMENTAL PROCEDURES

Materials

The same methyl oleate (98%) and linoleate (99%) were used as described previously (1,2). Autoxidations were carried out on 5 g samples stirred at 80 C with oxygen in 50-ml Erlenmeyer flasks. Oxidation was followed by oxygen uptake, peroxide value, and GC after silylation and $NaBH_4$ reduction (1).

HPLC

The same instrument and system reported by Scholfield (8) were used with sample size of 200 μl and mobile phases of H_2O:CH_3CN 15:85% (by weight) for reduced-oxidized oleate. With reduced-oxidized linoleate, the solvent system consisted of 40:60% by wt H_2O:CH_3CN, and after elution of oxidation fractions nonoxidized linoleate was removed with pure CH_3CN. The refractive index detector was used to monitor fractionation in both samples. The UV detector was only useful to monitor conjugated dienols in reduced-oxidized linoleate. Fractions were worked up by removing the solvent on a rotating evaporator below 40 C, transferring into sealed vials and storing at -20 C.

Methods

Infrared (IR) and nuclear magnetic resonance (NMR) analyses were preformed as previously (9) except as noted for GC, thin layer chromatography (TLC), and GC-MS. Analytical GC of the trimethylsilyl (TMS) ethers (10) was programmed between 180 and 250 C at 4 C per min and 25 ml carrier flow rate, on a 183 cm x 2 mm ID stainless-steel direct injection column packed with 3% JXR on Gas Chrom Q, 100/120 mesh (Applied Science Labs, State College, PA). The silica gel TLC plates containing an ultraviolet (UV) 254 nm fluorescence agent were developed with diethyl ether-hexane 1:1 (v/v). After examination of the plates with UV light, they were charred with 50% H_2SO_4 to visualize all the spots. Conditions for GC-MS (TMS ethers) inclued a GC program of 180 C for 12 min after injection; the temperature was then increased 5 C per min to 250 C, followed by 5 min 250 C hold. The GC column was glass,

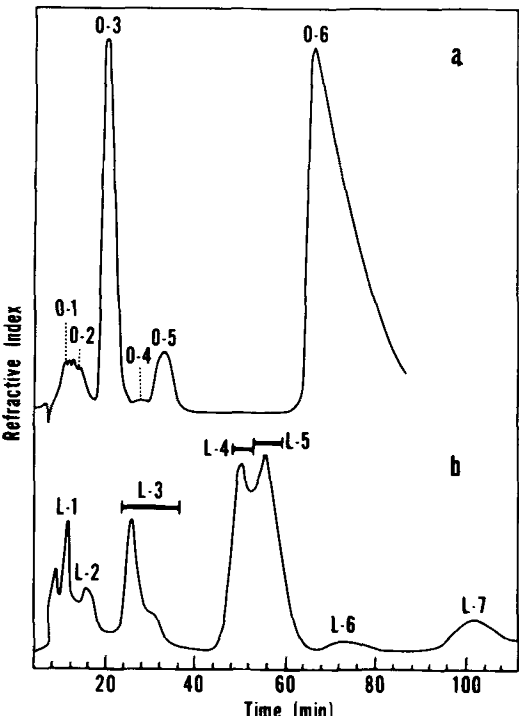

FIG. 1. Reverse phase HPLC of $NaBH_4$ reduced-oxidized lipids with H_2O:CH_3CN mobile phase (flow rate 5.0 ml per min; refractive index detector attenuation: 16X). (a) Reduced-oxidized methyl oleate, PV 1232. 0-1, diOH monoenes; 0-2, diOH saturates; 0-3, 8,9,10,11-OH allylic monoenes; 0-4, 9,10,11-OH saturates; 0-5, epoxy saturates; 0-6, methyl oleate + stearate. Mobile phase 15:85 wt % H_2O:CH_3CN. (b) Reduced-oxidized methyl linoleate, PV 1249. L-1, triOH monoenes; L-2, diOH monoenes; L-3, 9- and 13-epoxy-OH monoenes, L-4, 9- and 13-OH-*cis,trans*-dienes; L-5, 9- and 13-OH-*trans,trans*-dienes; L-6, 10- and 12-OH monoenes; L-7, epoxy monoenes. Mobile phase 40:60 wt % H_2O:CH_3CN. Nonoxidized methyl linoleate eluted with 100% CH_3CN.

183 cm x 4 mm ID, filled with the above JXR packing. Mass spectrometer scan range was m/e 0 to 600 every 9 sec. To avoid omission of positional isomers, the masses from the spectra recorded across each GC peak were summed by computer (1).

RESULTS

Chromatographic Fractionation and Analyses

HPLC of the reduced-oxidized oleate (Fig. 1a) yielded main fractions containing dihydroxy esters (0-1 and 0-2), hydroxy allylic monoenes (0-3), epoxy esters (0-5), and unoxidized methyl oleate (0-6). The main oxygenated component 0-3 is derived from the corresponding oleate hydroperoxides and consists of a mixture of isomers with hydroperoxide on carbon positions 8, 9, 10, 11 (1). HPLC of the reduced-oxidized linoleate produced a more complex chromatogram (Fig. 1b) including trihydroxy (L-1), dihydroxy (L-2), and epoxyhydroxy esters (L-3), hydroxy-conjugated dienes (L-4, L-5), hydroxy monoenes (L-6), and epoxy monoenes (L-7). The main hydroxy component derived from the corresponding 9- and 13-hydroperoxides (2) is partially separated into *cis,trans* (L-4), and *trans,trans* diene isomers (L-5).

The same reduced-oxidized samples were analyzed by GC after silylation. Gas chromatograms of the TMS derivatives (Fig. 2) showed the same distribution of products as obtained by HPLC. In the reduced-oxidized oleate sample, the peak due to epoxystearate (0-5) was not completely separated from the main peak due to the allylic OTMS ethers (0-3) (Fig. 2a). In the reduced-oxidized linoleate sample, the main component is separated into a single peak due to the 9- and 13-TMS-*cis,trans*-dienes (L-4) and a double peak due to the corresponding *trans-trans* isomers (L-5) (Fig. 2b) (2).

Weight compositions obtained by HPLC are compared to GC analyses in Table I. Recovery by HPLC was 92.3% with the oxidized oleate sample and 86.1% with the oxidized linoleate sample. Unknown materials presumed to be oxidation polymers were not eluted with the H_2O:CH_3CN solvent system but were eluted with $CHCl_3$. The $CHCl_3$ fraction was not examined further. HPLC fractions eluted with H_2O-CH_3CN were further characterized by GC, TLC, IR, NMR, and MS. Isolated components were shown by peak enhancement technique to have the same chromatographic behavior in excess silylating reagent as the corresponding components identified by GC-MS (1,2).

Reduced-Oxidized Oleate (Fig. 1a)

Fraction 0-1 (Dihydroxyoctadecenoates): GC of the TMS derivative showed one peak with retention 1.78 relative to methyl stearate (5.85 min). TLC gave one non-UV active spot with R_f 0.06 relative to methyl oleate. IR (CS_2) indicated the presence of olefinic unsaturation (2936 cm^{-1}), *cis* and *trans* unsaturation (720 and 960 cm^{-1}), and strong OH absorption (3688-3131 cm^{-1}). NMR (90 MHz, $CDCl_3$) showed absorption at 5.5 ppm (m) and 5.7 ppm (m) due to olefinic protons, and at 4.08 due to the carbinol methine protons. GC-MS of the TMS derivatives revealed a mixture of positional isomers of dihydroxyoctadecenoates. Although the molecular ion (TMS ether, m/e 472) did not appear, the M-15 (m/e, rel. int.) (457, 2.1) and the M-31 (441, 1.8)

ions were present. Characteristic fragments (TMS ethers) showed a mixture of positional diTMS isomers with one OH scattered between C-8 (245, 18), C-9 (259, 17; 257, 2.5), C-10 (271, 9.5), C-11 (285, 35), and the other OH between C-9 (227, 51), C-10 (213, 6.2; 215, 16.1), C-11 (201, 27), and C-12 (187, 7.4). This information confirms previous results obtained by GC-MS on unfractionated oxidized oleate (1) and provides evidence of one additional isomer 11,12-diOH octadecenoate.

Fraction 0-2 (Dihydroxyoctadecanoates): GC (TMS ethers) produced one peak with retention 1.88 relative to methyl stearate. TLC gave one non-UV active spot with R_f 0.13 relative to methyl oleate. IR showed strong hydroxy absorption (3140-3700 cm^{-1}). NMR gave very weak absorption at ~3.4 ppm for the carbinol methine protons and no olefinic proton absorptions at 5.5 or 5.7 ppm. GC-MS of the TMS ethers indicated a mixture of methyl dihydroxyoctadecanoate positional isomers with M-15 (459, 1.2) and M-31 (443, 1.4). Characteristic fragments for the positional isomers indicated that one OH was scattered between C-8 and C-10 and the other between C-9 and C-11.

These results confirm previous evidence obtained by GC-MS on unfractionated oxidized oleate showing that the 9,10-dihydroxyoctadecanoate is the major dihydroxyoctadecanoate found in autoxidized oleate (1).

Fraction 0-3 (Monohydroxyoctadecenoates): GC (TMS ethers) gave on peak with retention time of 1.44 relative to methyl stearate. TLC gave a non-UV active spot with R_f 0.43 relative to methyl oleate. IR indicated a mixture of both *cis* and *trans* unsaturation (645 and 958 cm^{-1}) and hydroxy absorption (3600 to 3410 cm^{-1}). NMR showed the presence of olefinic protons at 5.53 ppm (m) with a pattern suggesting *trans* configuration but J could not be calculated, allylic olefinic unsaturation at 2.02 ppm (m) (CH_2-C=C) and 4.00 ppm (m) absorption for the carbinol methine proton. GC-MS of fraction 0-3 after silylation showed that it was a mixture of 8-, 9-, and 10-, and 11-allylic-OH-octadecenoate as previously reported on the unfractionated sample (1).

Fraction 0-4 (Monohydroxyoctadecanoates): This minor component (less than 0.1%) was shown by mass spectrometry (TMS derivatives) to be a mixture of 9-, 10-, and 11-hydroxyoctadecanoates (MW 386): M-15 and M-31 ions are present at (371, 3.3) and (355, 7.2). Characteristic fragments for the silylated monohydroxyoctadecanoates are: 9-TMS (229, 17) (259, 19), 10-TMS (215, 100) (273, 95), and 11-TMS (201, 39) (287, 25). The relatively greater proportion of the 10-OH isomer and the absence of the 8-OH isomer indicates that these minor components are not derived from the allylic OH octadecenoates identified in fraction 0-3.

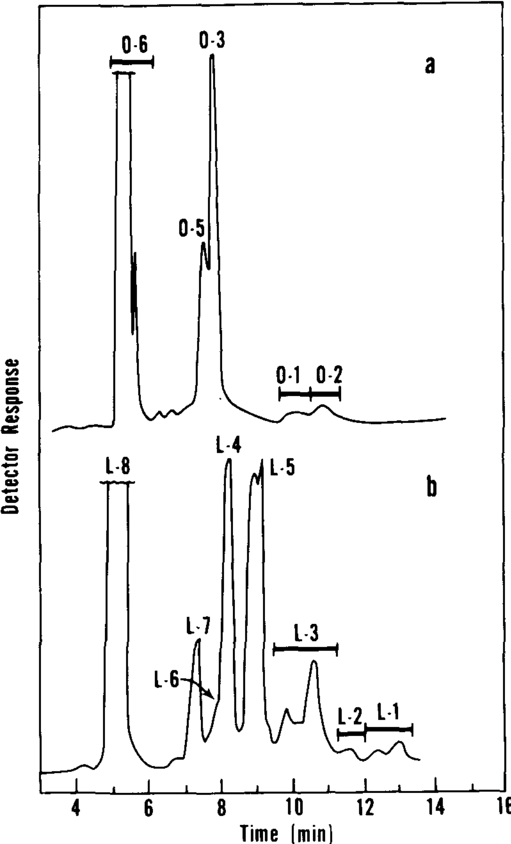

FIG. 2. Gas chromatograms of $NaBH_4$ reduced-oxidized lipids (TMS ethers) on 3% JXR packing in a 6 ft x 1/8 in. column, 180 to 250 C at 4 C/min temperature program. (a) Reduced-oxidized methyl oleate (PV 1232) (OTMS). 0-1, diOH monoenes; 0-2, diOH saturates; 0-3, 8,9,10,11-OH monoenes, 0-5, epoxy saturates; 0-6, methyl oleate + methyl stearate; (b) Reduced-oxidized methyl linoleate (PV 1249). L-1, triOH monoenes; L-2, diOH monoene; L-3, epoxy-OH monoenes; L-4, 9- and 13-*cis,trans*-OH-dienes; L-5, OH-*trans,trans*-9- and 13-dienes; L-6, 10- and 12-OH monoenes; L-7, epoxy monoenes; L-8, methyl linoleate.

Fraction 0-5 (Epoxyoctadecanoate): GC gave one peak with retention time of 1.36 relative to methyl stearate. Silylation caused no change in retention time. TLC gave a non-UV active spot with R_f = 0.85 relative to methyl oleate. NMR showed a broad triplet at 2.65 ppm indicating the presence of a *trans* epoxide

TABLE I

Composition of $NaBH_4$ Reduced-Oxidized Oleate and Linoleate

Fractions[a]	HPLC wt %[b]	GC wt %	Identification
		Oleate (PV 1232)	
0-1	1.0	0.7	DiOH monoenes
0-2	1.2	0.7	DiOH stearates
0-3 + 0-4	19.7	21.3	OH monoenes
0-5	5.0	4.3	Epoxy stearates
0-6	73.1	73.0	Oleate + stearate[c]
		Linoleate (PV 1249)	
L-1	1.7	1.2	TriOH monoenes
L-2	0.3	0.2	DiOH monoenes
L-3	5.2	5.4	Epoxy OH monoenes
L-4	5.6	7.0	9- and 13-OH-*cis,trans*-Dienes
L-5	8.9	11.6	9- and 13-OH-*trans,trans*-Dienes
L-6	1.5	1.2	OH monoenes
L-7	2.3	2.8	Epoxy monoenes
L-8[d]	74.4	70.6	Linoleate

[a]See Figures 1 and 2.

[b]Composition of H_2O-CH_3CH and CH_3CN eluted fractions normalized to 100%. Oleate 92.3% was recovered with H_2O-CH_3CN and linoleate: 86.1% was recovered with H_2O-CH_3CN and CH_3CN mobile phases.

[c]Present in starting materials and not shown in Figure 1a.

[d]Not shown in Figure 1b and eluted with CH_3CN.

ring (11). Mass spectrometry showed fraction 0-5 to be methyl 9,10-epoxyoctadecanoate with molecular ion (312, 0.9) and M-31 (281, 6.2). Characteristic fragments for the 9,10-epoxy isomer include m/e (155, 100) and (199, 23) (1). A small amount of 8,9-epoxy (185, 12), and 10,11-epoxy isomers (169, 5.3) also may be indicated in fraction 0-5. These results agree with the previous GC-MS evidence on unfractionated reduced-oxidized oleate (1).

Reduced-Oxidized Linoleate (Fig. 1b)

Fraction L-1 (Trihydroxyoctadecenoates): GC after silylation gave two peaks with retention times 2.35 and 2.46 relative to methyl linoleate (5.27 min). TLC showed two non-UV active spots with R_f 0.04 and 0.07 relative to methyl linoleate. IR indicated olefinic *trans* unsaturation (965 cm^{-1}) and hydroxy absorption (3620 cm^{-1}, free OH; 3640-3160 cm^{-1}, bonded C-OH). NMR confirmed the presence of olefinic protons, 5.76 ppm (m) and the hydroxy group from the carbinol methine proton, 4.11 ppm (m). MS of fraction L-1 was in accordance with spectra reported by Graveland (12) for methyl 9,10,13-tri(TMS)-11-octadecenoate and 9,12,13-tri(TMS)-10-octadecenoate (MW 560). Characteristic ions include: (M-15, 0.5), (M-31-90, 1.9), (M-100, 11.2), (173, 97.2), 259, 100), and (301, 4.2).

Fraction L-2 (Dihydroxyoctadecenoates): GC (TMS ethers) produced one peak with retention time 2.20 relative to methyl linoleate. TLC gave one non-UV active spot. IR denoted olefinic (3010 cm^{-1}, H-C=C-H) *trans* unsaturation (980 cm^{-1}) and hydroxyl absorption (3600, 3620-3500 cm^{-1}). NMR confirmed the IR assignment of a *trans* absorption centered at 6.22 ppm (J=12.1 Hz), broad olefinic absorption at 5.6 ppm, and methine absorption at 4.12 ppm (m) (C*H*OH). MS (TMS ether) was in agreement with previously reported spectra (13) for 9,13-di(TMS) 10-and/or 11-octadecenoate (MW 472). Characteristic fragments (TMS ethers) (13) include (M-43, 10), (173, 52), (259, 31), and (355, 9). We have thus confirmed methyl 9,13-dihydroxyoctadecenoate previously found by GC-MS (2) in reduced-oxidized linoleate and now established that the double bond is in the *trans* configuration.

Fraction L-3 (Epoxyhydroxyoctadecenoates): GC (TMS ethers) showed three peaks with retention 1.92, 1.97, and 2.01 relative to methyl linoleate. TLC gave three non-UV active spots with R_f 0.23, 0.27, and 0.28 relative to methyl linoleate. IR showed olefinic (3005 cm^{-1}, H-C=C-H) *trans* unsaturation (958 cm^{-1}, C=C). Also evident was absorption for hydroxy (3600 cm^{-1}, free COH; 3640-3380 cm^{-1}, bonded COH) and a *trans* epoxide group (874 cm^{-1}) (14). NMR supports the IR assignments with evidence for the *trans* double bond at 5.93 ppm (m) (J=15.5 MHz) and 5.53 ppm (J=15.6 MHz), *trans* epoxide 3.09 ppm (m) (J=2.2

MHz), and the carbinol methine proton at 4.17 ppm (m). MS indicates main fraction L-3 (Fig. 1b) to be a mixture of methyl 9-hydroxy-12, 13-epoxy-10- and 13-hydroxy-9,10-epoxy-11-octadecenoates with molecular ion (TMS ethers) (398, 2.4), (M-15, 4.8), and (M-31, 2.0) in the spectrum. Characteristic fragments (TMS ethers) correspond to those reported by Graveland (12) for the 9-/13-hydroxy-9,10-/12,13-epoxyoctadecenoates: (173, 76), (199, 15), (241, 99), (259, 61), and (327, 86). The small fraction from the rear shoulder of peak L-3 is apparently a mixture of 11-hydroxy-9,10-epoxy- and 11-hydroxy-12,13-epoxyoctadecenoates according to MS of the OTMS derivatives: M-15 ion (383, 1.1) and same characteristic fragments as reported by Hamberg and Gotthammar (15) for the 11-hydroxy-9,10/12,13-epoxyene: (199, 100), (71, 2.8), (113, 4.1), (285, 74), and (327, 4.0). IR showed the same absorption bands as previous fraction L-3, and NMR showed a similar spectrum with absorption band due to *trans* epoxide (3.09 ppm, J=2.2 MHz). These data confirm and supplement previous results determined by GC-MS (2).

Fraction L-4 (Hydroxy-cis,trans-Octadecadienoates): GC (TMS ethers) produced one peak with retention time 1.55 relative to linoleate. TLC gave two spots with R_f 0.54 and 0.50 which before charring were UV active indicating a conjugated double bond system. According to Sessa et al. (16), the spot with R_f 0.54 is due to the 13-hydroxy-*cis,trans*-dienol and that with R_f 0.50 is the 9-hydroxy-*cis,-trans*-dienol isomer. IR indicated olefinic (3005 cm^{-1}, HC=CH) conjugated *cis,trans* unsaturation (990 and 968 cm^{-1}) and hydroxy absorption (3600 cm^{-1}, free COH; 3700-3160 cm^{-1}, bonded COH). NMR supported the IR analysis with signals for the olefinic protons 5.91 ppm (m), 2.10 ppm (M) (CH_2-C=), and for the methine proton 4.15 ppm (m) on the carbinol methine proton. The absorption due to olefinic protons and coupling constants were in agreement with those reported in the literature (17,18). MS was identical to that previously determined by GC-MS for the mixture of 9- and 13-hydroxy-*cis,trans*octadecadienoates (2)..

Fraction L-5 (Hydroxy-trans,trans-Octadecadienoates): GC (TMS ethers) produced two peaks of retention times 1.69 and 1.73 relative to linoleate. TLC gave two UV active spots with R_f 0.45 and 0.50, which according to Sessa et al. (16) represent the *trans,trans*-9- and 13-dienols, respectively. The *trans,trans*-conjugated unsaturated system was confirmed by IR (985 cm^{-1}) together with hydroxy absorption (3600 cm^{-1}, free C-OH; 3695-3318 cm^{-1}, bonded C-OH). NMR gave signals for the *trans,trans* olefinic protons 5.41 (m), 2.07 ppm (m) (CH_2-C=), and 4.20 ppm for the carbinol methine proton. In agreement with observations of Chan and Levett (5), the olefinic protons of the *trans,trans* isomers exhibited a complex spectrum which could not be analyzed. MS was identical to that determined previously by GC-MS for a mixture of *trans,trans*-9- and 13-hydroxyoctadecadienoate (2). HPLC afforded separation and direct evidence for the *cis,trans* and *trans,trans* configuration of the diene systems in fractions L-5 and L-6.

Fraction L-6 (Hydroxyoctadecenoates): GC (TMS ethers) produced one peak of retention 1.39 relative to linoleate. IR indicated olefinic (3000 cm^{-1}, HC=CH) *cis* unsaturation (714 cm^{-1}) and hydroxy absorption (3600 cm^{-1}, free C-OH; 3695-3318, bonded C-OH). NMR supported the IR analysis with absorptions for the olefinic protons at 5.41 ppm (m) and the carbinol methine proton 4.20 (m). MS (TMS ether) gave M-15 (369, 3.2) and M-31 (353, 1.4) ions corresponding to hydroxyoctadecenoates (MW 384) and characteristic TMS-containing fragments for the 10-hydroxyoctadecenoate (213, 4.3) (273, 68) and for the 12-hydroxyoctadecenoate (187, 100) (299, 3.3). These minor compounds, not previously detected by GC-MS of reduced methyl linoleate (2), are apparently side-products from the $NaBH_4$ reduction of the 9- and 13-hydroperoxides (19).

Fraction L-7 (Epoxyoctadecenoates): GC produced one peak of retention time 1.39 relative to linoleate. Silylation caused no change in chromatographic behavior. TLC gave one spot, not UV active of R_f = 0.89 relative to linoleate. IR indicated an olefinic (3002 cm^{-1}, H-C=C-H) *cis* unsaturation (718 cm^{-1}) and a *cis* epoxide group 815-835 cm^{-1}. NMR supported the IR assignments with olefinic absorption centered at 5.44 ppm (m) and an isolated *cis* epoxide group 2.92 ppm (20). The pattern for the olefinic absorption suggested *cis* configuration, but coupling constant could not be obtained. MS showed the molecular (310, 1.9) and M-31 (279, 9.0) ions corresponding to epoxyoctadecenoate. Ions characteristic for methyl 9,10-epoxyoctadecenoate include (155, 27) and (197, 2.0). Evidence suggesting other epoxy monoenes includes: 8,9-epoxy- (167, 19), (185, 12); 9,10-epoxy- (153, 18), (199, 16); 10,11-epoxy- (139, 19), (213, 0.7); and 11,12-epoxy- (125, 16) (227, 0.4) octadecenoates. These epoxy monoenes were not detected previously by GC-MS of unfractionated reduced-oxidized methyl linoleate (2).

DISCUSSION

Hydroperoxides of methyl oleate and linoleate have been extensively investigated (1,2,4,5,21), but the secondary products of autoxidation have received less attention and may play an important part in flavor deterioration. The preparative reverse phase HPLC method used in this paper provided purified oxidation products in sufficient amounts to afford structural confirmation of previous GC-MS data (1,2) by IR, NMR, GC, TLC, and MS. Examination of HPLC fractions provided not only additional structural details regarding double bond and epoxide configuration of some of the secondary oxidation products but also quantitative information on their relative amounts in oxidized oleate and linoleate.

The monohydroxy esters derived from 8-, 9-, 10-, and 11-oleate hydroperoxides were isolated in 19.7% yield by HPLC (Table I). Secondary products from oxidized oleate previously indicated by GC-MS (1) and now confirmed by HPLC include dihydroxyoctadecenoate (hydroxy on C-8 through 12), dihydroxyoctadecanoate (hydroxy on C-8 through 11), 9-, 10-, 11-monohydroxy-, dihydroxy- (hydroxy on C-8 through 11), and epoxy- (mainly 9,10- and some 8,9- and 10,11-) octadecanoate isomers. Structural analysis of HPLC fractions indicated evidence of new minor products including the 11,12-dihydroxyoctadecenoate and the isomeric mixture of 9-,10- and 11-OH-octadecanoate. These monohydroxy esters are apparently minor side-products of the $NaBH_4$ reduction. The dihydroxyoctadecenoate esters were shown to be a mixture of *cis* and *trans* isomers. Quantitative HPLC and GC analyses (after silylation) showed the 9,10-epoxyoctadecanoate to be the most important of the nonvolatile secondary products. The abundance of 9,10-epoxy ester suggests that it is derived by secondary reaction of oleate with oleate hydroperoxides (22). The other epoxy isomers, 8,9-epoxy- and 10,11-epoxyoctadecanoates, which are found in much smaller quantities, apparently originate from the oleate hydroperoxides. The 9,10-dihydroxy and the other dihydroxy isomers may be formed either by cleavage of the corresponding epoxy esters or from dihydroperoxides (21).

The monohydroxy esters derived from 9- and 13-linoleate hydroperoxides were isolated in 14.5% yield by HPLC (Table I). Secondary products from linoleate tentatively identified by GC-MS (2) and now confirmed by analyses of HPLC fractions include: methyl 9,12,13-trihydroxy-10- and/or 9,10,13-trihydroxy-11-octadecenoates, 9,13-dihydroxy-10- and/or 11-octadecenoates and 9-hydroxy-12,13-epoxy-10-, 13-hydroxy-9,10-epoxy-11-, 11-hydroxy-12,13-epoxy-, and 11-hydroxy-9,10-epoxyoctadecenoates. The double bond and epoxy group of these oxidation products were *trans* in configuration. New linoleate oxidation components identified by HPLC include the 10- and 12-hydroxyoctadecenoates and *cis*-epoxy-*cis*-octadecenoates (with epoxy groups on carbons 8,9; 9,10; 10,11; 11,12). The hydroxy monounsaturated esters correspond to the side-products previously identified from $NaBH_4$-reduced linoleate hydroperoxides (19). HPLC and GC analysis (OTMS derivatives) showed epoxy- and epoxyhydroxyoctadecenoates to be the most important secondary oxidation products of $NaBH_4$ reduced-oxidized linoleate.

Because we used $NaBH_4$, any keto products are reduced, just like the hydroperoxides, to the corresponding hydroxy derivatives. Epoxy- and epoxyhydroxy esters can arise from linoleate hydroperoxides through oxy radicals (RO·) by cyclization with the α double bond and addition of a hydroxy radical on the remaining allylic propene system (23,24). The dihydroxy and trihydroxy esters can be explained by postulating cleavage of the epoxy ester, or by addition of a hydroxy radical to the conjugated diene system of the hydroperoxides, or by reduction of di- or trihydroperoxides (21), or keto-hydroxy or hydroxy-hydroperoxides (13). These suggestions need to be verified by chromatographic studies carried out directly on the nonreduced-oxidized fatty esters. Such studies are also needed to determine the role of secondary oxygenated products in flavor deterioration. For this work, partition chromatography by the reverse phase system used in this study would minimize decomposition of hydroperoxides to be expected with adsorption chromatographic systems (21). The next paper in this series will deal with HPLC separation of autoxidized linolenate.

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Synthesis of Acyl-S-Pantetheine by Rat Liver Microsomes

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ABSTRACT

The synthesis of acyl-S-pantetheine was found to occur in rat liver microsomal preparations. The reaction required ATP and a metal ion as cofactors, a fatty acid and the reduced form of pantetheine for optimal activity. The K_m for pantetheine was 0.8 mM, for ATP 0.8 mM, and for oleic acid 0.3 mM. Mg^{2+} (20 mM), Mn^{2+} (5 mM), Ca^{2+} (5 mM), and Fe^{2+} (5 mM) produced approximately equal activity when all other conditions were optimal. The characterization of the product and other properties of the enzyme are described. The acyl-S-pantetheine formed does not act as an acyl donor in the acylation of *sn*-glycerol-3-phosphate, 1,2-diacylglycerol, or lysolecithin.

INTRODUCTION

Several years ago Trams et al. (1) reported that acyl-S-pantetheine could be formed from acyl-CoA derivatives by rat liver plasma membranes. They suggested that an acyl-CoA derivative was first attacked by a nucleotide pyrophosphatase to yield 4′-phosphopantetheine acyl ester and 3′,5′-ADP. The acyl ester was then cleaved by a phosphomonoesterase to produce an acyl-S-pantetheine derivative.

Numerous other laboratories (2-4) have attempted to show that acyl-S-pantetheine could be formed by rat liver microsomal acyl-CoA ligase (EC 6.2.1.3) but failed to do so. This compound was shown to be synthesized by *Escherichia coli* acyl-CoA ligase at about 15% the rate of acyl-CoA formation (5).

We have recently re-examined the possibility that acyl-S-pantetheine is formed from ATP and pantetheine when we noted that these two compounds together inhibited the synthesis of phosphatidylcholine (6) and phosphatidylethanolamine (7). This paper describes the properties of an enzyme found in rat liver microsomes which carries out the synthesis of acyl-S-pantetheines.

MATERIALS AND METHODS

CoA, ATP, and other nucleotides were obtained from P-L Biochemicals, Milwaukee, WI; pantethine, dithiothreitol, bovine serum albumin, glutathione, cysteine, cysteamine, potato acid phosphatase (type IV, EC 3.1.3.2), calf intestinal mucosal alkaline phosphatase (type I, EC 3.1.3.1), and *Crotalus adamanteus* nucleotide pyrophosphatase (EC 3.6.1.9) were obtained from Sigma Chemical Co., St. Louis, MO. [1-^{14}C]Oleic acid, [1-^{14}C]palmitic acid, and [^{3}H]CoA were from New England Nuclear, Montreal, Quebec. Unlabeled fatty acids were from the Hormel Institute, Austin, MN.

^{3}H Pantetheine

[^{3}H]Pantetheine was prepared from [^{3}H]CoA by first treating it at pH 5.0 with acid phosphatase to remove the phosphate group at the 3′ position. The pH was then readjusted to 7.5 and the dephospho-CoA cleaved using nucleotide pyrophosphatase. The 4′-phosphopantetheine was then treated at pH 9.0 with alkaline phosphatase. [^{3}H]Pantetheine was finally isolated by paper chromatography using the solvent system n-butanol-acetic acid-water (5:2:1, v/v).

Enzyme Assay Procedure

The assay system for following the formation of acyl-S-pantetheine was carried out utilizing either [^{14}C]labeled fatty acids or [^{3}H]pantetheine. In either case, the incubation system contained the following in a total volume of 0.5 ml: 100 mM Tris-HCl buffer pH 7.7, 20 mM $MgCl_2$, 4.0 mM pantetheine, 5 mM ATP, 1.0 mM fatty acid (as the ammonium salt), 10 mM dithiothreitol, and 0.25-0.5 mg of microsomal protein.

After incubation for 20 min at 37 C, the reaction was terminated by the addition of 4.0 ml of methanol. The tubes were then centrifuged and the supernatant carefully decanted. The precipitated protein was again extracted with methanol, and after centrifugation the methanol extracts were combined. To the methanol, 10 ml of chloroform was added, followed by 30 ml of 2 M KCl. The tubes were then shaken and centrifuged to aid in phase separation. The top aqueous phase was removed by aspiration and the lower chloroform phase washed three more times with KCl and finally once with water. When the label utilized was [^{3}H]pantetheine, an aliquot of the chloroform phase was taken to dryness, scintillation fluid added, and the lipids counted. When the label was [1-^{14}C]oleate, carrier oleyl-S-pantetheine was added to each tube and an aliquot of the chloroform phase was applied to Silica Gel G

thin layer plates. The plates were then developed in the solvent systems chloroform-methanol (9:1, v/v) or petroleum ether-ether-acetic acid (70:20:4, v/v). After locating the oleyl-S-pantetheine by spraying the plates with Rhodamine 6B, the spots were scraped and counted as described by Webb and Mettrick (8).

Enzyme Preparation

Rat liver microsomes were prepared as described previously (9) utilizing Sprague-Dawley male rats weighing ca. 180 g. They were fasted for 18 hr prior to sacrifice. Protein was determined as described by Lowry et al. (10) using crystalline bovine serum albumin as standard.

S-Acyl Thioester Preparations

Palmityl and oleyl thioesters of pantetheine, glutathione, and thioglycolic acid were prepared essentially as described by Trams et al (1). Pantetheine was prepared from pantethine by reduction using mercaptoethanol and then lyophilization, while the other sulfhydryl compounds were utilized without any further reduction. The acyl thioesters were chromatographed twice on silicic acid columns and finally by thin layer chromatography (TLC) using the solvent system chloroform-methanol (9:1, v/v).

The thioesters were also characterized by treating with hydroxylamine (11). Authentic hydroxamates were prepared as described by Migrdichian (12).

RESULTS AND DISCUSSION

Shown in Table I are the results obtained when either [1-^{14}C]oleate or [^{3}H]pantetheine were incubated with rat liver microsomes as described in the Materials and Methods section. When labeled oleate was utilized, 39.0 nmoles/min/mg protein of oleyl-S-pantetheine was synthesized (Exp. 1, line 1). When either ATP or pantetheine was omitted from the incubation system, no radioactive oleate could be detected in oleyl-S-pantetheine (Exp. 1, lines 2 and 3). Similarly, if pantethine was utilized rather than pantetheine, no radioactivity was detectable.

When [^{3}H] pantetheine was utilized to follow the reaction, 15.7 nmoles of oleyl-S-pantetheine was formed (Exp. 2, line 1). In the absence of added oleate, 1.5 nmoles of acyl-S-pantetheine was formed from endogenous fatty acids. In the absence of added ATP or prior boiling of the enzyme, no radioactive lipid could be detected (Exp. 2, lines 3 and 4).

Characterization of Reaction Product

An experiment was performed on a ten-fold scale as described in the Methods and Materials section, utilizing [1-^{14}C]oleate and [^{3}H]pantetheine of equal specific activity. After incubation, 10 mg of carrier oleyl-S-pantetheine was added and the product isolated as described under enzyme assay procedure and then subjected to column chromatography on silicic acid. The product that was eluted from the column using chloroform-methanol (95:5, v/v) was collected and again subjected to column chromatography to ensure that complete separation of any unreacted [^{14}C]oleate occurred. The doubly labeled oleyl-S-pantetheine was further purified by TLC using the solvent system chloroform-methanol (9:1, v/v). The radioactive product migrated identically with that of authentic oleyl-S-pantetheine, having an R_f of 0.45, while oleic acid migrated with an R_f of 0.66. Pantetheine, pantethine, and CoA remained at the origin. The isolated product contained 13,500 dpm of [^{14}C] and 16,000 dpm of [^{3}H]. The lower amount of radioactivity in [^{14}C] was probably due to the dilution of oleate by endogenous fatty acids.

An aliquot of the labeled product was treated with hydroxylamine (11) at neutral pH and then extracted with ether and the extract subjected to TLC on Silica Gel G (13) using the solvent system toulene-methanol (8:2, v/v). The product was identified as an acyl hydroxamate by the fact that it gave a typical purple color on spraying the plate with ferric perchlorate. The radioactive product also migrated with an R_f identical to that of authentic oleyl hydroxamate (13).

Another aliquot of the radioactive oleyl-S-pantetheine was subjected to hydrolysis with 1 N HCl at 100 C for 5 min. The aqueous phase was extracted with diethyl ether and then subjected to paper chromatography using 3 mm Whatmann paper and the solvent system n-butanol-acetic acid-water (5:2:1, v/v). It had an R_f of 0.70, which was identical to that of pantetheine, while CoA had an R_f of 0.17. The ether extracts were also chromatographed on thin layer Silica Gel G and the radioactive product found to co-migrate with that of authentic oleic acid.

In contrast to palmityl-CoA, the doubly labeled product of the above reaction was readily soluble in diethyl ether, petroleum ether, chloroform, or methanol.

Kinetics of Acyl-S-Pantetheine Formation

Time course studies for the synthesis of oleyl-S-pantetheine indicated that the rate was

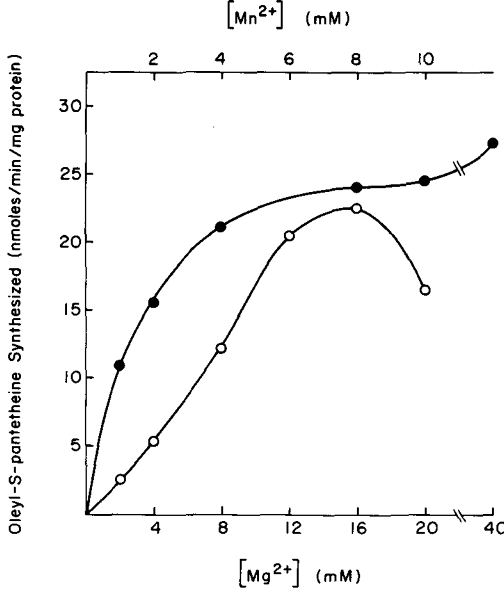

FIG. 1. Effect of varying Mg^{2+} or Mn^{2+} on oleyl-S-pantetheine synthesis. The conditions for assay were as described in the Methods and Materials section.

linear for at least 20 min. The enzyme usually lost ca. 50-70% of its activity when stored at -20 C for 2 weeks.

pH Profile

Utilizing Tris-HCl buffer, the optimum pH for the reaction was found to be between 7.8 and 8.5. When phosphate buffer was substituted for Tris-HCl, there was little apparent difference in activity.

Effect of Varying Protein Concentration

Studies on the effect of varying protein concentration on the rate of oleyl-S-pantetheine synthesis indicated that it was linear up to a concentration of 1.2 mg/ml.

Metal Ion Requirement

The effect of varying Mg^{2+} and Mn^{2+} is shown in Figure 1. Magnesium ions at 20 mM were optimal, while 5 mM manganese ions showed optimal activity. Shown in Table II are the effects of a variety of metal ions and for comparative purposes their activity with acyl-CoA ligase reaction. The major differences between the two enzymes was observed with respect to their activities with Ca^{2+} and Fe^{2+}. Calcium was 80% as effective as Mg^{2+} in the synthesis of oleyl-S-pantetheine, but only 30% in the case of oleyl-CoA formation. Similarly Fe^{2+} was considerably more effective in the formation of oleyl-S-pantetheine than oleyl-CoA.

Effect of Varying ATP Concentration

Figure 2 shows the effect of varying ATP on the synthesis of oleyl-S-pantetheine. The K_m was found to be ca. 0.8 mM and the optimal concentration ca. 5 mM. Other nucleotide triphosphates were inactive except UTP which was 15% that of ATP. ADP showed activity, but when glucose and hexokinase were included in the incubation system, no activity resulted with this nucleotide.

Analogues of ATP such as adenylylimidodiphosphate (AMP-PNP), α,β-methylene adenosine 5'-triphosphate (APCPP), and $\beta\gamma$-methylene adenosine 5'-triphosphate (APPCP) were unable to act together with pantetheine in the formation of oleyl-S-pantetheine. Likewise, these analogues did not inhibit when added in the presence of optimal concentration of ATP.

It has been shown that AMP (15) and adenosine (16) inhibit the synthesis of palmityl-CoA catalyzed by acyl-CoA ligase. When AMP (8

TABLE I

Synthesis of Oleyl-S-Pantetheine by Rat Liver Microsomes[a]

Exp. no.	Additions or deletions	Oleyl-S-pantetheine synthesized (nmoles/min/mg protein)
1	Complete	39.0
	−ATP	< 0.1
	−Pantetheine	< 0.1
	−Pantetheine + pantethine	< 0.1
2	Complete	15.7
	−Oleate	1.5
	−ATP	< 0.1
	Complete (boiled enzyme)	< 0.1

[a]The reaction was carried out using [1-^{14}C]oleate (Exp. 1) or [^{3}H]pantetheine (Exp. 2). All other conditions were as described under enzyme assay procedure.

TABLE II

Effect of Various Metal Ions on Oleyl-S-Pantetheine Formation[a]

Metal ion	Relative activity	
	Oleyl-S-pantetheine	Oleyl-S-CoA
Mg^{2+}	100	100
Mn^{2+}	95	100
Ca^{2+}	80	30
Zn^{2+}	15	17
Cd^{2+}	10	18
Co^{2+}	23	41
Fe^{2+}	100	30

[a]*In experiments testing for oleyl-S-pantetheine synthesis, the concentration of Mg^{2+} was 20 mM while all other metal ions were at 5 mM. In the experiments testing for oleyl-S-CoA formation, all metals were at 5 mM. Acyl-CoA ligase was assayed as described by Lloyd-Davies and Brindley (14), oleyl-S-pantetheine synthesis as described under enzyme assay procedure.*

mM) or adenosine (8 mM) were added to the complete incubation system, no significant inhibition of oleyl-S-pantetheine synthesis could be observed.

Effect of Pantetheine and Other Sulfhydryl Reagents

When the concentration of pantetheine was varied, it was found that the K_m for the reaction was 0.8 mM and the optimal concentration was ca. 6 mM (Fig. 3A). Other sulfhydryl compounds such as glutathione, cysteamine, glycollic acid, and mercaptoethanol were found not to act in the activation of fatty acids. The enzyme requires that the sulfhydryl group be in

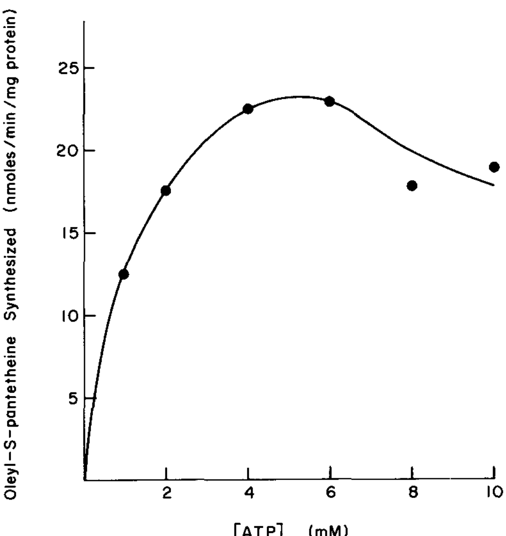

FIG. 2. Effect of varying ATP on oleyl-S-pantetheine formation. Assay conditions were as described as described in the Methods and Materials section.

the reduced state, because the replacement of pantetheine by pantethine did not result in the formation of any detectable oleyl-S-pantetheine (Table I). As can be seen from Figure 3A, ca. 8 nmoles of oleyl-S-pantetheine are formed /min/mg of protein when the concentration of pantetheine was 1 mM. When an identical experiment was performed using 1 mM CoA, a total of 41.5 nmoles of oleyl-CoA was formed.

The addition of 1 mM CoA in the presence of optimal concentration of pantetheine caused

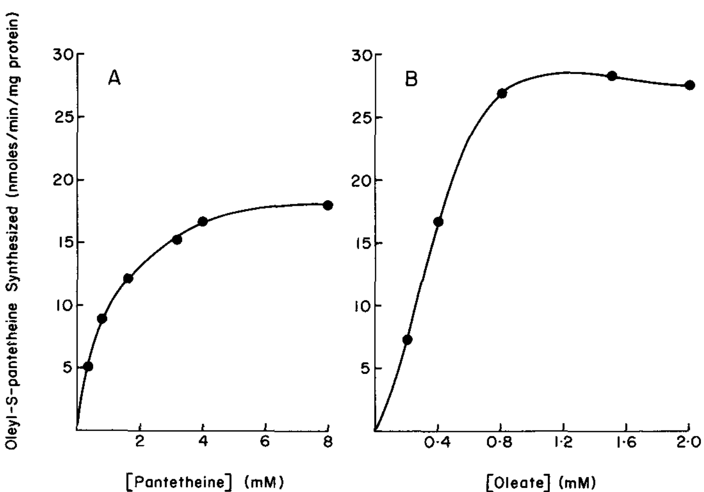

FIG. 3. (A) Effect of varying pantetheine concentration. (B) The effect of varying oleate concentration. Assay conditions were as described in Methods and Materials section.

TABLE III

Effect of Various Fatty Acids as Precursors of Acyl-S-Pantetheine[a]

Fatty acid added	Relative activity
Oleic	100
Palmitic	84
Lauric	104
Stearic	45
Octanoic	11
α-Bromopalmitic	8
α-Hydroxystearic	20
12-Hydroxystearic	21
Lignoceric	11
Palmitoleic	90
Linoleic	98
Arachidonic	150
Nervonic	17
Elaidic	97

[a]The concentration of all the fatty acids was 1 mM. All other conditions were as described in the Methods and Materials section. The activity obtained with oleic acid was arbitrarily set at 100.

an approximate 50% reduction in the formation of oleyl-S-pantetheine.

Effect of Varying Oleate and Other Fatty Acids

When oleate was varied, it was found that the apparent K_m was 0.3 mM, with optimal activity at ca. 1 mM (Fig. 3B). As can be seen in Table III, other fatty acids were readily incorporated into acyl-S-pantetheines. The unsaturated fatty acids appear to be more readily incorporated, but this is maybe a function of their solubility rather than specificity of the enzyme.

Role of Acyl-S-Pantetheine in Acylation Reactions

It was found that neither exogenously added or endogenously formed oleyl-S-pantetheine could act in acylation reactions leading to the formation of phosphatidic acid, triacylglycerol, or lecithin in the microsomal preparations utilized. It was found that when oleyl-S-pantetheine was incubated under a variety of conditions and concentrations with rat liver microsomes, it was not appreciably hydrolyzed, only 15% being hydrolyzed in 60 min.

Physiological Function of Enzyme

Previous attempts to show that acyl-S-pantetheine could be formed by rat liver microsomal acyl-CoA ligase were unsuccessful, presumably because the optimal pantetheine concentration for the reaction is ca. 6 mM. The K_m for CoA in acyl-CoA ligase reaction has been determined to be ca. 3.3 μM by Pande (3) and 7.2 μM by Marcel and Suzue (17) in the rat liver microsomal enzyme and 50 μM by Bar-Tana et al. (2) for the partially purified microsomal enzyme. The K_m for pantetheine in the reaction studied here is ca. 0.8 mM (Fig. 3A).

Brown (18) has reported that the amount of pantetheine in rat liver acetone powder preparations was 6.8 nmoles/g. This compares to 148 nmoles of 4′-phosphopantetheine and 1003 nmoles of CoA and dephospho-CoA. Pantetheine is formed primarily from the catabolism of CoA (19).

The physiological function of acyl-S-pantetheine derivatives formed as described above or from acyl-CoA (1) is not clear at the present time in light of the low concentration of pantetheine present in liver and the high concentration required in the activation of fatty acids.

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The Distribution of Dietary Plant Sterols in Serum Lipoproteins and Liver Subcellular Fractions of Rats[1]

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ABSTRACT

Rats were fed plant sterols containing campesterol and β-sitosterol in the different proportions, and their distribution in serum lipoproteins and in liver subcellular fractions was determined. In serum lipoproteins, the percentage as well as the concentration of plant sterols increased with the increase in the density of lipoproteins. Thus, high density lipoprotein (HDL) contained the highest and very low density lipoprotein (VLDL), the lowest. Also, there were distinct differences in the ratio of campesterol to sitosterol among lipoproteins, it was the highest in VLDL and lowest in HDL. Quantitatively, more than 75% of campesterol and 80% of sitosterol were carried in HDL; the values were significantly different from those of cholesterol (ca. 70%) in relation to total cholesterol. The distribution of plant sterols in liver subcellular fractions was virtually the same with that of cholesterol. Both nuclei and microsomes contained approximately 40% of total plant sterols.

INTRODUCTION

The intestinal absorption of plant sterols is significantly lower than that of cholesterol, and, in general, the sterols with longer side chains are absorbed to a lesser extent (1). Thus, only very small amounts of plant sterols are detectable in blood and tissues (2), although the usual diet may contain appreciable amounts of these sterols (3).

It is widely believed that the hypocholesterolemic effect of plant sterols is entirely due to their ability to inhibit cholesterol absorption in the intestine (1), but there is some evidence indicating an "extra absorptive" effect on cholesterol metabolism (4,5). In contrast, Bhattacharyya and Connor (6) have recently described patients with hyperphytosterolemia and have suggested that plant sterols in some way initiate the development of the xanthomas. Since hypocholesterolemic potency of plant oils might depend on their sterol composition (5) and the extent of absorption differs among plant sterols (1), tissue distribution of absorbed sterols should be determined in order to make clear the "extra absorptive" effect of plant sterols.

In this communication, we report the distribution of dietary plant sterols with different compositions in serum lipoproteins and in liver subcellular fractions of rats.

EXPERIMENTAL PROCEDURES

Male Wistar rats (Kyudo Co.) weighing an average of 104 g (experiment I) and 90 g (experiment II) were fed experimental diets for 64 and 44 days, respectively. The composition of the basal diet (7) was (%): casein, 20; safflower oil, 5; mineral mixture, 4; vitamin mixture, 1; cellulose powder, 4; choline chloride, 0.15; and sucrose to 100. Plant sterols were added at the expense of sucrose (7). In experiment I, one group of rats was fed the basal diet and the other received a diet containing 0.25% plant sterol. In experiment II, plant sterol was added at a 1.0% level with or without 0.5% cholesterol. Plant sterols (β-sitosterol, E. Merck, Lot Nos. 9905993 and 6408929 for experiments I and II, respectively) were repeatedly crystallized from ethyl acetate until over 98% purity as sterols was achieved as measured by gas liquid chromatography (GLC). The recrystallized sterol for experiment I consisted of 93% β-sitosterol and 7% campesterol, and that for experiment II, 60% β-sitosterol, 35% campesterol, and 5% stigmasterol. Rats fasted overnight were killed by decapitation. Lipoprotein fractions were isolated by ultracentrifugal flotation using a Spinco 40.3 rotor (8) into very low density (VLDL, $d<1.019$), low density (LDL, $1.019<d<1.063$), and high density (HDL, $1.063<d<1.21$) lipoproteins. Although there was considerable controversy for appropriate fractionation of rat plasma lipoproteins (9-11), the density limit suitable for human plasma was tentatively adapted. Livers were homogenized in 0.25 M sucrose, and nuclei (800 x g, 10 min), mitochondria (12,500 x g, 20 min), and microsomes (105,000 x g, 90 min) were separated by centrifugation. Lipids were extracted and purified by the method of Folch et al. (12). GLC analyses of the trimethylsilyl ethers of the unsaponifiable fraction were carried out using a Shimadzu Model 4CMPF gas chromatograph

[1]A preliminary part of the study was presented at the First Congress of the Federation of Asian and Oceanian Biochemists in Nagoya, October 1977.

TABLE I

Sterol Compositions of Serum Lipoproteins[a]

Experiments and groups[b]	Sterols	Whole serum	Lipoprotein fractions VLDL	LDL (% of total sterols)	HDL
I-1	Cholesterol (C_{27})	98.8 ± 0.1	99.5 ± 0.0[C]	98.3 ± 0.1[D]	98.1 ± 0.1[D]
	Campesterol (C_{28})	0.5 ± 0.1	0.3 ± 0.0[C]	0.8 ± 0.1[D]	0.7 ± 0.1[D]
	Sitosterol (C_{29})	0.7 ± 0.1	0.2 ± 0.0[C]	0.9 ± 0.1[D]	1.2 ± 0.1[D]
	Ratio, C_{28}/C_{29}	0.76 ± 0.08	1.50 ± 0.00[C]	0.87 ± 0.05[D]	0.60 ± 0.06[E]
I-2	Cholesterol (C_{27})	91.1 ± 0.5	97.1 ± 0.2[C]	92.5 ± 1.1[D]	89.2 ± 0.5[E]
	Campesterol (C_{28})	1.5 ± 0.1	1.1 ± 0.1[C]	1.3 ± 0.2[CD]	1.6 ± 0.1[D]
	Sitosterol (C_{29})	7.4 ± 0.4	1.8 ± 0.2[C]	6.1 ± 0.8[D]	9.2 ± 0.5[E]
	Ratio, C_{28}/C_{29}	0.20 ± 0.06	0.62 ± 0.03[C]	0.21 ± 0.01[D]	0.17 ± 0.01[E]
II-3	Cholesterol (C_{27})	86.0 ± 0.7	93.1 ± 0.7[C]	87.7 ± 0.4[D]	84.4 ± 0.5[E]
	Campesterol (C_{28})	7.4 ± 0.3	4.8 ± 0.5[C]	6.9 ± 0.2[D]	8.0 ± 0.3[E]
	Sitosterol (C_{29})	6.6 ± 0.4	2.1 ± 0.2[C]	5.4 ± 0.3[D]	7.6 ± 0.3[E]
	Ratio, C_{28}/C_{29}	1.12 ± 0.05	2.31 ± 0.02[C]	1.30 ± 0.06[D]	1.05 ± 0.02[E]
II-4	Cholesterol (C_{27})	88.7 ± 0.5	94.7 ± 0.2[C]	88.7 ± 0.4[D]	87.6 ± 0.4[D]
	Campesterol (C_{28})	6.1 ± 0.3	3.8 ± 0.5[C]	6.6 ± 0.3[D]	6.4 ± 0.2[D]
	Sitosterol (C_{29})	5.2 ± 0.3	1.5 ± 0.1[C]	4.7 ± 0.2[D]	6.0 ± 0.2[E]
	Ratio, C_{28}/C_{29}	1.17 ± 0.06	2.49 ± 0.08[C]	1.41 ± 0.07[D]	1.07 ± 0.02[E]

[a]Mean ± SEM of ten rats for whole serum or five rats for lipoproteins (one sample consisted of serum from two rats selected arbitrarily). Values in a same line not sharing the same letter are signisignificantly different at $p<0.001$-0.05.

[b]Group 1 was fed a diet containing no additional phytosterols and group 2 was fed a diet containing 0.25% phytosterols. Group 3 was fed a diet containing 1.0% phytosterols and group 4 was fed a diet containing 1.0% phytosterols + 0.5% cholesterol. The composition of phytosterol used in experiment I is 93% β-sitosterol and 7% campesterol, and that in experiment II is 60% β-sitosterol, 35% campesterol, and 5% stigmasterol.

TABLE II

Concentration of Sterols in Serum Lipoproteins[a]

Experiments and groups[b]	Sterols	Whole serum (mg/dl)	Lipoprotein fractions VLDL	LDL (mg/dl)	HDL
I-1	Cholesterol	121 ± 6.5	17.9 ± 1.00	16.6 ± 1.35	74.2 ± 7.00
	Campesterol	0.63 ± 0.08	0.04 ± 0.00	0.12 ± 0.02	0.48 ± 0.09
	Sitosterol	0.87 ± 0.08	0.03 ± 0.00	0.13 ± 0.01	0.80 ± 0.07
I-2	Cholesterol	95.7 ± 1.88[c]	10.7 ± 1.32[c]	16.7 ± 2.20	64.8 ± 3.10
	Campesterol	1.58 ± 0.13	0.12 ± 0.02	0.23 ± 0.04	1.15 ± 0.12
	Sitosterol	7.77 ± 0.66	0.20 ± 0.03	1.10 ± 0.17	6.20 ± 0.64
II-3	Cholesterol	86.0 ± 2.88	12.3 ± 0.75	11.6 ± 0.30	60.5 ± 1.10
	Campesterol	7.40 ± 0.34	0.63 ± 0.04	0.91 ± 0.03	5.74 ± 0.25
	Sitosterol	6.60 ± 0.37	0.28 ± 0.02	0.71 ± 0.04	5.45 ± 0.20
II-4	Cholesterol	97.6 ± 6.60	13.7 ± 1.70	12.0 ± 0.65	67.4 ± 2.93
	Campesterol	6.71 ± 0.41	0.55 ± 0.07	0.91 ± 0.08	4.92 ± 0.20
	Sitosterol	5.72 ± 0.32	0.22 ± 0.03	0.65 ± 0.05	4.61 ± 0.23

[a]Mean ± SEM of ten rats for whole serum or five rats for lipoproteins (one sample consisted of serum from two rats selected arbitrarily).

[b]See Table I.

[c]Differs from group 1 at $p<0.05$.

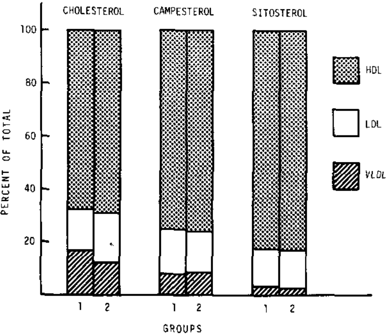

FIG. 1. The distribution of sterols in serum lipoproteins (experiment I). Diet compositions are given in Table I.

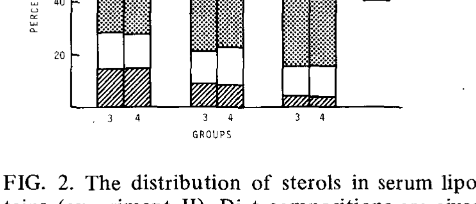

FIG. 2. The distribution of sterols in serum lipoproteins (experiment II). Diet compositions are given in Table I.

with a glass column (3 mm x 2 m) packed with 3% OV-17 on 60-80 mesh Gas Chrom Q (7,13). Column temperature was 260 C. Cholesterol was determined by the method of Sperry and Webb (14).

RESULTS

Growth and Liver Weight

In both experiments, the growth rate appeared normal. In rats fed cholesterol, liver weight was slightly but significantly higher than that from rats fed plant sterol alone (3.55 ±0.05 vs. 3.28±0.05 g/100 g body weight, $p<0.001$).

The Distribution of Plant Sterols in Serum Lipoproteins

Table I shows the composition of total sterol fractions in serum lipoproteins. Identical results were obtained when sterols were fractionated into the esterified and unesterified fractions by thin layer chromatography (15). A comparison of the composition of dietary and serum sterols revealed that campesterol was absorbed much

TABLE III

Sterol Compositions of Liver Subcellular Fractions[a]

Experiments and groups[b]	Sterols	Whole liver	Subcellular fractions: Nuclei	Mitochondria (% of total sterols)	Microsomes	Cell sap
I-1	Cholesterol (C_{27})	98.6 ± 0.1	98.7 ± 0.1^{C}	98.0 ± 0.1^{D}	98.8 ± 0.1^{C}	97.0
	Campesterol (C_{28})	0.6 ± 0.0	0.8 ± 0.1^{C}	1.2 ± 0.1^{D}	0.5 ± 0.0^{E}	2.2
	Sitosterol (C_{29})	0.7 ± 0.1	0.5 ± 0.0^{C}	0.8 ± 0.0^{D}	0.7 ± 0.1^{CD}	0.8
	Ratio, C_{28}/C_{29}	0.97 ± 0.03	1.47 ± 0.07^{C}	1.51 ± 0.14^{C}	0.71 ± 0.03^{D}	2.75
I-2	Cholesterol (C_{27})	92.0 ± 0.5	92.7 ± 0.5^{C}	90.6 ± 0.6^{D}	92.2 ± 0.5^{CD}	94.5
	Campesterol (C_{28})	1.7 ± 0.1	2.2 ± 0.1^{C}	2.9 ± 0.1^{D}	1.6 ± 0.1^{E}	2.2
	Sitosterol (C_{29})	6.3 ± 0.4	5.1 ± 0.4^{C}	6.5 ± 0.5^{D}	6.2 ± 0.4^{CD}	3.3
	Ratio, C_{28}/C_{29}	0.27 ± 0.01	0.47 ± 0.04^{C}	0.45 ± 0.02^{C}	0.25 ± 0.01^{D}	0.67
II-3	Cholesterol (C_{27})	85.6 ± 0.6	86.9 ± 0.6^{C}	86.5 ± 0.6^{C}	84.2 ± 0.7^{D}	ND
	Campesterol (C_{28})	8.4 ± 0.3	7.1 ± 0.3^{C}	7.7 ± 0.3^{C}	9.3 ± 0.3^{D}	ND
	Sitosterol (C_{29})	6.0 ± 0.3	6.0 ± 0.4^{C}	5.8 ± 0.4^{C}	6.5 ± 0.4^{C}	ND
	Ratio, C_{28}/C_{29}	1.44 ± 0.10	1.23 ± 0.06^{C}	1.35 ± 0.06^{CD}	1.45 ± 0.04^{D}	ND
II-4	Cholesterol (C_{27})	91.8 ± 0.1	91.9 ± 0.3^{C}	88.6 ± 0.4^{D}	86.4 ± 0.4^{E}	ND
	Campesterol (C_{28})	5.0 ± 0.2	4.9 ± 0.2^{C}	6.4 ± 0.2^{D}	7.5 ± 0.2^{E}	ND
	Sitosterol (C_{29})	3.2 ± 0.2	3.2 ± 0.2^{C}	5.0 ± 0.3^{D}	6.1 ± 0.2^{E}	ND
	Ratio, C_{28}/C_{29}	1.56 ± 0.14	1.53 ± 0.06^{C}	1.32 ± 0.08^{D}	1.23 ± 0.18^{CD}	ND

[a]Mean ± SEM of ten rats, except for eight rats for subcellular fractions in experiment I. Values for cell sap are from pooled samples. ND, not determined. Values in a same line not sharing a common letter are significantly different at $p<0.001$-0.05.

[b]See Table I.

TABLE IV

Concentration of Sterols in Liver Subcellular Fractions[a]

Experiments and groups[b]	Sterols	Whole liver (mg/g)	Subcellular fractions (mg/g)			
			Nuclei	Mitochondria	Microsomes	Cell sap
I-1	Cholesterol	3.68 ± 0.17	1.42 ± 0.05	0.35 ± 0.02	1.10 ± 0.03	0.47
	Campesterol	0.02 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.01
	Sitosterol	0.03 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.00
I-2	Cholesterol	2.83 ± 0.12[c]	1.14 ± 0.03	0.30 ± 0.02	0.99 ± 0.04	0.28
	Campesterol	0.05 ± 0.00	0.03 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.01
	Sitosterol	0.19 ± 0.01	0.06 ± 0.01	0.02 ± 0.00	0.07 ± 0.00	0.01
II-3	Cholesterol	2.92 ± 0.07	0.91 ± 0.03	0.42 ± 0.02	1.03 ± 0.01	ND
	Campesterol	0.29 ± 0.01	0.07 ± 0.00	0.04 ± 0.00	0.11 ± 0.00	ND
	Sitosterol	0.20 ± 0.01	0.06 ± 0.00	0.03 ± 0.00	0.08 ± 0.00	ND
II-4	Cholesterol	4.42 ± 0.22[c]	1.44 ± 0.09[c]	0.44 ± 0.01	1.05 ± 0.02	ND
	Campesterol	0.24 ± 0.01	0.08 ± 0.00	0.03 ± 0.00	0.09 ± 0.00	ND
	Sitosterol	0.15 ± 0.01	0.05 ± 0.00	0.02 ± 0.00	0.07 ± 0.00	ND

[a]Mean ± SEM of ten rats, except for eight rats for subcellular fractions in experiment I. Values for cell sap are from pooled samples. ND, not determined.

[b]See Table I.

[c]Differs from the corresponding groups at $p<0.01$.

more preferentially than sitosterol (1). Stigmasterol was hardly detected and thus it was omitted from the table. There were two distinct features in the distribution of plant sterols among lipoproteins. First, the percentage of plant sterols increased with the increase of the density of lipoproteins. Thus, HDL contained the highest level of plant sterols, and VLDL, the lowest. Second, the ratio of campesterol to sitosterol was very characteristic in each lipoprotein. The difference in the percentage of sitosterol was prominent among lipoproteins, whereas that of campesterol appeared relatively moderate. Consequently, the ratio was the highest in VLDL and the lowest in HDL; LDL was the intermediate. When dietary sterols contained large amounts of campesterol (experiment II), this sterol was incorporated markedly into LDL and HDL. However, again the same tendency as in experiment I was demonstrated. Also, there were no demonstrable effects of dietary cholesterol on the distribution of plant sterols.

The concentration of these sterols in lipoproteins, based on the data by the Liebermann-Burchard reaction (14) and by GLC analysis, is shown in Table II. In experiment I, the feeding plant sterol resulted in a significant decrease in serum cholesterol and this was due to the decrease in the VLDL fraction. HDL cholesterol also tended to decrease by dietary plant sterol, but was not statistically significant. In experiment II, no significant difference was found in the concentration of serum cholesterol between the two groups, indicating that plant sterols inhibited an expected rise due to feeding cholesterol.

Since rat serum contains more than two-thirds of sterols in HDL, the concentration of plant sterols in this fraction was distinctly high, followed by LDL and VLDL in decreasing order. The concentration of VLDL plant sterols was very low. From these values, the distribution of sterols in serum lipoproteins was estimated (Fig. 1). More than 80% of sitosterol and 75% of campesterol recovered occurred in HDL; the corresponding value for cholesterol was 70%, and the differences were statistically significant (C_{27} vs. C_{28}, $p<0.05$; C_{27} vs. C_{29}, $p<0.001$, and C_{28} vs. C_{29}, $p<0.01$). This was compensated by the difference in the distribution of these sterols in VLDL. In LDL, the relative distribution of sitosterol (13-15%) was slightly lower than that of cholesterol and campesterol (16-19%). Again, similar but more distinct trends were demonstrated in experiment II (Fig. 2).

The Distribution of Plant Sterols in Liver Subcellular Fractions

The distribution of plant sterols in liver subcellular fractions is given in Table III. It was clear that, though not so marked as in serum lipoproteins, plant sterols did not distribute equally in liver organelles. In experiment I, the percentage of plant sterols in mitochondria was somewhat higher, while that in the soluble cell

fraction was low. The ratio of campesterol to sitosterol was apparently the same between nuclei and mitochondria, but was the lowest in microsomes. In experiment II, microsomes contained the highest percentage of plant sterols and this was mainly due to the increase in campesterol. The ratio of campesterol to sitosterol was slightly different from that of the preceding experiment. In general, cholesterol feeding did not influence the distribution pattern of plant sterols.

The concentration of these sterols in liver subcellular fractions is shown in Table IV. In experiment I, plant sterol lowered the concentration of liver cholesterol and this was attributed to the decrease in nuclei and cell sap. Both nuclei and microsomes contained about 40% of sitosterol present in the liver. The corresponding values for campesterol were around 45% in nuclei and 30% in microsomes. The concentration of plant sterols in mitochondria was considerably low. These distribution patterns resembled those of cholesterol. In experiment II, feeding cholesterol resulted in a significant rise of cholesterol in nuclei and mitochondria, but the relative distribution of plant sterols in subcellular organelles remained unchanged and resembled that of the preceding experiment.

DISCUSSION

In the present experiments, we simultaneously measured the distribution of plant sterols in several tissues such as epididymal adipose tissue, adrenals, and testes. All these tissues contained plant sterols in the concentration and composition almost similar to those observed in the liver. Abdominal aorta also contained plant sterols but to a lesser extent. Unpublished experiments showed that red blood cells were also labeled with dietary plant sterols.

The physiological significance of the observation that dietary plant sterols distribute unevenly in serum lipoproteins and in liver subcellular fractions is not apparent at present. Incorporation of plant sterols in the place of cholesterol may influence the integrity and consequently the metabolism of lipoproteins (16), though the chemical structure of these sterols resembles each other. The concentration of plant sterols in each lipoprotein was roughly similar to, but significantly different from, that of cholesterol in relation to total cholesterol. In hyperphytosterolemic patients (6), β-sitosterol was mainly transported in LDL and HDL; VLDL contained only trace amounts of plant sterols. The distribution of campesterol in LDL was a little higher than that of cholesterol and sitosterol. On considering differences in the distribution of cholesterol in serum lipoproteins between human and rats (17), it seems likely that in rats a major transportation system of plant sterols is HDL, followed by LDL, and that, in a relative sense, campesterol is to some extent carried preferentially as LDL. VLDL may not be a significant carrier of plant sterols.

In rats, the appearance of labeled β-sitosterol intravenously injected in the bile acid fraction is lower and the excretion of this compound as neutral sterols is higher than those of cholesterol (16), indicating that these two sterols are metabolized relatively independently. These observations may relate to the different distribution of plant sterols in each serum lipoprotein.

Although Subbiah and Kuksis (16) have claimed that in rats intravenously injected plant sterols have no demonstrable effect upon cholesterol metabolism, evidence has been obtained to show an "extra absorptive" effect of plant sterol on cholesterol metabolism in rats (4) as well as in man (18) and chicks (5). Some specific distributions of plant sterols not only in serum lipoproteins, but also in liver subcellular fractions, suggest a possibility of successful use of these sterols as injectable hypocholesterolemic agents. The fact that plant sterols exist predominantly in HDL may relate to its hypocholesterolemic potency, since HDL is considered to have an antiatherosclerotic activity (19,20).

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Effects of Ethanol Intake on Lipoprotein Lipase Activity in Adipose Tissue of Fasting Subjects

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ABSTRACT

Ethanol (ca. 1 g/kg body weight) was given alone or together with glucose or lipid (mixed triglycerides) perorally to young, fasting subjects. The changes with time (0-6 hr) of lipoprotein lipase activity (LLA) in adipose tissue, plasma glycerol, triglyceride, insulin, blood glucose, and alcohol concentrations were followed. A maximal mean blood alcohol concentration of 0.09% (w/v) was obtained 1 hr after ingestion with no apparent intoxicating effects. Ethanol intake prevented the previously observed [Nilsson-Ehle, P., S. Carlström, and P. Belfrage, *Scand. J. Clin. Lab. Invest.* 35:373 (1975)] glucose-induced rapid elevation of adipose tissue LLA but had small effects on this enzymatic activity when given alone or together with lipid. Confirming results by others, ethanol intake decreased plasma glycerol concentration and increased plasma triglycerides, especially after intake of lipid. It is suggested that ethanol intake interferes with the normal carbohydrate-induced elevation of adipose tissue LLA after a mixed meal, thereby decreasing the removal capacity for circulating dietary lipid and causing enhanced and prolonged alimentary hyperlipemia.

INTRODUCTION

Ethanol intake by man leads to elevation of the plasma triglyceride concentration (1,2). This elevation is partly due to increased synthesis and secretion of very low density lipoproteins from the liver (3). Since the alcoholic hyperlipemia is markedly enhanced and prolonged by prior intake of fat (4,5), it has been suggested that ethanol intake would also cause a triglyceride removal deficiency by decreasing the activity of the key enzyme, lipoprotein lipase (6). Decreased lipoprotein lipase activity (LLA) has been reported in experimental animals after ethanol administration (7), but results from others have failed to confirm this finding (8,9). No comparable data from experiments with human subjects are available.

We have worked out a method for the determination of LLA in needle biopsy specimens of human adipose tissue (10,11). The method allowed us to follow the time-course of LLA changes after dietary carbohydrate and lipid intake by human subjects (12). With these data available, we have determined the time-course of ethanol effects on adipose tissue LLA and some related variables in fasting volunteers. We have also studied how ethanol modified the effects from carbohydrate and lipid intake on these variables.

MATERIALS AND METHODS

Experimental Design

Six to eight healthy, male students (age 20-27) took part in each of the four experiments, which will be referred to as the "control," "ethanol," "glucose plus ethanol," and "lipid plus ethanol" experiments. Due to the inconvenience caused by the experimental procedure, especially the repeated needle biopsy of adipose tissue, each subject generally took part in only a single experiment. Thus, each of these was performed with different individuals. Comparison will frequently be made with the previous work (12) in which the effects of glucose or lipid intake on adipose tissue LLA were studied. The previous experiments were designed in the same way as the present ones and will be referred to as the "glucose" and the "lipid" experiment.

All subjects had fasted 14 hr overnight before the start of the experiments (9:00-9:30 a.m.). They were given nothing but the indicated substances and kept quietly at rest during the experiments. Administration of the various compounds at zero time was performed according to the following: "control," 270 ml of very dilute low caloric juice; "ethanol," 70 g of ethanol in the solution above; "glucose plus ethanol," as in the "ethanol" experiment but with the addition of 30 g of glucose/m^2 calculated body surface; "lipid plus ethanol," as in the "ethanol" experiment but with the addition of 50 g of mixed triglyceride (Intralipid, a phospholipid-stabilized soybean oil emulsion from AB Vitrum, Stockholm, Sweden). The volumes were the same in all cases, and the fluids were taken within 5-10 min. Samples of capillary and venous blood (antecubital vein) and adipose tissue specimens (10,12) were taken immediately before the start of the experiments and at the time intervals indicated thereafter. The

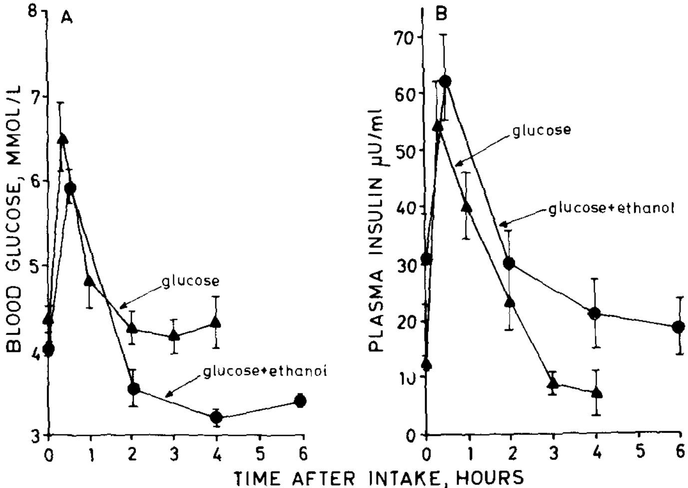

FIG. 1. Blood glucose and plasma insulin concentrations after intake of glucose or glucose in combination with ethanol. The "glucose" data were obtained in a previous work (12). Amount of glucose was 30 g/m^2 calculated body weight in both experiments and ethanol was 70 g. Values are mean ± SEM for 8 ("ethanol + glucose") or 8-11 fasting subjects.

"glucose" and "lipid" experiments of the previous study (12) had been performed as the corresponding experiments described above but without ethanol and, sometimes, with other time intervals for blood and tissue sampling.

Analyses

Adipose tissue LLA was determined in acetone-ether preparations of needle biopsy specimens of buttock fat as previously described (10,11). One milliunit (mU) of enzyme activity represents the release of one nanomole of fatty acid per min, and tissue levels are expressed as mU/g wet weight of adipose tissue. Capillary plasma glycerol was determined with an enzymatic fluorimetric method (13). Plasma triglycerides were determined colorimetrically (14), plasma insulin radio-immunologically (15), blood glucose enzymatically (16), and blood ethanol by gas chromtography. In some cases, lipoprotein electrophoresis was performed in agarose gel (17), and α- and β-lipoproteins were determined immunochemically by electroimmuno assay (18). Precision for several of the methods used has been reported in a previous work (12) and was similar in the present experiments.

Statistical Calculations

Statistical significance for the mean individual difference between a zero time and a later value has been calculated using Student's t-test for paired observations.

RESULTS

Blood Ethanol Concentration

For the six subjects taking part in the "ethanol" experiment, the following values for blood ethanol concentration expressed as parts per thousand (w/v) were obtained: At 20 min after the start of the experiment, 0.7 ± 0.06 (0.4-0.8) (mean ± SE [range]); at 1 hr, 0.9 ± 0.04 (0.8-1.1); and at 2 hr, 0.7 ± 0.07 (0.5-0.9). Similar values were found in the "glucose plus ethanol" experiment and slightly lower values in the "lipid plus ethanol" experiment, probably due to a delayed absorption of ethanol.

Effects of Ethanol Intake on Blood Glucose and Plasma Insulin Concentration

Intake of ethanol alone or in combination with lipid did not significantly change blood glucose and plasma insulin concentration during the 6 hr studied (data not presented). Ethanol taken in combination with glucose resulted in slightly lower blood glucose concentrations than after glucose alone [Fig. 1A, "glucose" values obtained in the previous work (12)]. Ethanol intake had no obvious effect on the plasma insulin peak concentration after glucose intake, but the insulin concentration after the peak value was elevated compared to the values obtained after glucose alone [Fig. 1B, "glucose" data obtained previously (12)].

Effects of Ethanol Intake on Lipoprotein Lipase Activity (LLA) of Adipose Tissue

Adipose tissue LLA decreased after intake of

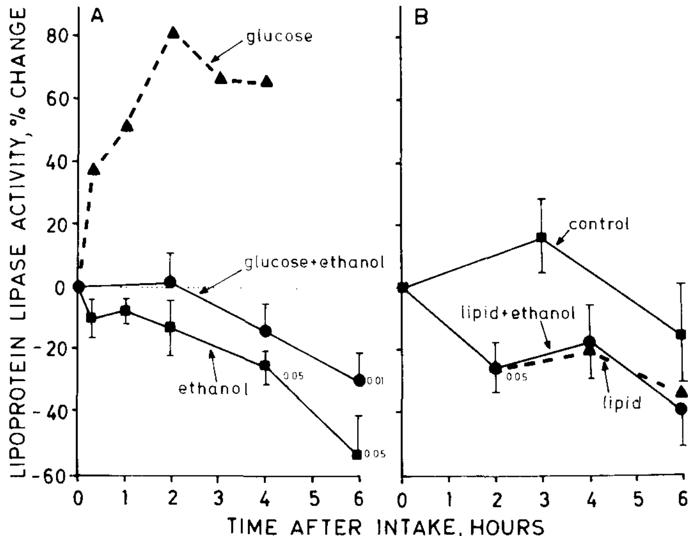

FIG. 2. Effects on lipoprotein lipase activity (LLA) in adipose tissue specimens from intake of ethanol, alone, or in combination with the indicated dietary components. Values for LLA after intake of the same amounts of glucose and lipid (broken lines) redrawn from a previous work (12) by permission of the publisher. Amount of lipid was 40 g, control group was given only the diluting fluid; glucose and ethanol as is stated in Figure 1. Data points indicate mean ± SEM individual change from initial value. Numbers indicate P-values for statistically significant changes. Initial values (mU/g of adipose tissue) were: control, 7.9 ± 1.4 (6) (mean ± SEM) (number of subjects); ethanol, 11.4 ± 2.1 (6); glucose + ethanol, 7.4 ± 1.6 (8); and lipid + ethanol 9.3 ± 3.5 (6).

ethanol (Fig. 2A). The decrease seemed to be more pronounced than that obtained in the "control" group (Fig. 2B) representing continued fasting. Ethanol taken with glucose prevented the rapid increase of adipose tissue LLA previously shown to occur after glucose alone (12) (redrawn in Fig. 2A, for convenience). Intake of ethanol together with lipid resulted in similar changes with time as those shown in the previous work (12) to take place after lipid (Fig. 2B).

Effects on Plasma Glycerol Concentration

The plasma glycerol concentration, which increased steadily in the "control" group during their continued fasting (Fig. 3B), instead decreased rapidly after ethanol intake (Fig. 3B). The previously (12) noted "rebound" effect after glucose intake (redrawn in Fig. 3A) was completely abolished by the simultaneous intake of ethanol (Fig. 3A).

No data on plasma glycerol concentrations were obtained in the "lipid plus ethanol" experiments.

Effects on Plasma Triglyceride and Lipoprotein Concentration

After ethanol intake, plasma triglyceride concentration increased continuously (Fig. 4A) while a slight decrease was observed in the "control" experiment (Fig. 4B). Plasma triglyceride concentration had returned to initial values 24 hr after ethanol intake (data not shown). Ethanol taken together with glucose resulted in a change with time similar to that obtained after ethanol alone and contrasting with the decrease shown previously (12) to occur after glucose (redrawn in Fig. 4A). Ethanol markedly changed the plasma triglyceride pattern after lipid intake (Fig. 4B). A continuous increase up to 2.5 times the initial value at the end of the 6 hr observation period was obtained instead of the transient increase previously (12) shown to take place after lipid alone (redrawn in Fig. 4B).

In the "ethanol" experiment, α- and β-lipoprotein concentrations were determined at each time interval. The α-lipoproteins varied within the range of 123-128% and the β-lipoproteins within 72-76% of pooled plasma standards with no significant difference between zero time and any other time point. [The length of the immunoprecipitates was measured and compared with a series of standards obtained from a pool representing 400 male blood donors. The value obtained was expressed relative to the lipoprotein concentration of the blood donor pool.]

DISCUSSION

The most significant result of this study was

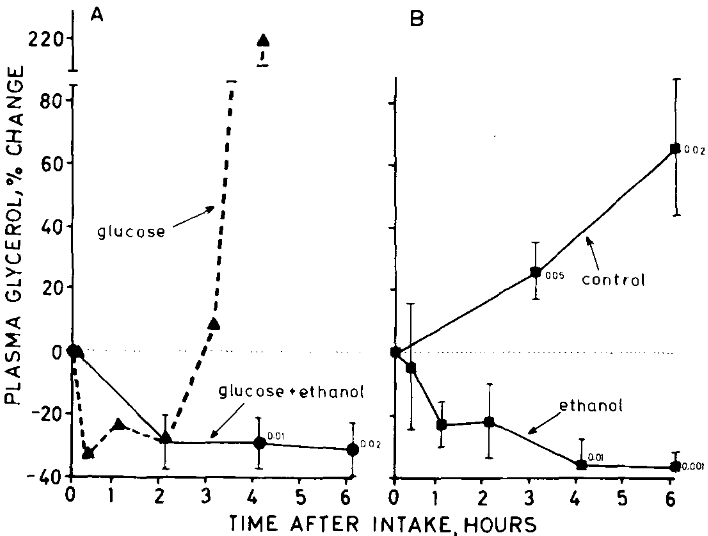

FIG. 3. Effects on plasma glycerol concentration from intake of ethanol alone or in combination with the indicated dietary components. All conditions and symbols as stated in the legends for Figures 1 and 2. Initial values (mmol/l) were: control, 0.096 ± 0.011 (6); ethanol, 0.078 ± 0.005 (6); and glucose + ethanol, 0.077 ± 0.008 (8). No data were obtained after intake of lipid and ethanol in combination.

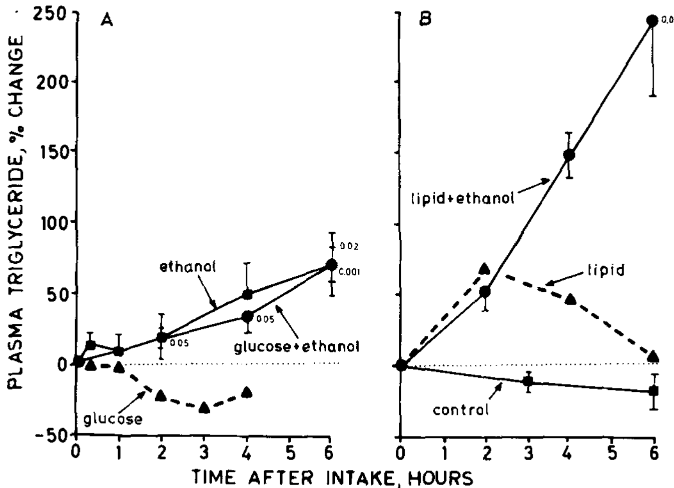

FIG. 4. Effects on plasma triglyceride concentration from intake of ethanol, alone, or in combination with the indicated dietary components. All conditions and symbols as stated in the legends for Figures 1 and 2. Initial values (mmol/l) were: control, 0.72 ± 0.10 (6); ethanol, 0.63 ± 0.080 (6); glucose + ethanol, 0.48 ± 0.065 (8); and lipid + ethanol, 0.43 ± 0.057 (6). P-value of < 0.02 in Figure 4A refers to the "ethanol" experiment, the others to "glucose + ethanol" experiment.

the finding that intake of a moderate amount of ethanol (ca. 1 g/kg body weight) prevented the rapid increase of adipose tissue LLA which has been shown to occur after glucose intake (12). This may be important as a mechanism for the enhanced and prolonged elevation of plasma triglyceride concentration when ethanol is ingested in a mixed meal, with both carbohydrate and lipid. The high removal capacity for circulating plasma triglyceride caused by increased LLA after carbohydrate intake may be a prerequisite for the rapid clearing of dietary fat from the circulation. Thus, the most important effect of ethanol intake could be to

interfere with the normal adjustment of the lipid removal mechanism in response to a mixed meal.

The rapid increase of the plasma triglyceride concentration found after intake of ethanol alone is more likely to have been due to an increased rate of triglyceride secretion into the circulation from the liver (19) since the relatively low plasma triglyceride concentration obtained would not saturate the removal capacity (20). On the other hand, in the experiments where lipid had been ingested, the influx to the plasma lipid pool should be much larger, and the pronounced hyperlipemia when ethanol was added is fairly good evidence for a decreased removal capacity for plasma triglyceride. This decrease may have been caused by an inhibition of LLA. Adipose tissue LLA did not decrease more after ethanol plus lipid than with lipid alone, but in this fasting condition LLA of other tissues, e.g., muscle, may be quantitatively more important for the total plasma triglyceride removal capacity (21).

Ethanol intake did not seem to prevent the activation of LLA from dietary glucose intake through interference with insulin release since plasma insulin values were slightly elevated when ethanol was taken with glucose. Also, plasma glycerol concentration was always decreased when ethanol was taken, confirming the results of others (19,22). If the ethanol intake would have caused release of catecholamines or glucagon, which are known to decrease adipose tissue LLA (21), an increased intracellular lipolysis rate would have been expected. This should have caused an increased plasma glycerol concentration (23) in contrast to what was found.

However, acetate, which is the main metabolite of ethanol oxidation, has been shown to inhibit adipose tissue lipolysis (24). Thus, it cannot be excluded that ethanol effects on other hormones than insulin could have caused the ethanol-induced prevention of the stimulation of LLA by glucose intake.

In experiments with long term ethanol intake (25,26) and in chronic alcoholics (27), adipose tissue LLA has been found to be markedly elevated. This also indicates that ethanol-induced alterations of the plasma lipids are directly coupled to this enzyme system even if the relationship is still obscure.

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The Effect of Dietary Erucic Acid on Cardiac Triglycerides and Free Fatty Acid Levels in Rats[1]

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ABSTRACT

Male Sprague-Dawley rats, 3 weeks of age, were fed semisynthetic diets containing test oils at 20% by weight for 3 days, 1 week, and 16 weeks. The test oils contained up to 22.3% erucic acid. Growth retardation was evident in rats fed rapeseed oil high in erucic acid, and soybean oil and Tower rapeseed oil diets containing about 5% erucic acid. Cardiac triglyceride accumulation was found in rats fed diets containing about 5% erucic acid but not in rats fed Tower rapeseed oil which contains 0.2% of this acid. The cardiac free fatty acid levels were low, 50-100 μg/g of wet heart tissue, and were not affected by feeding diets containing about 5% erucic acid. Feeding a diet containing a high erucic acid rapeseed oil did result in higher free fatty acid levels but only at 3 days and 1 week; the level at 16 weeks was similar to the other oils. The fatty acid analysis of cardiac triglycerides and free fatty acids showed high percentages of erucic acid at 3 days and 1 week; at 16 weeks these levels had declined significantly. The results indicate that the accumulated erucic and eicosenoic acids, at 3 days and 1 week, accounted for the increase in cardiac free fatty acids when rats were fed the high erucic acid rapeseed oil. There appears to be no evidence that the early cardiac triglyceride or free fatty acid accumulation is related to the formation of the long term myocardial lesions.

INTRODUCTION

Rats fed diets containing rapeseed oil (RSO) high in erucic acid (22:1), developed early myocardial lipidosis (1-6), characterized by pronounced intracellular lipid deposition mainly in the form of triglycerides (TG) (2,6-11) and free fatty acids (FFA) (2,7,9). Fatty acid analysis showed the relative concentration of 22:1 to be very high in cardiac TGs (2,6-11) with lower levels reported in cardiac FFAs (2,7,9). On the other hand, rats fed diets containing RSO low in 22:1 showed no myocardial lipidosis as determined gravimetrically (4), and no TG accumulation (6,10), although, histologically a scanty fat deposition was demonstrated by oil redO staining (3-5). The cause of the early myocardial lipidosis was attributed to 22:1 (1-8,12) and to a lesser extent to eicosenoic acid (12). It was suggested that the TG and/or the FFA accumulation could lead to myocardial fibrosis (13).

The purpose of the present study was to determine quantitative and qualitative changes in the fatty acid composition of cardic TGs and FFAs during the first week of feeding and at 16 weeks using an improved extraction procedure minimizing autolysis of cardiac lipids (14). The diets included control oils, a low erucic acid RSO, oils to which about 5 % free erucic acid was added, and a high erucic acid rapeseed (HEAR) oil. The possibility that the changes in these lipid classes might lead to myocardial fibrosis will be discussed.

[1]Contribution No. 739 Animal Research Institute.

MATERIALS AND METHODS

Animals and Diets

Male Sprague-Dawley rats, 3 weeks of age, and weighing 40-50 g, were distributed randomly to eight dietary treatments, 15 animals per treatment, and fed ad libitum the test diets for 3 days, 1 week, or 16 weeks. Water was available at all times. In addition, five animals were killed at the start of the experiment to serve as 0 day control. The semisynthetic diets, prepared as described previously (15), contained 20% by weight of the following oils: soybean oil, olive oil, *Brassica napus* cv. Tower RSO, oil extracted from a seed mixture of *B. campestris* cv. Echo (85%) and Arlo (15%) designated HEAR (high erucic acid rapeseed). Free erucic acid was blended into soybean oil, olive oil, and Tower RSO to give the indicated level (Table I) of this acid in the dietary oil.

Extraction and Analysis of Neutral Lipid Classes

Rats were killed by exsanguination under CO_2 anesthesia. The hearts were immediately removed, weighed, and frozen between two blocks of dry ice. The hearts were quickly pulverized at dry ice temperature, and the lipids extracted with chilled $CHCl_3/CH_3OH$ (2:1) as previously described (14). Total lipids were determined gravimetrically. A known aliquot of total lipids was applied with a thin layer chromatography (TLC) sample streaker

TABLE I

Fatty Acid Composition of the Diets

Diet[a]	Fatty acid[b] (wt %)							
	16:0	16:1	18:0	18:1	18:2	18:3	20:1	22:1
Soybean	12.4	0.1	3.7	25.4	50.6	7.9		
Soybean + EA (5.7%)	13.5	0.1	4.3	25.4	44.1	6.0	0.8	5.7
Olive	11.6	1.2	2.5	75.5	7.3	0.7	0.4	0.1
Olive + EA (4.5%)	11.5	1.2	2.7	71.2	6.8	0.6	0.7	4.5
Tower RSO[c]	6.1	0.1	2.0	56.5	26.0	7.1	1.5	0.3
Tower RSO + EA (0.8%)	6.5	0.1	2.4	55.7	25.7	6.5	1.6	0.8
Tower RSO + EA (5.9%)	7.1	0.1	2.5	54.2	22.2	5.7	1.9	5.9
HEAR[d]	4.0	0.3	1.7	36.2	15.1	5.9	12.3	22.3

[a]Semisynthetic diets containing the vegetable oils at 20% by weight of the diet. Free erucic acid (EA) was blended into the vegetable oils; final 22:1 level is indicated in parentheses.

[b]The major fatty acids are listed; minor amounts of 14:0, 15:0, 17:0, 20:0, and 20:2 are not shown.

[c]Tower RSO = *Brassica napus* cv. Tower rapeseed oil.

[d]HEAR = high erucic acid rapeseed; oil extracted from a seed mixture containing 85% *B. campestris* cv. Echo and 15% *B. campestris* cv. Arlo.

(Applied Science Laboratories Inc., State College, PA) on Silica Gel G plates (Fisher Scientific Co., Ottawa, Ontario), 500 microns in thickness, and developed using the solvent hexane-diethyl ether-acetic acid (85:15:1). Bands were visualized under UV light after spraying the chromatogram with Rhodamine B in methanol. The TG and FFA bands were identified by co-chromatography with authentic standards. The bands corresponding to TG and FFA were removed, methyl heptadecanoate was added as an internal standard and the mixture was transesterified with anhydrous HCl/CH_3OH (5% m/m). The methyl esters were purified by TLC using 1,2-dichloroethane as developing solvent.

The fatty acids in the TG and FFA fraction were resolved with a Hewlett-Packard Model 5830A gas liquid chromatograph, equipped with a flame ionization detector and digital integrator. Glass columns (1.8 m x 2 mm) were packed with 5% butanediol succinate on 80/100 mesh Chromosorb G, high performance (Chromatographic Specialties Ltd., Brockville, Ontario). The chromatograph was operated isothermally at 190 C. Peaks were identified by comparing the retention time to authentic methyl esters standards (Nu-Chek-Prep., Elysian, MN).

Analysis of variance was performed on body and heart weights, cardiac lipid content, cardiac TG and FFA concentration, and methyl ester data. The least significant difference at the 1% level was determined from the pooled error estimates of the analysis of variance (16).

RESULTS

The fatty acid compositions of the diets are shown in Table I. The erucic acid level of the new cultivar of rapeseed (Tower RSO) was only 0.3%, while that of the old cultivar (HEAR) was 22.3%. Addition of free 22:1 to each of the oils, soybean, olive, and Tower RSO, brought the level of this acid to about 5%, the maximum level recommended in an edible oil in Canada.

Rats fed diets containing HEAR oil for 3 days and 1 week showed increased heart weights possibly due to elevated fat levels (Table II). After 16 weeks of feeding, the heart weight and cardiac fat level appeared normal, but the body weights were significantly lower ($P<0.01$) compared to rats fed soybean oil, olive oil, or Tower RSO. No differences in body weight, heart weight, or percent cardiac fat were observed in rats fed Tower RSO, soybean oil, or olive oil. The incorporation of about 5% erucic acid into soybean oil or Tower RSO resulted in significantly ($P<0.01$) lower body and heart weights at 16 weeks, but no differences were observed in their cardiac fat levels. On the other hand, olive oil containing 4.5% erucic acid did not cause growth depression or lower heart weights compared to olive oil.

The level of TG in the cardiac lipids of rats fed HEAR oil was high after 3 days and still higher after 1 week (Table III). After 16 weeks on this diet, the level of TG decreased considerably from the 1 week level, but it was still significantly higher than that found in all other groups. With only one exception (Tower RSO, 1 week), the cardiac TG level did not increase significantly from the 0 day value by feeding rats diets containing soybean oil, olive oil, or Tower RSO throughout the experiment. How-

TABLE II

Body and Heart Weights, and Percent Cardiac Lipids of Rats Fed Experimental Diets

Diet[a]	Body wt (g) 16 weeks	Heart weight (g)				Percent lipid (of wet wt)			
		0 day	3 day	1 week	16 weeks	0 day	3 day	1 week	16 weeks
Initial kill[b]		0.25[c]				2.9			
Soybean	478.4		0.25	0.33	1.28		2.5	2.9	4.4
Soybean + EA (5.7%)	412.7		0.25	0.38	1.09		3.0	3.3	3.9
Olive	453.2		0.26	0.35	1.22		2.6	2.7	3.9
Olive + EA (4.5%)	465.8		0.24	0.36	1.15		2.8	2.7	3.8
Tower RSO[d]	457.4		0.26	0.34	1.25		2.5	2.6	4.0
Tower RSO + EA (0.8%)	454.1		0.25	0.34	1.12		1.9	2.4	3.9
Tower RSO + EA (5.9%)	382.9		0.27	0.35	1.07		2.8	3.1	4.0
HEAR[e]	415.9		0.32	0.46	1.34		6.3	7.4	4.0
LSD[f]	29.3		0.03	0.04	0.12		0.5	0.5	0.5

[a]Semisynthetic diets containing the vegetable oils at 20% by weight of the diet. Free erucic acid (EA) was blended into the vegetable oils; final 22:1 level is indicated in parentheses.

[b]Initial kill = male rats examined at 3 weeks of age.

[c]All values in the table are means of five animals.

[d]Tower RSO = *Brassica napus* cv. Tower rapeseed oil.

[e]HEAR = high erucic acid rapeseed; oil extracted from a seed mixture containing 85% *B. campestris* cv. Echo and 15% *B. campestris* cv. Arlo.

[f]LSD = least significant difference obtained from pooled error estimates of analysis of variance. Means within a group differing by more than the LSD are significantly different at the 1% level.

TABLE III

Triglyceride and Free Fatty Acid Levels (per g of Wet Tissue) in Cardiac Lipids of Rats Fed Experimental Diets[a]

Diet	Triglycerides (mg/g)				Free fatty acids (μg/g)			
	0 day	3 days	1 week	16 weeks	0 day	3 days	1 week	16 weeks
Initial kill	1.3				39			
Soybean		2.0	2.0	1.7		95	113	47
Soybean + EA (5.7%)		2.7	3.9	1.9		82	63	34
Olive		0.9	2.1	2.1		45	57	47
Olive + EA (4.5%)		2.4	2.4	2.2		85	49	46
Tower RSO		1.4	3.2	1.5		73	98	46
Tower RSO + EA (0.8%)		2.0	1.7	1.8		55	64	53
Tower RSO + (5.9%)		3.5	3.7	1.4		95	66	67
HEAR ± SD		14.4 ± 4.3	29.3 ± 2.2	4.5 ± 1.8		210 ± 65	210 ± 48	68 ± 24
LSD[b]			1.3				36	

[a]See footnotes Table II.

[b]LSD of all diets except HEAR for which individual standard deviations (SD) are given.

TABLE IV

Fatty Acid Composition of Rat Heart Triglycerides[a]

Diet	Time on diet	Fatty acids[b] (wt %)								
		14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:1	22:1
Initial kill	0	3.0	32.4	2.1	16.7	33.3	12.0			
Soy	3d	0.8	18.6	0.9	8.4	29.8	33.6	2.1	0.8	0.6
	1 wk	1.6	21.4	1.4	7.4	28.8	32.1	2.2	0.7	0.3
	16 wks	0.7	16.9	1.5	5.8	30.6	39.6	1.9	0.6	0.1
Soy + EA (5.7%)	3d	1.1	16.4	1.0	6.9	24.7	26.3	1.5	2.2	16.5
	1 wk	0.9	14.9	1.3	5.1	29.4	29.5	2.4	1.9	12.6
	16 wks	0.9	21.7	1.5	7.6	30.0	31.7	1.1	1.2	2.1
Olive	3d	2.5	22.8	2.1	7.1	54.6	5.6	0.1	1.4	0.2
	1 wk	1.7	22.2	1.5	6.0	60.1	5.9	0.1	0.7	0.2
	16 wks	0.6	17.6	1.8	4.1	69.3	4.5	0.1	0.6	0.1
Olive + EA (4.5%)	3d	1.4	17.6	1.2	5.3	51.7	4.7	0.1	1.8	14.0
	1 wk	1.2	18.2	1.1	5.5	56.2	5.4	0.1	1.4	8.2
	16 wks	0.8	17.1	2.1	4.3	67.2	4.0	0.1	1.0	2.2
Tower RSO	3d	1.7	16.8	1.3	8.3	42.9	17.9	1.9	2.2	1.8
	1 wk	1.2	12.1	1.0	4.4	54.1	17.7	2.1	2.6	1.3
	16 wks	0.8	15.0	1.8	5.0	53.7	18.2	1.5	1.2	0.4
Tower RSO + EA (0.8%)	3d	1.4	15.9	1.1	5.4	46.9	16.5	1.6	2.4	4.1
	1 wk	1.5	15.2	1.4	6.2	47.3	18.3	2.6	2.1	1.8
	16 wks	0.8	14.6	1.7	5.3	52.6	18.9	1.5	1.4	0.7
Tower RSO + EA (5.9%)	3d	1.2	14.6	1.1	5.5	43.1	13.9	1.2	3.1	13.4
	1 wk	1.0	11.4	1.2	4.1	45.5	16.5	1.6	3.1	13.2
	16 wks	0.9	18.0	2.4	5.4	52.0	15.3	0.9	1.6	1.9
HEAR	3d	0.5	7.6	0.8	3.3	34.5	8.8	1.1	12.8	25.6
	1 wk	0.2	5.0	0.7	2.6	36.7	9.4	1.2	14.4	25.3
	16 wks	0.1	10.3	2.1	4.3	42.8	10.6	1.4	10.2	13.7
LSD		0.8	4.8	0.6	1.6	4.5	3.1	0.9	0.7	2.9

[a]See footnotes Table II.
[b]Minor amounts of 15:0, 17:0, 20:0, 20:2, 20:4, and 22:0 are not shown.

TABLE V

Fatty Acid Composition of Rat Heart Free Fatty Acids[a]

Diet	Time on diet	Fatty acid[b] (wt %)								
		14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:1	22:1
Initial kill	0	8.0	33.0	3.3	36.1	12.5	3.8			
Soy	3d	3.7	27.7	3.8	24.2	19.0	12.3	1.0	0.4	0.5
	1 wk	2.0	19.5	2.3	19.5	20.1	22.8	3.5	0.6	0.5
	16 wks	2.3	22.6	3.7	24.4	14.6	22.5	1.8	0.5	0.5
Soy + EA (5.7%)	3d	3.6	22.8	2.0	26.1	15.9	13.2	1.4	0.8	6.8
	1 wk	2.6	18.4	1.8	15.4	14.8	20.2	2.8	1.2	11.0
	16 wks	5.2	31.2	2.9	19.0	10.0	20.5	3.3	0.2	2.1
Olive	3d	4.6	27.7	3.0	24.7	28.9	5.9	0.3	0.6	0.1
	1 wk	4.0	26.0	2.7	21.4	32.2	6.2	0.3	0.5	0.5
	16 wks	4.3	27.3	3.9	19.8	27.5	7.1	0.4	0.8	0.6
Olive + EA (4.5%)	3d	3.1	23.4	2.8	20.4	30.2	4.6	0.1	1.2	6.9
	1 wk	3.0	22.3	2.3	21.9	28.5	5.5	0.1	1.6	9.2
	16 wks	3.6	23.0	3.7	16.8	35.4	7.2	0.3	0.9	2.6
Tower RSO	3d	1.9	20.0	2.4	21.1	29.2	12.9	2.0	1.9	1.2
	1 wk	2.0	19.1	1.8	22.5	30.9	12.3	2.9	1.7	0.8
	16 wks	3.4	25.4	3.0	24.4	16.4	14.4	3.9	0.7	0.8
Tower RSO + EA (0.8%)	3d	2.7	22.4	3.2	23.9	23.7	10.8	1.4	1.3	1.1
	1 wk	3.9	19.4	2.8	20.1	26.4	12.8	3.1	1.5	1.7
	16 wks	4.3	23.5	3.4	16.6	21.3	18.2	4.7	1.1	1.3
Tower RSO + EA (5.9%)	3d	3.2	20.2	2.0	17.9	23.2	11.5	2.1	2.3	10.8
	1 wk	2.9	18.9	3.1	15.9	21.9	13.3	3.4	2.1	11.2
	16 wks	3.3	20.7	3.6	11.0	25.7	20.4	5.8	0.9	2.0
HEAR	3d	2.1	17.3	1.0	12.2	20.5	5.1	0.8	5.9	29.5
	1 wk	1.0	8.1	1.3	4.6	18.6	6.2	1.4	7.5	42.5
	16 wks	4.3	23.9	3.5	15.8	14.9	5.6	1.1	4.0	14.5
LSD		2.7	7.4	1.5	10.1	8.2	8.1	2.4	0.8	2.3

[a]See footnotes Table II.
[b]Minor amounts of 15:0, 17:0, 20:0, 20:2, 20:4, and 22:0 are not shown.

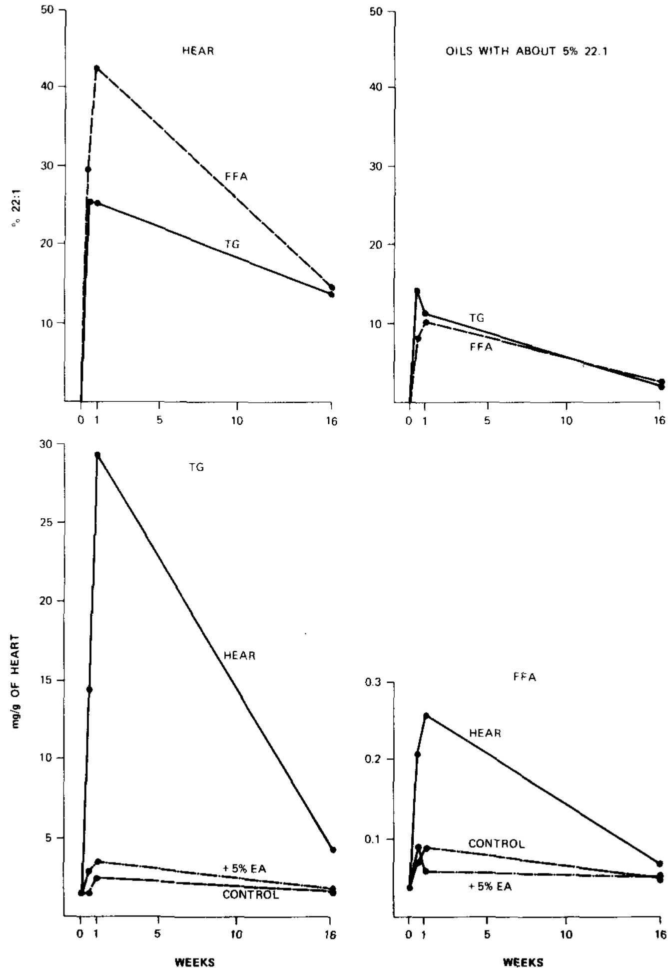

Fig. 1. Upper graphs: The relative concentration of erucic acid (22:1) in the triglyceride (TG) and free fatty acid (FFA) fractions of the hearts of rats fed a high erucic acid rapeseed oil (HEAR) and vegetable oils to which about 5% 22:1 was added. Lower graphs: The absolute amount (mg/g of wet weight) of TG and FFA in rats fed soybean oil or olive oil (control), vegetable oils to which 5% 22:1 was added and HEAR oils.

ever, the inclusion of about 5% erucic acid in the vegetable oils resulted in higher cardiac TG levels at 3 days and 1 week.

The levels of cardiac FFAs for rats fed the experimental diets (Table III) were generally very low. Even though an occasional significant difference existed among diets and sampling times, none of the differences appears to be consistent, except the HEAR-fed groups which had significantly elevated FFA levels during early feeding (3 days and 1 week) only. The level of FFAs in the hearts of rats fed HEAR oil decreased at 16 weeks to a level not significantly different from all other diets.

The relative fatty acid composition of cardiac TGs was greatly influenced by the dietary fatty acids as evidenced from a comparison of the 0 day and any 3 day value (Table IV). In most cases, changes in the relative abundance of fatty acids derived from dietary sources were attained within the first week: i.e., 18:2 when diets containing soybean oil were fed; 18:1 when diets containing olive oil or Tower RSO were fed; and 18:3 when diets containing soybean oil, Tower RSO, or HEAR oil were fed. High percentages of long chain monoenes (20:1 and 22:1) were found in the cardiac TGs of rats fed diets containing either free or esterified erucic acid for 1 week. At 16 weeks, however, the relative abundance of these monoenoic acids had decreased significantly from the level at 1 week.

The fatty acid composition of the FFA fraction of cardiac lipids is given in Table V.

Changes in the relative concentration of the FFAs due to diet were less pronounced than fatty acid changes observed for TGs, e.g., the level of 18:2 when soybean oil containing diets were fed, or 18:1 when olive oil or Tower RSO containing diets were fed. The level of 18:3 in cardiac FFAs, although similar to that found in cardiac TG at 3 days and 1 week, appeared to increase in the groups fed diets containing Tower RSO or soybean + EA. The pattern for 22:1 in cardiac FFAs was similar to that in cardiac TGs, a rise during the first week of feeding followed by a decline to a much lower level at 16 weeks (Fig. 1). However, differences were observed between these two lipid classes. For example, the relative abundance of 22:1 peaked at 1 week in FFAs compared to 3 days in TGs. Rats fed the diet containing HEAR oil showed the same pattern of 22:1 accumulation in the cardiac FFAs as seen in the other groups, but at 1 week the content of 20:1 and 22:1 totaled 50% of the FFAs. Although the relative abundance of saturates (16:0 and 18:0) for the HEAR group was significantly less than for all other groups during the early feeding, the absolute amount was not significantly different owing to the increase in FFA concentration for the HEAR group at this time.

DISUCSSION

The present study confirms previously published results that rats fed diets containing HEAR oil will show depressed growth response (17), lipidosis mainly in the form of TGs, high levels of 22:1 in the TG fraction (2,6-11), and the general pattern of lipid and 22:1 accumulation represented graphically in Figure 1 (3,6). However, it is evident from this study that cardiac FFA levels are much lower (about 1/20) than previously reported (2,7,9) because a proper extraction procedure was employed which minimized autolysis of the cardiac lipids (14). It is of interest to note that the high levels of cardiac FFAs due to autolysis diluted the high concentration of 22:1 actually present in this lipid fraction. For example, the cardiac FFA level found in the present trial for rats fed HEAR oil for 1 week was 210 μg/g of heart tissue with 22:1 comprising 42.5% of the total fatty acids, whereas by the old extraction method, a level of 1,700 μg/g was found and the concentration of 22:1 was only 16.1% (14). The temporary accumulation of FFAs in the hearts of rats fed HEAR oil could be explained entirely by the accumulation of 20:1 and 22:1. These acids are known to be oxidized at a slower rate than the common fatty acids (13), a fact which could account for their accumulation until such time as the heart tissue has adapted to metabolize these long chain monoenes.

It is concluded from this study that rats fed Tower RSO could not be distinguished from those fed soybean oil or olive oil in weight gain, percent cardiac fat, cardiac TG, and FFA levels. The dietary fatty acids, however, markedly influence the fatty acid composition of cardiac TGs, while changes in the composition of cardiac FFAs are less pronounced.

The result of feeding rats vegetable oil blends containing about 5 % 22:1 demonstrated the growth retarding effect of this acid (17). A temporary increase in cardiac TGs was evident, which could not be detected gravimetrically (% lipid), but had been demonstrated histologically by use of oil red 0 staining (4). The level of cardiac FFAs remained similar to that found when rats were fed control oils (soybean or olive oil). The incorporation of 22:1 into cardiac TGs and FFAs of rats fed these diets followed the general pattern observed with HEAR oil (Fig. 1).

Several hypotheses have been proposed to explain the cause of the long term myocardial lesions in rats fed RSO. Investigators have suggested a continuum of events from lipidosis, mainly as TGs to necrosis and fibrosis on continued feeding of RSO (5,13). However, such an hypothesis cannot explain why rats of both sexes accumulate similar levels of TG while long term myocardial lesions occur primarily in the male rat (4,18-20). It cannot explain why both the Sprague-Dawley male rat and the Chester Beatty (Hooded) male rat accumulated similar levels of TGs (unpublished results) and yet myocardial lesions occur only in the former strain of rats (21). It does not explain why RSOs low in 22:1 and producing no TG accumulation (Tower RSO) show a higher incidence of lesions than control oils (15).

Likewise, high levels of cardiac FFAs in rats fed RSO have been implicated as the cause of the long term lesions (13). This hypothesis was based on earlier studies showing extremely high levels of cardiac FFAs in rats fed RSO (2), which subsequently has been attributed to incorrect extraction techniques (14). Results from the present study indicate that only the feeding of HEAR oil caused a temporary increase in cardiac FFAs, and this would not explain the cause of lesions in rats fed olive oil and Tower RSO containing erucic acid blends (15). Furthermore, the comparison study between Sprague-Dawley and Chester Beatty (Hooded) rats did not indicate differences in cardiac FFA levels (unpublished results) despite the differences in lesion response (21). There-

fore, it must be concluded that the cardiac FFA levels found in the present trial represent physiological concentrations of this metabolic intermediate, and do not appear to be the cause of myocardial lesions.

It is evident from this study that neither cardiac TG nor FFA accumulation appear to be related to the cause of myocardial lesions in rats fed diets containing 22:1. Accumulation of both these lipid classes appears to be transitory in rats fed HEAR oil. For diets containing about 5% of 22:1, there was evidence of a temporary TG accumulation, but no increase in FFAs was found.

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The Effect of Protein Deficiency on Some Rat Liver Lipid Metabolic Enzymes and CoA

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ABSTRACT

Two groups of male Wistar rats were fed normal (i.e., 18%) and protein-free diets, respectively, for 7 weeks. In vivo incorporation of [1-^{14}C] acetate into palmitic, stearic, oleic, and arachidonic acids by the liver was reduced in the protein-deficient rats. In vitro incubation of liver microsomes with labeled palmitate or linoleate revealed no change in the specific activities of chain elongating or desaturating enzymes. Protein deficiency resulted in a decrease in specific activity of short chain acyl-CoA synthetase and in total CoA, accompanied by the virtual disappearance of acyl-CoA and an increase in free CoA. Furthermore, there was less microsomal fatty acid synthetase and mitochondrial β-hydroxybutyrate dehydrogenase activity. These results are discussed in relation to fatty acid synthesis and the changes in liver fatty acid composition.

INTRODUCTION

It is increasingly evident that the level of dietary protein influences lipid metabolism. De Gómez Dumm et al. (1) recently reported that in rats, a high protein diet caused enhanced oxidiative desaturation of linoleic (18:2) to γ-linolenic (γ-18:3) acid, but had no effect on chain elongation. Protein deprivation was shown by Williams and Hurlebaus (2) to result in arachidonic acid (20:4) improverishment in liver phospholipids while at the same time 18:2, a dietary precursor of 20:4, accumulated in liver neutral lipids. Plasma 20:4 was also reduced. Since then it has been shown by Gerson (3) that protein deprivation resulted in the depletion of phospholipid C_{20} and C_{22} fatty acids, particularly 20:4, in outer mitochondrial membranes and microsomes of rat liver, accompanied by an accumulation of 18:2. It was postulated that 18:2, which is desaturated and elonagated by microsomal enzymes to 20:4, accumulated because chain elongation and/or desaturation was impaired in protein-depleted rats. So far no direct evidence to support this hypothesis has appeared. In addition, little is known concerning fatty acid synthesis during acute protein deprivation.

The present paper concerns itself with the activities of some of the enzymes involved in these reactions as well as the availability of coenzyme A, without which activation of fatty acids cannot take place.

It is now recognized that ketone body metabolism is an important means for the liver to regulate fatty acid metabolism and citric acid cycle activity (4,5) and to make acetate available for fatty acid synthesis (6). Furthermore, it has been established that β-hydroxybutyrate is the preferred substrate for fatty acid and sterol biosynthesis in the nervous system of young rats (7). We, therefore, determined the effect of protein deprivation on the activities of β-hydroxybutyrate dehydrogenase. This is a key enzyme in the conversion of D-β-nydroxybutyrate to acetoacetate.

METHODS

Preparation of Microsomes and Mitochondria

These particulates were prepared and purified as previously described (3). The mitochondria were sonically disintegrated for 2 min at O C in a 100 watt sonic disintegrator.

Chain Elongation, Desaturation, and Elongation and Desaturation

The incubation procedures used were based on those described by De Gómez Dumm et al. (1), although ten times the total volume and cofactors were incubated with 20 mg microsomal protein for 20 min at 25 C using 5.6 μCi [1-^{14}C] 16:0 (150 μg) or 5.6 μCi [1-^{14}C] 18:2 (28 μg). The substrate for chain elongation was labeled 16:0 and for desaturation as well as elongation and desaturation was labeled 18:2. The reaction products, i.e., 18:0, γ18:3, and 20:4, were isolated by gas liquid chromatography (GLC) and counted.

Acyl-CoA Synthetases (EC 6.2.1.1, EC 6.2.1.2, and EC 6.2.1.3)

Microsomal protein (5 mg) was incubated with 5 μmol acetic, butyric, palmitic, and linoleic acids, respectively, and the disappearance of -SH measured over a period of 20 min as described by Mahler et al. (8). The linearity of ΔE_{520} during this period had previously been verified with 7.5, 15, and 20 min incubations.

TABLE I

Caloric Intakes and Rat and Liver Data at the Time of Sacrifice in Normal and Protein-deficient Rats (Mean ± SE of 11 Animals)

	Normal	Protein deficient
Caloric intake (K cals/day)	64 ± 2	60 ± 4
Body weight (g)	466 ± 9	234 ± 9
Liver weight (g)	13.6 ±0.8	6.3 ± 0.3
Lipid/g liver (mg)	31 ±1	57 ± 2
Liver microsomal protein isolated per rat (mg)	195 ± 12	102 ± 5

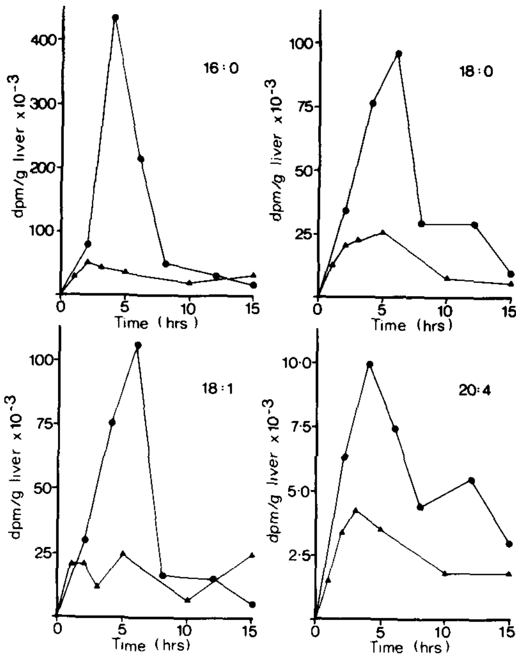

FIG. 1. The in vivo incorporation of [1-^{14}C] acetate into liver fatty acids of normal (●) and protein-deficient (▲) rats killed at intervals of up to 15 hr after intraperitoneal injection.

Microsomal Fatty Acid Synthetase

Microsomal protein (200 μg) was incubated for 10 min at 37 C with [1-^{14}C] acetyl-CoA (0.05 μmol = 4.0 x 10^5 d/min) according to Craig et al. (9). It had previously been confirmed that the rate of reaction is constant over this period (10,11). The reaction was terminated by the addition of chloroform-methanol (2:1). The lipids were then extracted from the mixture and counted for ^{14}C to determine the activity of the enzyme complex.

TABLE II

Liver Fatty Acids Shown in Figure 1 as % of Total Fatty Acids in Normal and Protein-deficient Rats (Mean ± SE of Six Animals)

Fatty acid	Normal	Deficient
16:0	25.3 ± 1.0	29.2 ± 1.2
18:0	19.3 ± 0.9	10.5 ± 0.8
18:1	23.1 ± 1.4	34.7 ± 3.7
20:4	4.0 ± 0.3	1.7 ± 0.2

D-β-hydroxybutyrate Dehydrogenase (EC 1.1.1.30)

The forward and reverse reactions of this enzyme were determined with sonicated mitochondria by monitoring changes in NADH$^+$ concentration with a Beckman D.U. spectrophotometer at 20 C. The assay used was described by Lehninger et al. (12).

Coenzyme A

One gram portions of liver were homogenized with 6 ml 0.25 M sucrose, 0.15 M KCl, and 0.1 mM EDTA. The proteins were removed by acidification with HCl to pH 4 and heating to 100 C. Free CoA was determined by the nitroprusside method (13). This method was found to give a $\Delta E_{520} = 0.123 \pm 0.004$ (mean of eight determinations ± SE) per 100 nmole CoASH and a linear response up to that concentration. Recovery, when tested by the addition of 6 nmole CoASH, was found to be 104 ± 12%.

Total CoA was determined at pH 9.0 by the method of Wakil and Hübscher (14), ΔE_{295} per mole being $1.90 \pm 0.16 \times 10^7$. This compares with $1.91 \pm 0.08 \times 10^7$ found by the above authors.

Protein Determination

Proteins were determined by the method of Lowry et al. (15) using bovine serum albumin as standard.

Extraction of Lipids and Conversion to Methyl Esters

Lipids were extracted from fresh material with 2:1 chloforom-methanol (16). They were then converted to methyl esters with 2 ml BCl_3-methanol complex, which does not form unwanted by-products (17,18), for 2 hr at 100 C. The esters were extracted twice with 10 ml hexane and the combined extracts washed six times with water. The purity of the product was confirmed by thin layer chromatography, using hexane-ether-acetic acid (85:15:1) as solvent system and methyl oleate as standard.

TABLE III

Elongation of Labeled 16:0 to 18:0, Desaturation of 18:2 to γ-18:3, and Elongation and Desaturation of 18:2 to 20:4 by Liver Microsomes of Normal and Protein-deficient Rats after 20 min Incubation at 25 C (Mean ± SE of Five Animals)

	Normal (d/min x 10^{-4})	Deficient (d/min x 10^{-4})
Elongation of [1-^{14}C] 16:0		
Incorporation into 18:0		
per mg microsomal protein	0.213 ± 0.024	0.203 ± 0.019
per g liver	2.82 ± 0.44	3.25 ± 0.45
Desaturation of [1-^{14}C] 18:2		
Incorporation into γ-18:3		
per mg microsomal protein	0.134 ± 0.018	0.140 ±0.016
per g liver	1.83 ± 0.21	2.22 ± 0.14
Elongation and desaturation of [1-^{14}C] 18:2		
Incorporation into 20:4		
per mg microsomal protein	0.113 ± 0.018	0.137 ± 0.019
per g liver	2.02 ± 0.31	1.93 ± 0.18

TABLE IV

Activities of Microsomal Acyl-CoA Synthetase and Fatty Acid Synthetase and of Mitochondrial D-β-Hydroxybutyrate Dehydrogenase in Normal and Protein-deficient Rats (Mean ± SE of Five Animals)

	Per mg protein	
Enzyme	Normal	Deficient
Acyl-CoA synthetase		
μmoles CoA-SH used per min		
Substrate		
Acetic acid	0.23 ± 0.05	0.13 ± 0.01[a]
Butyric acid	0.22 ± 0.01	0.13 ± 0.03[a]
Palmitic acid	0.21 ± 0.02	0.20 ± 0.02
Linoleic acid	0.24 ± 0.03	0.19 ± 0.05
Fatty acid synthetase		
nmoles [1-^{14}C] acetyl-CoA incorporated per min	0.65 ± 0.05	0.31 ± 0.08[a]
D-β-hydroxybutyrate dehydrogenase		
nmoles $NADH^+$ liberated per min	22.0 ± 3.1	2.4 ± 0.4[a]
nmoles $NADH^+$ used per min	11.4 ± 6.8	2.3 ± 0.4[a]

[a]Significantly different from normal ($P<0.05$).

Gas Chromatography and Counting of ^{14}C

The gas chromatograph (F & M, Model 5750) used was provided with exit stream splitter and flame ionization detector. Methyl esters were separated on a 2 m x 2 mm (ID) EGSSX on Chromosorb W (acid washed, silanized) column, using N_2 as carrier gas. The method of recovery was that of Schlenk and Sand (19). The amount of fatty acid collected for ^{14}C counting could be determined by measuring the response of the gas chromatograph and the split ratio of the stream splitter.

The counter used was a Packard Tri-Carb liquid scintillation counter (Model 2002) operating at an efficiency of ca. 72%.

EXPERIMENTAL AND RESULTS

Animals and Diets

Twenty-two male Wistar rats were grown on a balanced diet containing 18% protein for 10 weeks after weaning. After this, half the animals were transferred to a protein-free diet for a further 7 weeks while the remaining 11 rats were kept as controls. The diets were composed as previously described (3).

Table I summarizes caloric intake, rat and liver weights and lipid and microsomal protein content of the livers at the time of sacrifice.

The In Vivo Incorporation of [1-^{14}C] Acetate into Rat Liver Fatty Acids

Six normal and six protein-deficient rats

TABLE V

Coenzyme A in the Livers of Normal and Protein-deficient Rats (Mean ± SE of Five Animals)

	Per g liver (μmoles)	
	Normal	Deficient
Total CoA	0.70 ± 0.09	0.49 ± 0.05[a]
Free CoA	0.14 ± 0.06[b]	0.44 ± 0.04[a]
Acyl CoA (difference)	0.58 ± 0.09	0.02 ± 0.05[a]

[a]Significantly different from normal ($P<0.05$).
[b]Significantly different from total CoA ($P<0.05$).

were injected intraperitoneally with 100 μCi [1-^{14}C] sodium acetate dissolved in 0.1 ml water. They were killed at intervals as shown in Figure 1, their livers removed, and the liver fatty acids separated by gas chromatography. Palmitate (16:0), stearate (18:0), oleate (18:1), and arachidonate (20:4) were recovered for counting (Fig. 1). Their proportions are shown in Table II.

The remaining ten rats, five normal and five deficient, were used for in vitro determinations of the following enzymes and of CoA:

Microsomal Elongation, Desaturation, and Elongation/Desaturation

The substrates used were [1-^{14}C] 16:0 aerobically elongated to ^{14}C-18:0; [1-^{14}C] 18:2, desaturated anaerobically to ^{14}C-γ18:3; and [1-^{14}C] 18:2, aerobically desaturated and elongated to ^{14}C-20:4 (See Table III).

Microsomal Acyl-CoA Synthetase, Microsomal Fatty Acid Synthetase and Mitochondrial β-Hydroxybutyrate Dehydrogenase

These determination are presented in Table IV.

Table V summarizes the results of the CoA determinations.

In the deficient group acyl-CoA, estimated as the difference between free and total CoA is less than the standard error. This indicates that there are no significant amounts in the liver.

DISCUSSION

From Table I, one can see that the decrease in liver weights of protein-deficient rats was accompanied by an increase in the concentraion of lipid. It has been suggested that this is due to impaired lipid transport (20). Table II shows that protein deficiency has affected fatty acid composition as well. As a result, 16:0 and 18:1 have approximately doubled on a per g liver basis.

Figure 1 shows less ^{14}C incorporation into liver lipids in vivo which suggests a decrease in fatty acid synthesis due either to deficiencies in the necessary enzyme systems or the channeling of acetate into other metabolic pathways.

It appears that long term protein deprivation has less effect on microsomal desaturation of 18:2 than the short term deprivation studied by De Gómez Dumm et al. (21), who found a small decrease of 18:2 desaturation in vitro after 96 hr.

The results summarizied in Table III thus provide no explanation of the decrease in long chain fatty acids previously observed (3,22,23). However, this might be partially explained by the decrease in specific activities of the short chain acyl-CoA synthetases (Table IV) which were reduced to ca. 60% of the normal levels. The specific activity of long chain acyl-CoA synthetase was not affected by protein deficiency.

Our findings, summarizied in Table V, of reduced CoA and the virtual disappearance of acyl-CoA, may well be much more significant since CoA is obligatory as a cofactor in all fatty acid syntheses, elongations, and desaturations.

Methods for the isolation of CoA from tissues are not totally specific, and redisual -SH contributed by other acid-soluble compounds, e.g., traces of cysteine, may be present. However, the method used by us was found satisfactory not only by Wakil and Hübscher (14) but also by Allred and Guy (24). These authors tested acificiation and heating separately and found that heating alone gave higher values. They consequently relied on acidification.

The accumulation of free CoA and the simultaneous disappearance of acyl-CoA are in keeping with reduced short chain acyl-CoA synthetase activity. The resultant diminished acetyl-CoA synthesis may be one of the reasons for the reduction in ^{14}C incorporation into 16:0, 18:0, and 20:4 observed in the in vivo experiment.

The loss of microsomal fatty acid synthetase activity during protein deficiency could also contribute to the decrease in C_{20} and C_{22} fatty acids previously recorded (2,3). This enzyme appears to be of major significance since it has been shown that microsomes account for 50-70% of in vivo fatty acid synthesis in the liver. The mitochondrial and soluble systems contribute the remainder (25,26).

Our results suggest that during protein deficiency changes in liver fatty acid composition, particularly the reduction of the essential fatty acid 20:4, may be explained at least in part by reduced availability of short chain acyl-CoA synthetase and of CoA and a decrease in

microsomal fatty acid synthetase.

Information on the effect of protein deficiency on β-hydroxybutyrate dehydrogenase, although not directly relevant to the above, is included in Table IV. The enzyme catalyzes the conversion of β-hydroxybutyrate to acetoacetate, which is in turn converted to acetyl-CoA, available for fatty acid biosynthesis (6,7). In the livers of protein-deficient rats, the activity of the enzyme was reduced to ca. 10-20% of normal. A reduction of this magnitude probably precludes this pathway as an alternative source of acetyl CoA.

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COMMUNICATIONS

Gangliosides of Cultured Cells of a Rat Mammary Carcinoma Cell Line[1]

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ABSTRACT

Ganglioside content of rat mammary carcinoma-derived cells grown in layers in vitro was nearly as high as that of apical plasma membrane-derived milk fat globule membrane and nearly four times higher than the content of normal, lactating mammary tissue on a protein basis. The major ganglioside of these carcinoma-derived cells was identified as GDla (sialic acid-Gal-GalNAc-(sialic acid)-Gal-Glc-Cer. Relative to carcinoma-derived cells, rat mammary tissue and milk fat globule membrane had more complex ganglioside patterns but appeared to lack substantial quantities of GDla.

INTRODUCTION

Gangliosides, sialic acid-containing glycosphingolipids, are concentrated in brain and central nervous system tissues but have also been identified in many mammalian extraneural tissues (e.g., 2-5). Because ganglioside patterns are frequently altered in malignantly transformed cells and solid tumors, these glycolipids have become the object of much experimental attention (reviews, 6-10). Generally, transformed cells and tissues have larger amounts of total gangliosides, and most, but not all, transformed cells also display a simplification in pattern of ganglioside distribution. In all cases specifically examined, this simplification in ganglioside pattern was found to be due to diminished activity of a glycosyltransferase functioning in biogenesis of the higher ganglioside homologs.

We recently established in culture a cell line derived from a dimethylbenz(α)anthracene-induced (11) rat mammary carcinoma that displayed a transformed growth pattern in vitro (12). In view of the observation that ganglioside pattern and synthesis are altered in chemically induced rat mammary carcinomas in vivo (13), it was of interest to determine the ganglioside composition of this cell line. Results of this study are given in the present communication.

MATERIALS

Ganglioside GM3 was isolated from dog erythrocytes (14), GM2 was from brain tissue taken at autopsy from a human with Tay-Sachs disease (15), GM1 was isolated from a neuraminidase-treated preparation of bovine brain gangliosides (14), and GD1a and GD1b were from normal human brain (16). Rat milk was obtained, with the aid of oxytocin injections, as described (17), and milk fat globule membranes were isolated from this milk (18). Mammary tissue was removed from lactating rats (Holtzman Company, Madison, WI) which were sacrificed by cervical dislocation. The origin and conditions used for culturing the rat mammary carcinoma cell line have been described (12). Cells were harvested as layers at high cell densities by brief treatment with trypsin, washed three times with phosphate buffered saline, and lyophilized.

METHODS

Gangliosides were recovered from tissue homogenates or from water suspensions of cells or membranes by the method of Ledeen et al. (19). In our hands, recovery of ^{3}H-GM1, added to samples, was consistently more than 90%.

[1] Abbreviations: Gal, galactose; Glc, glucose; GalNAc, N-acetylgalactosamine; Cer, ceramide (N-acylsphingosine). Gangliosides abbreviated according to Svennerholm (1): GM3, sialic acid-Gal-Glc-Cer; GM2, GalNAc-(sialic acid)-Gal-Glc-Cer; GM1, Gal-GalNAc-(sialic acid)-Gal-Glc-Cer; GDla, sialic acid-Gal-GalNAc-(sialic acid)-Gal-Glc-Cer; GD1b, Gal-GalNAc-(sialic acid)$_2$-Gal-Glc-Cer.

TABLE I

Ganglioside-bound Sialic Acid Content of Cultured Rat Mammary Carcinoma-derived Cells, Rat Mammary Tissue, and Milk Fat Globule Membrane.

Fraction	Ganglioside sialic acid[a]
Carcinoma-derived cells	4.4 nmoles/mg protein
	8.8 nmoles/10^6 cells
	23.1 nmoles/mg DNA
Mammary tissue	1.1 nmoles/mg protein
	21.0 nmoles/mg phospholipid
	26.3 nmoles/mg DNA
Fat globule membrane	5.0 nmoles/mg protein
	19.2 nmoles/mg phospholipid

[a]Values are averages for two samples; individual values were within 10% of each other.

Thin layer chromatography (TLC) was performed on plates coated with a 0.5 mm layer of Silica Gel G. Plates were developed with chloroform-methanol-28% ammonium hydroxide-water (60:35:7:3), by volume) or with 1-propanol-28% ammonium hydroxide (7:3, v/v) (1). Neutral glycolipids were separated with the chloroform-containing solvent system only. Separated constituents were visualized by treating plates with resorcinol reagent (20) or with 50% aqueous sulfuric acid. When individual gangliosides were to be recovered for subsequent characterization, plates were sprayed with 0.005% aqueous Rhodamine 6G and viewed under ultraviolet light. Dye was removed and gangliosides were recovered as described (14).

Protein was determined according to Lowry et al. (21) with bovine serum albumin as the standard. After release by acid hydrolysis (0.1 N sulfuric acid, 80 C, 60 min), sialic acid was determined by the method of Warren (22). Sphingosine was measured according to Lauter and Trams (23), and phospholipids were measured, in washed lipid extracts, as in previous studies (14). DNA was estimated according to Burton (24).

Structural characterization was accomplished by carbohydrate and thin layer chromatographic analysis. Carbohydrates were released by hydrolysis for 15 hr in 1 M HCl at 100 C. After addition of 2-deoxy-D-glucose as an internal standard, alditol acetates were prepared (25) and separated in a 2 mm x 2.4 m glass U-tube packed with 3% SP-2340 on 100/120 mesh Supelcoport (Supelco, Inc., Bellefonte, PA). The chromatograph, a F & M Model 402 with flame ionization detectors, was programmed from 140 to 240 C at 3 C/min with a nitrogen carrier gas flow rate of 25 ml/min. To determine the core carbohydrate, gangliosides were subjected to partial acid hydrolysis (0.1 N HCl, 100 C, 3 hr), and the neutral glycosphingolipid fraction generated was recovered (14) and analyzed for carbohydrate composition as above.

Sialic acids were released by mild acid hydrolysis (0.005 N sulfuric acid, 100 C, 1 hr) or by treatment with neuraminidase from *Clostridium perfringens*. For the latter, gangliosides were incubated in 50 mM acetate buffer, pH 5.2, 1% Triton X-100 for 18 hr at 37 C. Following incubation, neutral glycolipids were recovered (13) and examined by TLC.

Fatty acids were analyzed as methyl esters on a column packed with OV-1 as in previous studies (14). Methyl esters were prepared by treating gangliosides with 0.05 N methanolic HCl for 18 hr at 80 C.

RESULTS

The rat mammary carcinoma cell line analyzed contained 4.4 nmoles of ganglioside sialic acid/mg protein (Table I). Identical results were obtained for confluent cell cultures of separate clones and preparations. This content, which is similar to that reported with other cultured cell lines (cf. references in introduction and 26,27), is equivalent to approximately 8 nmoles of ganglioside sialic acid/10^6 cells or 23 nmoles/mg DNA. By comparison, pooled mammary tissue from 11 lactating rats contained 1.05 nmoles of ganglioside sialic acid/mg protein (Table I). Rat milk fat globule membrane, which is derived from the apical plasma membrane of mammary epithelial cells (e.g., 28), had about 5 nmoles ganglioside sialic acid/mg protein (this value was obtained with a pooled sample from 10 rats). Among cellular membranes, gangliosides are concentrated in plasma membranes but are also found in Golgi apparatus membranes (29-32). That gangliosides are present in the rat mammary carcinoma-derived cells in nearly the same level as encoutered in apical plasma membrane-milk fat globule mem-

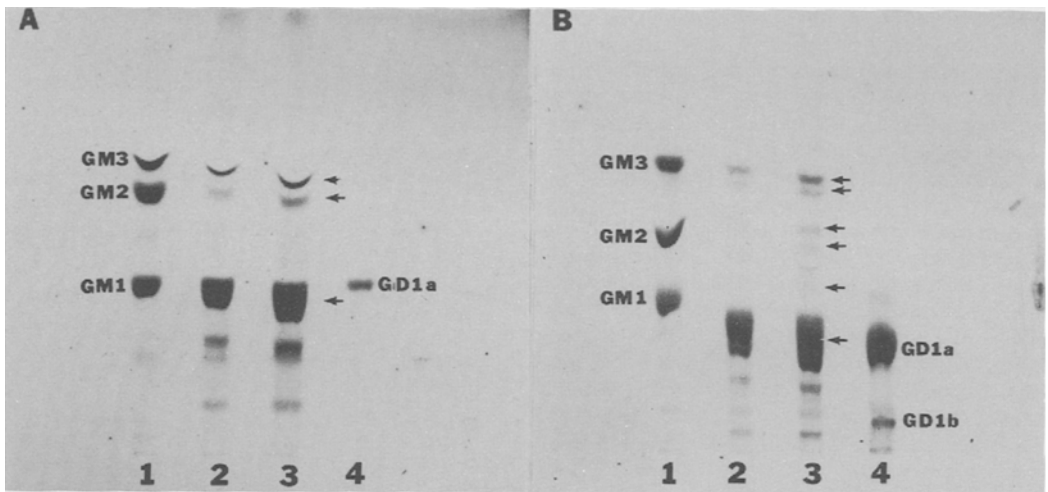

FIG. 1. Chromatographic separation of the gangliosides from a rat mammary carcinoma-derived cell line. (A) Plate developed in chloroform-methanol-ammonium hydroxide-water (60:35:7:3, by volume). (B) Plate developed in 1-propanol-ammonium hydroxide (7:3, v/v). Lanes 1 and 4, reference gangliosides as indicated, lanes 2 and 3, 30 and 60 nmoles of ganglioside sialic acid from carcinoma cells. Plates were treated with resorcinol reagent, and the resorcinol-positive constituents are indicated by arrows.

brane suggests that these cells are considerably enriched in gangliosides relative to the "average" untransformed cells of mammary tissue.

Thin layer separation revealed the presence of a total of four gangliosides in the carcinoma cells (Fig. 1). In both solvent systems, the major ganglioside migrated near authentic GDla, and, by densitometric analysis, this major ganglioside was found to account for about 75% of the sialic acid applied onto plates. Other gangliosides in the carcinoma cells displayed chromatographic mobilities similar to those of GM3, GM2, and GM1.

Rat mammary gland and milk fat globule membrane ganglioside patterns were similar to each other but were considerably different than the pattern for the carcinoma-derived cells grown in vitro (not shown). In particular, there was but a trace of ganlioside with GDla mobility in the globule membrane and mammary tissue fractions.

Results from partial structural characterization of the major ganglioside of the rat mammary carcinoma-derived cells gave ratios for sphingosine, glucose, galactose, galactosamine, and sialic acid of 1.10:1.00:1.99:0.80:2.20, respectively (calculated relative to glucose). These are very close to the values expected for GDla. Mild acid hydrolysis of the presumed GDla yielded as a major product a neutral glycolipid with chromatographic mobility identical to asialo-GMl. Treatment of the presumed GDla with neuraminidase yielded a sialic acid-containing product with chromatographic mobility identical to that of GMl. Partial acid hydrolysis of the ganglioside led to an enrichment of glucose in the neutral glycolipid fraction obtained, suggesting that glucose is the carbohydrate attached directly to ceramide in this ganglioside. While the sequence of carbohydrates and positions of linkages of sugars were not determined, these results leave little doubt that the major ganglioside in this line of rat mammary carcinoma-derived cells in GDla.

Fatty acid analyses revealed that the major ganglioside from the carcinoma-derived cells was similar to other mammary gland sphingolipids (e.g., 14,33) in that fatty acids were of long chain length and exclusively saturated or monounsaturated. This ganglioside was characterized by a high content of 20 and 24 carbon chain length fatty acids.

DISCUSSION

Results presented herein show that this rat mammary carcinoma-derived cell line differs from rat mammary tissue in ganglioside content and composition. In that the majority of cells in lactating mammary gland are epithelial cells (34), it appears reasonable to suggest that rat mammary carcinoma cells, which are of epithelial cell origin (12), may differ from their progenitor cells in ganglioside composition. The predominance of a complex disialoganglioside in cultured cells with a transformed growth pattern does not fit the frequently noted tendency toward an enrichment of less complex gangliosides (for references, see Introduction). How-

ever, our finding of the predominance of GDla in the cells of a line derived from a mammary tumor that exhibits transformed growth characteristics in vitro is not without precedent in cultures of other transformed cells. Yogeeswaran et al. (26,27) have described clones of SV 40 transformed mouse 3T3 cells that showed extremely high concentrations of GDla, representing 63 to 66% of the total ganglioside sialic acid. In contrast, other clones and/or lines of transformed mouse 3T3 cells had a predominance of simpler gangliosides, in particular GM3. Elevated levels of GDla have also been found in some transplantable Morris hepatomas (for review, see 35). Interesting in this connection is the observation of Chatterjee et al. (36) that GDla is the predominant ganglioside in monolayer cultures of KB cells, a line derived from human epitheloidal carcinoma, but is reduced relative to other gangliosides when these cells are grown in suspension culture. Whether or not a relationship exists between the high levels of GDla and the growth behavior or other properties of such cells remains to be clarified. In contrast to our cultured cell line, the major ganglioside in dimethylbenzanthracene-induced rat mammary carcinomas is GM1 (13). The sialyltransferase activity responsible for conversion of GMl to GDla is greatly diminished in these solid tumore relative to mammary tissue. The reason for the predominance of GDla in this cell line is not known but may be due to some selection or selective growth advantage for this particular cell type.

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1,2-Propanediol-Induced Changes in Plasma and Tissue Lipids of Rats

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ABSTRACT

Oral administration of 1,2-propanediol to rats in a daily dose of 1 ml of 28.4% aqueous solution per 100 g body weight for 30 days caused a significant decrease in the total lipids, fatty acids, phospholipids, and triglycerides of plasma, liver, and heart, The cholesterol content in plasma decreased while that in the tissues increased significantly. The accumulation of cholesterol in tissues tends to discourage long term use of 1,2-propanediol even by the oral route.

1,2-Propanediol has diverse applications as in pharmaceutics and cosmetics, in preservatives, as a humectant and as a vitamin stabilizer because it had been considered as nontoxic in nature (1). Recently it has been reported that 1,2-propanediol, hitherto considered as an "inert" substance, could not be recommended when administered by other than the oral route (2,3). Metabolic and chronic studies demonstrated that it could be used as a substitute for carbohydrate in the diet of rats (4,5) and young chicks (6,7) as it is biotransformed entirely by normal pathways to lactate or pyruvate (1,8). In the course of our investigations on the effect of a lipid-soluble oleoresin of gum guggul (from Commiphora mukul) on lipid metabolism using 1,2-propanediol as the vehicle, we observed significant decreases in the total lipids, fatty acids, phospholipids, and triglycerides of plasma, liver, and heart. The cholesterol content in plasma decreased while that in the tissues increased significantly. The results reported here suggest that long term use of 1,2-propanediol even by the oral route should be discouraged.

MATERIALS AND METHODS

Normal adult male albino rats (Kasauli strain, 150∓20 g in weight) were divided into two groups of 12 animals each. They were fed a standard laboratory diet (Hindustan Lever rat diet pellets) ad libitum. An oral dose of 1 ml of 1,2-propanediol (28.4% v/v in distilled water) per 100 g body weight was administered daily for a period of 30 days to one group, while the other group served as control and received similarly 1 ml of distilled water daily for the same period. Weights were recorded at the beginning and a day prior to sacrifice. Blood samples from fasting rats (18-20 hr) were collected into tubes containing potassium oxalate. The animals were decapitated, liver and heart were removed

TABLE I

Amount of Lipids in Plasma, Liver, and Heart of Rats Fed 1 ml of 28.4% Aqueous Solution of 1,2-Propanediol for 30 Days[a]

Source	Group[b]	Total lipids	Fatty acids	Phospholipids	Triglycerides
Plasma (mg/100 ml)	Control	279.3 (±8.7)	165.60 (±5.3)	84.8 (±3.5)	107.5 (±5.3)
	1,2-Propanediol fed	257.8[d] (±5.7)	147.3[d] (±4.1)	69.6[d] (±3.2)	93.3[d] (±3.4)
Liver (mg/100 mg wet tissue)	Control	6.7 (±0.16)	3.6 (±0.25)	3.0 (±0.15)	836.5[c] (±21.5)
	1,2-Propanediol fed	5.93[d] (±0.20)	3.0[d] (±0.13)	2.6[d] (±0.12)	751.5[d] (±25.3)
Heart (mg/100 mg wet tissue)	Control	5.9 (±0.20)	3.0 (±0.14)	2.9 (±0.27)	505.5[c] (±21.2)
	1,2-Propanediol fed	5.0[d] (±0.19)	2.5[d] (±0.11)	2.5[d] (±0.24)	434.7[d] (±19.4)

[a]Mean ± SD.
[b]Each group consists of 12 rats.
[c]Expressed as mg/100 g wet tissue.
[d]t_{22} = 2.819; p = 0.001.

immediately, washed and cleaned with ice cold physiological saline, drained between folds of filter paper, weighed, sliced and used for extraction of lipids according to the method of Folch et al. (9). Total lipids (10), fatty acids (10), phospholipids (11), triglycerides (12), total cholesterol and free cholesterol (13) were estimated by standard methods. The data were analyzed for statistical significance by students t-test. All the chemicals used were of analytical grade.

RESULTS AND DISCUSSION

1,2-Propanediol was administered to male rats orally at a dose equivalent to 0.284 ml/100 g body weight. The growth rate and activity of the animals during the period of observation were normal. The amounts of plasma and tissue lipids after 1,2-propanediol administration for 30 days are presented in Tables I and II. A statistically significant (P∠0.001) reduction in plasma and tissue total lipids, fatty acids, phospholipids, and triglycerides was observed in the 1,2-propanediol-fed group of animals as compared to their control (Table I). The total (P∠0.001), free (P∠0.001), and ester cholesterol (P∠0.05) content (Table II) decreased in plasma and increased in liver (P∠0.001, 0.001, and 0.001, respectively) and heart (P∠0.001, 0.05, and 0.001, respectively). These changes were reflected in the decrease in the ratios of the free to esterified cholesterol, and the increase in that of total cholesterol to phospholipids. The changes in the ratios of the former were statistically significant only in plasma and liver, whereas they were significant in all the cases in the latter.

It has been stated (1) that 1,2-propanediol in large quantities appears to be innocuous, since cumulative effects, both functional and morphological, are not demonstrable in the organism as a whole. The metabolism of 1,2-propanediol in experimental animals has been shown to take place by a series of sequential biological oxidations contributing energy by passing through lactaldehyde, methylglyoxal, lactate, and pyruvate and entering the citric acid cycle (1). It also participates in anabolic reactions by being incorporated into hepatic glycogen (4,5,7,14-18). In contrast to 1,2-propanediol, the isomeric 1,3-propanediol is several times more toxic because of the metabolic formation of the intermediary malonic acid (19).

The significant decrease in tissue and plasma lipids observed in the present study suggests that the contribution of 1,2-propanediol to fatty acid synthesis might be rather small.

TABLE II

Amount of Cholesterol in Plasma, Liver, and Heart of Rats Fed 1 ml of 28.4% Aqueous Solution of 1,2-Propanediol for 30 Days[a]

Source	Group[b]	Cholesterol Total	Cholesterol free	Ester	Free/Ester cholesterol	Total cholesterol/ Total phospholipids
Plasma (mg/100 ml)	Control	59.0 (±8.9)	20.7 (±2.1)	38.0 (±8.3)	0.56 (±0.10)	0.70 (±0.08)
	1,2-Propanediol fed	52.2[y c] (±6.0)	16.7[y] (±1.6)	35.6[x] (±4.7)	0.46[y] (±0.05)	0.75[y] (±0.07)
Liver (mg/100 g wet tissue)	Control	336.6 (±44.3)	138.8 (±18.4)	193.8 (±30.5)	0.71 (±0.06)	0.11 (±0.01)
	1,2-Propanediol fed	472.1[y] (±56.8)	168.4[y] (±20.9)	303.2[y] (±43.1)	0.57[y] (±0.12)	0.19[y] (±0.02)
Heart (mg/100 g wet tissue)	Control	282.2 (±36.2)	114.9 (±14.1)	167.1 (±11.6)	0.70 (±0.08)	0.10 (±0.01)
	1,2-Propanediol fed	309.5[y] (±28.4)	120.3[x] (±8.7)	188.2[y] (±6.5)	0.67 (±0.11)	0.12[y] (±0.03)

[a]Mean ± SD.
[b]Each group consists of 12 rats.
[c]x: $t_{22} = 2.074$; p = 0.05; y: $t_{22} = 2.819$; p = 0.001.

Besides, the decrease in the ratio of free to esterified cholesterol suggests a shift toward esterification in liver, heart, and plasma by 1,2-propanediol administration. The increase in the tissue cholesterol, however, is suggestive of an alternative pathway for 1,2-propanediol, which seems to be further supported by an earlier finding that ingestion of radioactive acetone in rats gave a ratio of the specific activities, carcass cholesterol to carcass fatty acids, about 6:1, whereas that in the case of ^{14}C-acetate it ranged from 0.4:1 to 1.7:1 (20). As the metabolism of acetone is known to take place via 1,2-propanediol or its 1-phosphate (21), it could possibly be postulated that a C_3 oxidation product of 1,2-propanediol, such as methyglyoxal, may condense with a C_2 unit to produce an intermediate capable of further condensation to a sterol (1,22,23). This possible pathway of 1,2-propanediol metabolism specifically promoting cholesterol biosynthesis or storage seems unique. Emmanuel (7), however, observed that 1,2-propanediol did not cause changes in plasma glucose, free fatty acids, cholesterol, and liver glycogen in broiler chicks, which might be due to species difference. By intravenous route, on the other hand, 1,2-propanediol displayed antiarrhythmic and antifibrillatory effect in rat and dog heart (24). The feeding of ethanol (3 ml of 30% solution for 8 weeks) to rat also caused a significant increase in the cholesterol content of the heart without affecting its content in liver and kidney, with a concomitant marked increase in the fatty acid content of all the organs (25).

The accumulation of cholesterol in tissues as observed in the present study, when considered along with the observed decrease in clotting time and in lymphocyte count (1), sensitization of lymphocytes (26), and increase in the platelet polymorphonuclear leucocyte count (1), tends to disfavor the use of 1,2-propanediol as a solvent even by the oral route for long term contrary to the proposals from some other laboratories (2,3).

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ERRATUM

In "Oxygenated Fatty Acid Constituents of Soybean Phosphatidylcholines," by D.J. Sessa, H.W. Gardner, R. Kleiman, and D. Weisleder [*Lipids* 12:613 (1977)], Figure 2 was printed incorrectly and Figure 3 was a poor representation of the original. The corrected figures appear below.

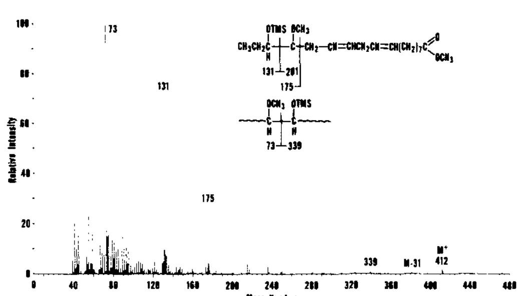

FIG. 2. Mass spectrum of an epoxy fatty acid methyl ester from Fraction B after reaction with BF_3/methanol and trimethylsilylation with bis(trimethylsilyl)-trifluoroacetamide.

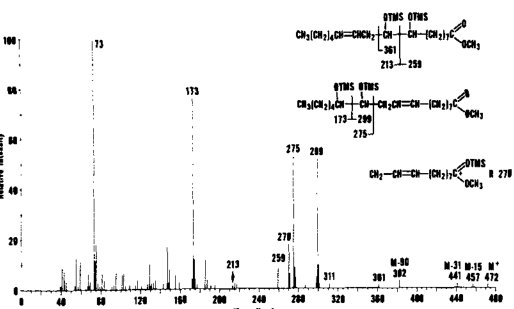

FIG. 3. Mass spectrum of TMS ether derivatives of dihydroxyoctadecenoates from Fraction E.

Effects of Cold Stress on Rats Fed Different Levels of Docosenoic Acids

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ABSTRACT

Male Sprague-Dawley rats, 4 weeks old, were subjected to an ambient temperature of 4 C for periods up to 24 days and fed a synthetic diet containing one of the following oils: peanut oil (PO), rapeseed oil (RO), low erucic acid rapeseed oil (LO), and partially hydrogenated marine oil (HO), each at 20% w/w. A parallel experiment using the same oils was performed at room temperature (23 C). During cold stress, animals on the RO diet showed higher mortality than all other groups; all 20 animals in this group died within 5 days. At room temperature, however, all animals survived. The lipid accumulation in the heart reached its peak in all groups after 3 days and then gradually declined. The accumulation was most pronounced in the RO animals and coincided with the high mortality at 4 C. The fatty acid composition of the cardiac triglycerides reflected that of the diet, while the composition of the cardiac lecithin was only marginally modified.

INTRODUCTION

Ingestion of rapeseed oil containing high concentrations of erucic acid has been reported to cause cardiac lipidosis and necrotic lesions in the heart of rats (1). Erucic acid in rapeseed oil has also been suggested to cause growth retardation (2,3).

It has been proposed that factors other than the high 22:1 content might be responsible for the cardiac lesions, e.g., high amounts of 18:1 and 18:3 and a low proportion of saturated fats (3,4). With regard to the effect of rapeseed oil during cold stress, Beare-Rogers and Nera (5) found 80% mortality after 3 weeks in a group of rats on a high erucic acid diet. In a similar experiment, Hulan et al. (6) reported no mortality.

The following study was made to elucidate the contradicting results reported on the effects of rapeseed oil containing high amounts of erucic acid on rats during cold stress. Further, these effects were compared with those of two other vegetable oils containing small amounts of erucic acid and a marine oil of animal origin. A parallel experiment with the same oils was performed at room temperature.

METHODS

Animals and Diets

Male weanling rats from a Sprague-Dawley strain (Anticimex) were placed in cages (59 x 39 x 20 cm) in groups of five in the animal unit for 7 days and fed water and pellets (Astra-Ewos) ad libitum. The animals were randomly assigned to individual cages (27 x 21 x 14 cm) with a wire mesh bottom, and transferred to a climate chamber at 4 C and 90% relative humidity (Expt. I) or 23 C and 65% relative humidity (Expt. II). They were kept on a schedule of 12 hr light: 12 hr dark, and fed a synthetic diet containing 20% w/w of one of the following oils: rapeseed oil (RO) (22:1=42% of the total fatty acids), low erucic acid rapeseed oil (LO) (22:1=6%), hydrogenated marine oil (HO) (22:1=3%), and peanut oil (PO) (22:1=0.5%). The composition of the diets is given in Table I. The fatty acid composition of the oils is given in Table II. The synthetic food was stored at -20 C before use.

In each experiment, 80 rats were used. They were divided into groups of 20 with the same mean body weight in each group. From each group, five animals were killed at days, 3, 7, 14,

TABLE I

Composition of the Synthetic Diets

Ingredients	g/kg diet
Casein	200
Dextrose	528
Oil	206
Powdered cellulose	10
Salt mixture[a]	50
Vitamin mixture[b]	4
Choline chloride	2

[a]Salts in 1 kg of diet: (g) NaCl 6.97; KH_2PO_4 19.45; $MgSO_4$ ($7H_2O$) 2.87; $CaCO_3$ 19.07; $FeSO_4$ ($7H_2O$) 1.35; (mg) $MnSO_4$ (H_2O) 203; $ZnSO_4$ ($7H_2O$) 27.4; $CuSO_4$ ($5H_2O$) 23.9; $CoCl_2$ ($6H_2O$) 1.15; KI 39.6.

[b]Vitamins in 1 kg of diet: (mg) thiamin 5; riboflavin 6; pyridoxin 5; niacinamide 40; pantothenate 40; p-aminobenzoic acid 100; biotin 0.4; folic acid 2; cyanocobalamin 0.03; inositol 100; menadione 5; dl-tocopherylacetate 1560; retinol 625 IU; ergocalciferol 100 IU.

TABLE II

Fatty Acid Composition of the Various Oils Included in the Diets

Fatty acids	Weight % of total			
	PO	RO	LO	HO
14:0	---	---	---	8.3
16:0	10.7	4.2	4.4	21.2
16:1	---	0.3	0.3	11.4
18:0	2.8	1.3	1.6	7.1
18:1	34.2	15.4	51.4	19.5
18:2	42.1	17.2	19.9	5.4
18:3	1.2	8.6	10.1	0.7
20:0	1.5	0.8	0.8	1.5
20:1	1.6	8.2	3.6	7.2
20:2	0.3	0.6	0.3	4.6
22:0	3.0	0.4	0.4	0.5
22:1	0.7	41.6	6.5	3.2
22:2	---	0.8	0.2	1.3
24:0	1.2	---	---	---
Saturated fatty acids	19.2	6.7	7.2	38.6
Monounsaturated fatty acids	36.5	65.5	61.8	41.3
Polyunsaturated fatty acids	43.6	27.2	30.5	13.0
S/U[a]	0.24	0.07	0.08	0.71
18:1+18:3/S	1.84	3.58	8.54	0.52

[a]S = saturated fatty acids; U = unsaturated fatty acids.

TABLE III

Cumulative Mortality Rates in Male Rats during Cold Stress Fed Diets with Different Levels of Docosenoic Acids

	22:1[a]	Days of feeding		
		0-7	0-14	0-24
PO	0.1	4/20[b]	5/20	5/20
RO	8.4	20/20[c]		
LO	1.2	1/20	3/20	3/20
HO	0.6	2/20	2/20	2/20[d]

[a]In percent of the diet, w/w.
[b]Three dead animals had tail wounds.
[c]All animals died within 5 days.
[d]Both dead animals had tail wounds.

and 24 at 4 C (Expt. I) or at days 3, 7, 14, and 42 at 23 C (Expt. II). Additionally, five animals (untreated) were killed on day 0 and used as a reference group in both experiments. All rats were weighed at intervals of 2-3 days, and their general condition noted. Food and water consumption was measured twice weekly.

Preparation of Fatty Acids for Analysis

Animals were killed by decapitation, and the heart, liver, and adrenals excised immediately and weighed on an analytical balance. The heart apex was cut off (3-4 mm) and quickly frozen for histological examination (see below). The remaining part of the heart (about 0.5 g) was weighed and used for the lipid analysis.

The lipid extraction was performed according to Folch et al. (7) as modified by Bruce and Svennerholm (8). The myocardium was homogenized in a Waring blendor with 50 ml redistilled chloroform-methanol (2:1, v/v). The homogenate was centrifuged at 2,600 x g for 20 min, and the supernatant decanted in a flask. The pellet was resuspended in 10 ml $CHCl_3$:CH_3OH and recentrifuged as above. The two supernatants were pooled and evaporated to almost dryness in a rotary evaporator at 35 C. The residue was dissolved and partitioned in 10 ml $CHCl_3$:CH_3OH:NaCl 0.85% (8:4:3, v/v/v). The organic phase containing the lipids was diluted to 6 ml with CH_3OH, and the extract stored in darkness at 4 C.

Lipid Separation and Analysis

An amount of the lipid extract, corresponding to 12 mg total lipid, was evaporated to dryness and dissolved in 0.4 ml $CHCl_3$:CH_3OH (2:1, v/v). Each sample was divided in two equal portions and applied to thin layer chromatography (TLC) plates (DC Fertigplatten Silica Gel F_{254}; 20 x 20 cm, 0.5 mm thickness). For separation of triglycerides, a solvent system of n-hexane-diethyl ether-acetic acid (80:20:1, v/v/v) was used (9), and for lecithin $CHCl_3$:CH_3OH:H_2O (65:25:4, v/v/v) (8). The plates were run for an hour, dried, and read in UV-light. The triglycerides and lecithin bands were scraped off and extracted from the silica gel with $CHCl_3$:CH_3OH (2:1, v/v) followed by centrifugation at 1,000 x g for 10 min and evaporated to dryness. After addition of 0.5 ml n-hexane, the extract was methylated according to Appelqvist (10) and gas chromatography (GC) performed on a HP

TABLE IV

Amount of 18:1 and 22:1 in Rat Heart Triglycerides and Degree of Lipidosis at 4 C and 23 C in Male Rats Fed Diets with Varying Amounts of 22:1
(Figures are Expressed as Percent of Total Fatty Acids [Mean of Five Animals])

			4 C				23 C			
			Days on diet				Days on diet			
	Group	In diet	3	7	14	24	3	7	14	42
18:1	PO	34.2	32.5	35.8	36.0	37.0	33.5	34.7	39.3	43.3
	RO	15.4	21.4	---	---	---	27.4	26.6	25.5	28.0
	LO	51.4	42.7	42.0	47.4	48.6	39.2	45.1	51.4	57.1
	HO	19.5	27.0	30.5	34.2	33.1	31.1	27.9	32.3	35.2
22:1	PO	0.7	0	0.3	0	0	0.4	1.1	0.5	0.5
	RO	41.6	37.4	---	---	---	18.5	26.5	33.6	33.2
	LO	6.5	9.4	6.9	1.0	4.0	6.3	4.7	4.5	4.5
	HO	3.2	2.1	0.8	0.1	0	2.6	2.0	1.6	2.2
Lipidosis[a]	PO		+	±	±	0	0	0	0	0
	RO		+ + +				+ + +	+ + +	+ +	+
	LO		+ +	0	±	+	+	±	0	0
	HO		+ + +	+	0	±	+	±	±	0

[a]0 = No fat droplets; ± = trace; +, + +, and + + + = increasing infiltration of fat droplets. + + Hearts were macroscopically slightly discolored, while + + + hearts were yellow-white.

5830A in the following conditions: column 6% EGA on 110-120 mesh Anakrom ABS, length 1.8 m, ID 2 mm, temperature programmed 130-190 C injector temperature 225 C, oven temperature max. 200 C.

Histological Analysis

The heart apex was quickly frozen with solid CO_2, and freeze-sectioned at -18 C. The sections (10 μm) were stained for lipid with Oil Red O (11). Three sections from the apex, each at a different level, were studied and the lipid accumulation estimated on a five-graded scale.

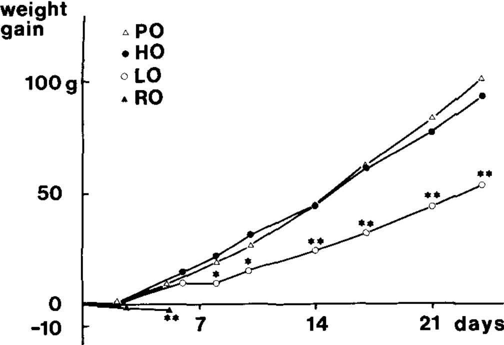

FIG. 1. Weight gain in groups of male rats during cold stress at 4 C fed diets containing different amounts of 22:1. The group mean body weight was the same for all groups initially. Abscissa: time in days from start of experiment. Ordinate: mean weight gain in grammes. * $p<0.05$, ** $p<0.005$ compared to the PO group.

RESULTS

Experiment I: Effects of the Various Diets at 4 C

The mortality during Expt. I shown in Table III. The highest mortality (100%) and the earliest deaths (all within 5 days) occurred in the RO group. The mortality in the LO and HO groups was similar to the PO group. Dissection of the RO animals revealed a yellow-white discoloration of the heart caused by the high accumulation of lipid (see also Table IV).

After 3 weeks, all surviving animals showed some signs of cold injury. The LO group was the worst affected, with scars on ears and extremities, while the HO group was the least affected. Some animals in all groups had self-inflicted wounds on their tails (see Table III).

Body growth curves are shown in Figure 1. The RO group lost weight up to day 5, when all animals had died. Their food consumption during days 4 and 5 was lower than that of the PO group (11.7 ± 1.8 g/day as compared to 13.9 ±1.8 g/day). The LO group also showed a significantly lower weight gain ($p<0.05$) than the PO group from day 8 onwards, while the growth of the HO group was similar to that of the PO group. Between days 18 and 24, the food consumption in the LO group was smaller than that in the PO group (155.0 ± 3.7 g as compared to 166.5 ± 1.6 g).

The amount of 18:1 and 22:1 in cardiac triglycerides at different times during the experiment is shown in Table IV. In the triglyceride fraction, the amount of 18:1 increased during the experiment in all groups. The 22:1 was quickly accumulated up to day 3, notably

among the RO animals, and then fell during the experiment in the other groups. Lipid accumulation, as revealed by histological analysis (Table IV) was largest at day 3 in all groups and then decreased, except in the RO group, where the accumulation was still maximal at day 5, when the last of the animals in this group died. A slight lipidosis could be seen at day 24 in the LO and HO groups.

Experiment II: Effects of the Various Diets at 23 C

In Expt. II, the same parameters as above were studied. This experiment was run for 42 days. No mortality was found in any of the groups, and there were no significant differences in growth or food consumption between the groups. The weight gain of all groups after 24 days was also similar to that of the HO and PO groups during cold stress. The fatty acid composition of cardiac lecithin and triglycerides showed a similar pattern as in the cold stress experiment, i.e., little change in the lecithin fraction and rapid changes in the triglyceride fraction. The changes in the lecithin fraction were correlated to the diet as far as 22:1 is concerned and to a minor extent also for 18:2. The triglyceride fraction throughout the experiment reflected the composition of the various diets (Table IV), except with regard to 18:1 in the HO group.

The most pronounced lipid accumulation (Table IV) was found in the RO group after 3 days. A gradual decline was seen after day 7. The LO and HO animals showed only a weak accumulation after 5 days, while in the PO animals there was no accumulation of lipids in the heart.

DISCUSSION

The most striking difference between the two experiments described above is the high mortality in the RO group during cold stress and the complete survival at room temperature on the same diet. The dose of erucic acid in the RO group was about 8%, expressed as a percentage of the diet. Two recent Canadian studies performed in a similar manner are of interest in this context. One of them found 80% mortality of male weanling rats fed erucic acid during cold stress (5), while the other group found no mortality in similar conditions (6). In the study by Hulan et al. (6), the dose of erucic acid was about 5%, while the other Canadian group, Beare-Rogers and Nera (5), gave 6%. In the study by Beare-Rogers and Nera, there were some deaths in all groups, e.g., in the "control" group which received a mixture of lard and corn oil, 3 animals of 15 had died after 3 weeks. They also found that 6 animals of 15 died in the low erucic acid rapeseed oil group. These results point to a similar sensitivity toward cold stress in the strains of rats used by them and by us. Hulan et al., on the other hand, reported that, of 20 animals in the rapeseed oil group, only 3 died of self-mutilation while there were no deaths in the control group. It is, therefore, possible that Hulan et al. used a more resistant strain of rats. Another possible interpretation of the different results might be that the toxic effects of erucic acid upon the myocardium during cold stress only become fatal at levels above 5% of the diet.

Other adverse effects of erucic acid reported by others (2,3) include growth inhibition and lipid accumulation in the myocardium. The growth inhibition was in the present experiments seen at 4 C in the two rapeseed oil groups. The lipid accumulation in the different groups seemed to be related to the 22:1 content of the diet. In spite of an intense and prolonged lipid accumulation in the hearts of the RO animals at room temperature, however, none of the animals died, nor did they show any outward signs of stress or injury. Thus, a combination of cold stress and high erucic acid diet seems to be necessary for fatal effects to appear. Although the cold stress caused the death of some animals in the HO and PO groups, the weight gain of the survivors was similar to that of the same groups at room temperature. Cold stress in itself, therefore, was not sufficient to cause growth inhibition in this experiment.

The fatty acid composition of the cardiac triglycerides was largely related to the diet after 3 days with a few expections. So, for example, did the 18:1 content increase with time in all groups at 4 C as well as at 23 C. The reason for this is not clear. The levels of 22:1, on the other hand, reflected the levels in the diet. An interesting observation was the constant increase with time of 22:1 in the RO group (23 C), while at the same time the lipid accumulation in the heart decreased. It has been proposed that erucic acid inhibits the oxidation of other fatty acids, while at the same time the oxidation of erucic acid itself increases with time of exposure, indicating induction of the necessary enzymes (12). The relatively low level of 22:1 in the RO group at the beginning of the experiment indicates that the lipid accumulation at this time to a large extent is due to other fatty acids, absorbed, but not metabolized. At 4 C, on the other hand, the level of 22:1 in heart triglycerides was close to that of the diet after 3 days.

The marine oil (HO) used in our experiments

contained various 22:1 isomers though at a low level (3.2% of total fatty acids). The mortality found during cold stress in the group fed this oil, might be due to the 22:1 acids in this diet. Other reports have shown that marine oils with high levels (15 to 20% of total fatty acids) of 22:1 acids may induce the same symptoms as rapeseed oil with high levels of erucic acid, i.e., cardiac lipidosis (13), pathological changes in the myocardium (14), and increased mortality during cold stress (5). A careful evaluation of the effects of marine oils should, therefore, be carried out before recommending their use as alternatives to rapeseed oil.

ACKNOWLEDGMENT

Our sincere thanks are due to Prof. L. Albanus and Dr. A. Bruce at the National Food Administration for many helpful suggestions and constructive criticisms. Thanks are also due to Mr. L–B. Croon for help with the gas chromatography, and to Mrs. I–L. Dahlén for the histological work.

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Liver and Thymus Lipid Composition in AKR Mice with and without Lymphomas

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ABSTRACT

Lipid composition of liver and thymus in controls, early stage lymphoma, and advanced stage lymphoma-bearing AKR mice was studied. There was a significant decrease in the liver total lipid content in mice with advanced lymphoma, whereas in the early stages, no quantitative change was seen. In livers of mice with advanced stage lymphoma, there was a significant decrease in the nonpolar fraction. The decrease was in triglyceride, whereas the cholesterol fractions were relatively increased though highly variable. There was an increase in the polar lipid/nonpolar lipid ratio in the advanced lymphoma livers and a very large increase in the polar lipid/triglyceride ratio, indicating that the decrease in total lipid in these livers was largely in the triglyceride fraction. Similar changes were seen in the thymus, in which the lipid composition reflected the transformation from normal to malignant cells.

INTRODUCTION

It has been shown in experimental animals (1-5) and in human patients (6,7) that the presence of a growing tumor influences the lipid metabolism of tissues, such as liver and adipose tissue, even though remote from the site of the tumor. Much more profound changes will obviously take place as the tissue is invaded by malignant cells until ultimately the metabolism measured will be that of the invading cells (3,5,8,9). With the initiation of the malignant transformation, however, it may be possible to measure metabolic alterations in the host tissues before their involvement with tumor.

The model chosen in the present experiments involves a study of liver and thymus lipid composition occurring in AKR mice before and after developing lymphoma, with the expectation that metabolism would be reflected in composition. In these animals, lymphomatosis is initiated in the thymus and then disseminates to other tissues, including the liver. It was considered possible that alterations might be seen prior to invasion and that ultimately the metabolism of the lymphoma cells would express itself. In any event, the changes in composition must serve as a background for the metabolic changes investigated by different techniques.

The study consists of measurement of lipid composition of thymus and liver in controls, in early stage lymphoma-bearing mice, and in cases of advanced lymphoma.

MATERIALS AND METHODS

Chloroform, methanol, petroleum ether, and

TABLE I

Distribution of Liver Total and Nonpolar Lipids of Lymphoma-bearing and Control AKR Mice

Condition of mice	Lipid fractions (mean ± SD): Total lipid (mg/g liver wet wt)		Nonpolar lipid (mg/g liver wet wt)		
	Polar	Nonpolar	Cholesteryl ester	Triglyceride	Cholesterol
Control (10)[a]	18.8 ± 2.9	8.2 ± 2.8	0.6 ± 0.3	4.6 ± 1.1	1.6 ± 0.2
Early stage lymphoma (8)[a]	20.9 ± 2.3	14.3 ± 5.1	1.8 ± 0.6	9.3 ± 3.1	2.2 ± 0.1
Advanced stage lymphoma (9)[a]	14.2 ± 5.5[c]	4.0 ± 1.2[c]	0.9 ± 0.7	1.2 ± 0.7[b]	2.4 ± 1.1

[a]Numbers of mice in parentheses.
[b]Significantly different from controls at the 0.01 level.
[c]Significantly different from controls at the 0.02 level (Student's t-test).

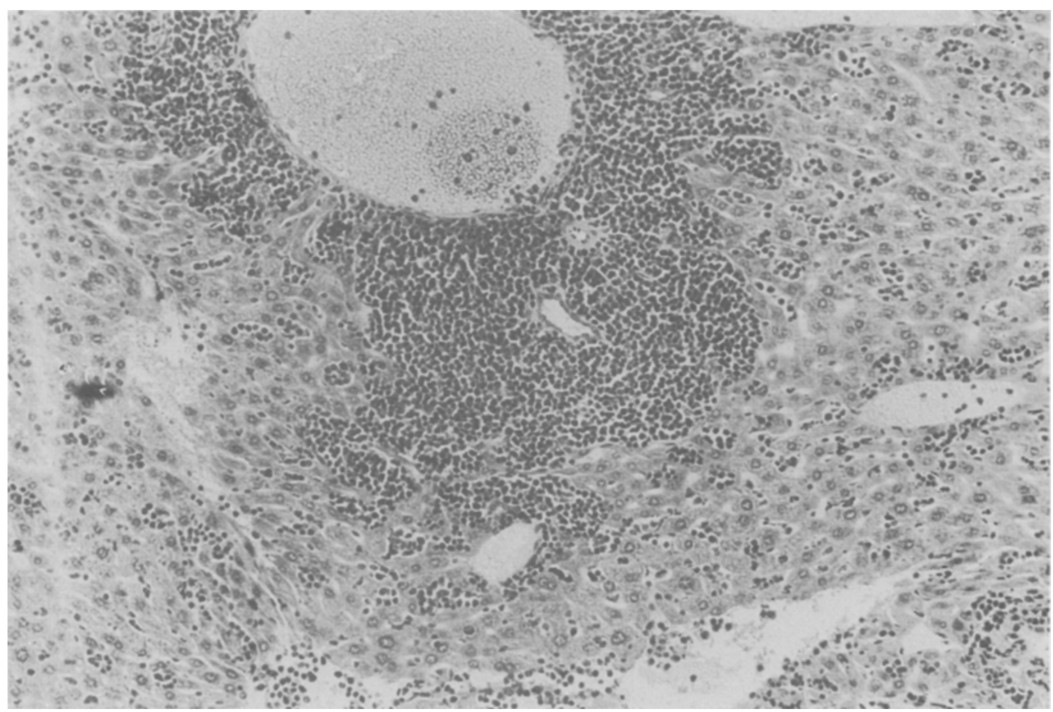

FIG. 1. Hematoxylin and eosin staining. X80. Liver of advanced lymphoma-bearing mice. Extensive invasion of lymphoma cells can be seen around the blood vessels and in parenchyma.

pentane were reagent grade and were distilled before use. Ethyl ether was used directly from a freshly opened can of reagent grade ether. Other reagents were also reagent grade.

Treatment of Animals

The experimental animals were both male and female AKR mice. Nearly all the animals of this inbred strain die of thymic lymphoma between the ages of 6 and 14 months with a peak incidence at 9-10 months of age (10). If injected with Gross Murine Leukemia Virus at 3 days, the onset of the disease is accelerated and peak incidence occurs at 2.7 months (11). Animals were housed in groups of 6-7 and fed laboratory mouse chow ad libitum.

Ten controls were sacrificed at 10-16 weeks, prior to the onset of visible lymphoma. Early stage lymphoma-bearing mice were sacrificed at the first signs of lymphoma and consisted of two groups of four each, sacrificed at 7 weeks (0.1 ml virus injected intraperitoneally) and at 38-44 weeks (not injected). The groups of advanced lymphoma-bearing animals were sacrificed when it became obvious that they were nearly terminal. These groups consisted of mice of 11-13 weeks (virus accelerated) and mice of 26-32 weeks (not injected). Immediately after sacrifice, thymus and liver tissues were collected and frozen at -80 C until extracted.

Extraction and Analysis of Lipids

Tissue samples, after weighing, were extracted by the method of Bligh and Dyer (12). Total lipid was separated into nonpolar and polar fractions by elution from small silicic acid columns with chloroform and methanol, respectively. The nonpolar fractions were separated into cholesteryl ester, triglyceride, free fatty acid, and cholesterol by thin layer chromatography (TLC) on Silica Gel 60 (Merck, Darmstadt, West Germany) by development with petroleum ether-diethyl ether-acetic acid (80:20:1). All techniques were checked with standard mixtures and were found to be accurate and reproducible within 3%.

The polar fractions were also separated into their component phospholipids by TLC, but since no changes were seen in the composition of these fractions, the results are not reported here. Spots were visualized and quantitated by spraying with cupric acetate-phosphoric acid reagent (13), charring at 140 C for 1 hr, and measuring on a Kontes Densitometer.

RESULTS

Histological Findings in Thymus and Liver

Hematoxylin and eosin sections of thymus

TABLE II

Distribution of Thymus Total and Nonpolar Lipids of Lymphoma-bearing and Control AKR Mice

Condition of mice	Lipid fractions (means ± SD)				
	Total lipid (mg/g thymus wet wt)		Nonpolar lipid (mg/g thymus wet wt)		
	Polar	Nonpolar	Cholesteryl ester	Triglyceride	Cholesterol
Control	17.8 ± 4.1	27.1 ± 14.7	N.S.	23.2 ± 14.7	3.9 ± 1.9
Early stage lymphoma	14.3 ± 6.5	29.2 ± 10.3	1.8 ± 0.5	24.3 ± 9.9	3.1 ± 0.7
Advanced stage lymphoma	9.4 ± 2.8[a]	5.9 ± 4.7[a]	2.2 ± 1.6[b]	1.8 ± 2.3[a]	1.9 ± 0.9

[a]Significantly different from controls at the 0.01 level.
[b]Significantly different from controls at the 0.02 level (Student's t-test). N.S. = not seen.

and liver were examined in controls, early stage lymphoma, and advanced stage lymphoma. Examination of sections from the early stage mice confirmed the gross observation of a slightly enlarged thymus in that some lymphoma cells were seen, whereas the livers appeared normal. In the advanced lymphoma cases, however, thymus cells were replaced by lymphoma cells and in the liver, extensive invasion by lymphoma cells was seen particularly around the blood vessels (Fig. 1). In these mice, it was apparent that adipose tissue was severely depleted, a condition usually seen in animals with advanced malignancy (2).

Lipid Analysis of Livers

No differences in lipid composition of livers due to differences in age or sex could be seen nor did the data from the tissues of virus-treated mice differ from those of nonvirus-treated ones. The total lipid content (in percent of wet weight) of livers extracted as described above was 3.4 ± 0.51 for controls, 4.0 ± 0.76 for early stage lymphoma, and 1.9 ± 0.6 for advanced stage lymphoma. It is evident that there was a significant decrease ($t = 5.46$, $P < 0.01$) in liver lipid in advanced lymphoma.

The distribution of total lipid and nonpolar lipid among the different fractions is shown in Table I. It can be seen that the lipid compositions of these fractions in liver control and early stage lymphoma mice were not significantly different, whereas in livers of advanced stage lymphoma-bearing mice there was a significant decrease in the triglyceride.

Similar data for the thymus are shown in Table II. For the thymus, total lipid as percent of wet weight of tissue was 5.1 ± 2.36 for controls, 7.0 ± 5.69 for early stage lymphoma, and 1.3 ± 0.76 for advanced stage lymphoma.

In the thymus, as in the liver, the distribution of total lipid into polar and nonpolar fractions was not much affected by early stage lymphoma, whereas in advanced stage lymphoma, there was a profound decrease in total lipid, which appears to be mainly the result of a decrease in the triglyceride fraction. Cholesteryl ester is of special interest in that it increased in stages with advancing lymphoma, first apparently at the expense of free cholesterol and then possibly at the expense of triglyceride.

DISCUSSION

The original aims of this research were first, to reveal changes in liver lipid composition brought about by a growing tumor, in this case a lymphoma, and second, to provide a background for metabolic studies.

It was thought that the early stages of lymphoma growth might bring about similar, though not so extensive, changes as were apparent in the advanced stages. However, this latter aim was not realized for two reasons. First, the direction of the slight changes seen in the early stage liver and thymus lipids was in the direction opposite to that in the later stages. It is possible that these changes could reflect preliminary alteration of metabolism as reported by Stein et al. (2) who observed a sharp fall in triglycerides in livers of C57 black mice on implantation of glioma followed by a transient increase and then a return to low levels. It is possible that the early stage lymphomas produced changes in the invaded tissues similar to those reported by Stein et al. It is also possible that in the mice with advanced lymphomas, the thymus and liver cells were conditioned by some factor from the tumor cells to furnish fatty acids, as triglycerides, as substrates and energy sources for cell membrane synthesis by the proliferating lymphoma cells. However, in the advanced stages, the thymus cells were almost totally replaced by lymphoma cells, and in the livers,

extensive replacement took place. Thus, in this stage, the lymphoma cells probably contributed most of the lipid composition data in the case of the thymus and a good part of it in the liver.

In addition to furnishing data on the lipid composition of an interesting tumor, these experiments raise several important questions for consideration in future studies. Can it be shown in this case, as in others (1-7), that triglyceride is indeed the source of fatty acids for the malignant cells? What is the meaning of the progressive increase in cholesteryl esters and what are their functions in the lymphoma cells? Finally, what enzymes and control factors are involved and could they be influenced with consequent alteration of tumor growth?

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Identification of Vasopressor Phospholipid in Crude Soybean Lecithin

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ABSTRACT

The vasopressor phospholipid in crude soybean lecithin was isolated by column chromatography on Sephadex LH-20. It represented 0.1% of crude soybean lecithin. The isolated phospholipid was identified to be lysophosphatidic acid by gas chromatography-mass spectrometry analysis of TMS-deacylated product and acetolysis product. Nuclear magnetic resonance analysis favored the 1-monoacyl isomer over the 2-isomer. By enzymic determination with L-3-glycerophosphate dehydrogenase, the isolated phospholipid was identified as 1-monoacyl-L-3-glycerophosphate. Gas chromatographic examination revealed that it was composed of a large percentage of unsaturated fatty acids, especially linoleic acid. The activity of isolated lysophosphatidic acid was slightly less than that of synthetic 1-linoleoyl-L-3-glycerophosphate.

INTRODUCTION

In our previous paper (1), we reported that soybean lecithin was separated by silicic acid column chromatography into two active fractions containing the principle with a potent hypertensive effect. The more polar fraction containing about two-thirds of total activity was further purified by fractionation over a Sephadex LH-20 column. The partition and chromatographic behaviors suggested that the purified pressor substance was an acidic phospholipid and more polar than naturally occurring vasoactive fatty acid analogs, such as slow reacting substance c(2), arachidonic acid (3), and prostaglandins (4-6).

The present investigation was undertaken to study the structure of this pharmacologically interesting phospholipid.

EXPERIMENTAL PROCEDURES

Crude soybean lecithin was treated with cold acetone, and the acetone-insoluble materials were separated into two active fractions [eluted with a mixture of chloroform-methanol (9:1, v/v) and (4:6, v/v), respectively] by column chromatography over silicic acid. The more polar fraction was further purified by chromatography eluted with a chloroform-methanol mixture (1:1, v/v) as previously described (1), and rechromatography eluted with chloroform-methanol-water (60:35:8, v/v/v) over Sephadex LH-20.

Deacylation and silylation of the isolated phospholipid were carried out by the procedure reported by Duncan et al. (7). The deacylated product was converted to the free acid form with Dowex 50W (H-form) and analyzed on a cellulose plate (Avicel SF) with the use of different developing solvent systems. An aliquot of deacylated phospholipid was analyzed with a Shimadzu LKB-9000 GC-MS. The column used was a glass column (1 m x 3 mm ID) packed with 1% OV-17 on Neopack AS (60-80 mesh). The column temperature was 100-250 C at the rate of 6 C/min, and the flow rate of carrier gas

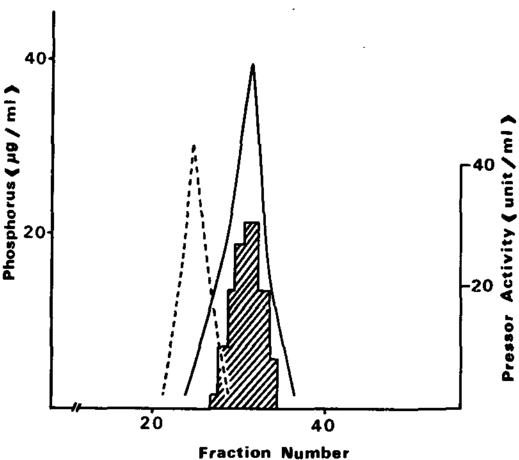

FIG. 1. Isolation of the vasopressor phospholipid by Sephadex LH-20 column chromatography. The partially-purified active fraction was chromatographed on a column of Sephadex LH-20 (100 g). Solvent system: chloroform-methanol-water (60:35:8, v/v/v). Fractions of 10 ml were collected. ----- = the inactive phospholipid, —— = the vasopressor phospholipid, ////// = pressor activity (units). One unit of activity corresponds to rise in blood pressure produced by 0.4 µg/kg of noradrenalin.

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TABLE I

TLC of Vasopressor Phospholipid on Silica Gel Plate

Phospholipid[a]	R_f in solvent system[b] Acidic	Neutral	Basic
Vasopressor phospholipid[c]	0.71	0.33	0.25
Lysophosphatidic acid	0.71	0.34	0.26
Phosphatidic acid	0.88	0.64	0.50
Phosphatidylinositol	0.78	0.53	0.56
Cardiolipin	0.93	0.75	0.92
Phosphatidylcholine	0.79	0.54	0.83
Lysophosphatidylcholine	0.37	0.17	0.38
Sphingomyelin	0.70	0.34	0.71

[a]Reference phospholipids were purchased from Sigma except for lysophosphatidic acid (Serdary). Phosphatidic acid (from egg lecithin, sodium salt), cardiolipin (from bovine heart, sodium salt), phosphatidylinositol (from soybean, sodium salts) lysophosphatidylcholine (from egg yolk), sphingomyelin (from bovine brain), lysophosphatidic acid (synthetic, palmitoyl-), phosphatidylcholine (synthetic, dipalmitoyl-).

[b]Solvent systems: Acidic = chloroform-methanol-acetic acid-water (60:35:1:8); neutral = chloroform-methanol-water (60:30:5); basic = chloroform-methanol-10% ammonia (60:35:8).

[c]Fraction number 30-33 (Fig. 1).

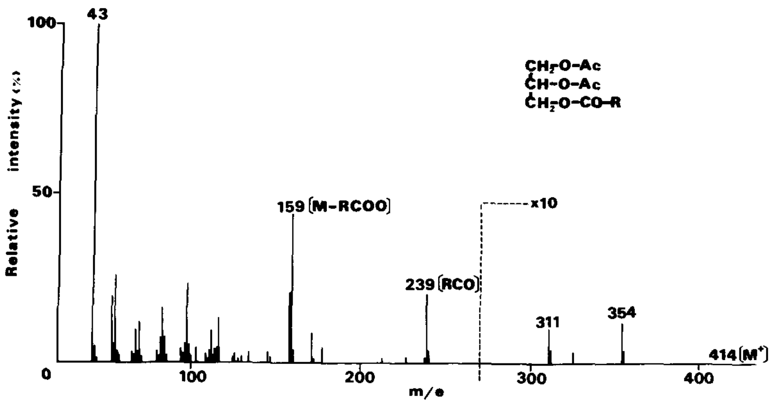

FIG. 2. The 70 eV mass spectrum of the acetolysis product from the isolated vasopressor phospholipid. Ac: acetyl-, R: $C_{15}H_{31}$.

(He) was 30 ml/min. Spectra were obtained at 70 eV or 20 eV ionizing potential, a trap current of 60 μA, accelerating voltage of 3.5 kV, ion source 290 C, and flash heater, 280 C.

Isolated phospholipid was acetolyzed in a mixture of acetic acid-acetic anhydride (3:2, v/v) at 130 C for 3 hr (8). The reaction mixture was partitioned with a mixture of chloroform-methanol-water (8:4:5, v/v/v). After centrifugation, the separated lower phase was evaporated to dryness and analyzed by a Shimadzu LKB-9000 GC-MS equipped with a glass column packed with 1% OV-17. The condition for GC-MS was the same as in the analysis of TMS-deacylated phospholipid.

Fatty acid methyl esters from the phospholipid were prepared by methanolysis (9) in acid-methanol, and then separated and quantified by a Shimadzu gas chromatograph GC-5A equipped with a column (2.5 mm x 3 m) packed with 15% DEGS on 60-80 mesh Shimalite W. The column oven temperature was 189 C, and the nitrogen flow rate was 60 ml/min.

Nuclear magnetic resonance (NMR) spectra of the isolated phospholipid (ca. 7.5 mg) were obtained on JNM-PS-100 (Nihon Denshi, Tokyo) with 5% solution in deuteriochloroform. The ambient temperature was 23 C. Chemical shifts are expressed in parts per million relative to tetramethylsilane at 10 ppm (τ scale).

Deacylated phospholipid was incubated with L-3-glycerophosphate dehydrogenase (EC 1.1.1.8) and NAD as reported by Long et al. (10), and the increase in extinction at 340 nm was determined. Authentic L-3-glycerophos-

TABLE II

TLC of Deacylated Vasopressor Phospholipid in Crude Soybean Lecithin

Compound[a]	R_f in solvent system[b]		
	A	B	C
Deacylated vasopressor phospholipid	0.44	0.65	0.64
Glycerophosphate	0.45	0.65	0.64
Glycerophosphorylinositol	0.20	0.29	0.34
Bis-(Glycerophosphoryl)-glycerol	0.26	0.44	0.46

[a]These compounds were prepared by mild alkali hydrolysis described in Experimental Procedures from the vasopressor phospholipid, phosphatidic acid (Sigma, from egg lecithin), phosphatidylinositol (Sigma, from soybean), and cardiolipin (Sigma, from bovine heart).

[b]Solvent systems: A = phenol saturated with water-acetic acid-ethanol (50:5:6, v/v/v); B = n-butanol-98% formic acid-water (80:13:7, v/v/v); C = t-butanol-water (62:38, v/v) + trichloroacetic acid, 10% (w/v).

phate was used as a standard substance.

Wistar rats weighing 200-250 g were anesthetized with the intraperitoneal administration of 1.8 g/kg of urethane. The assay procedure used was as described previously (1). Mean arterial pressure was measured with a mercury manometer connected to a catheter placed in the left carotid. Materials to be examined were dissolved in 50 μl of Ringer solution and injected from a microsyringe into the catheter placed in the jugular vein.

Phosphorus determination was performed according to the method of Chalvardjian and Rudnicki (11). Glycerol content was measured by the procedure described by Renkonen (12).

RESULTS AND DISCUSSION

Isolation of the Vasopressor Phospholipid

The purified active fraction in the previous study (1) contained two phospholipids. Satisfactory separation of the vasopressor phospholipid from another inactive one was achieved with use of chloroform-methanol-water (60:35:8, v/v/v) in Sephadex LH-20 column chromatography (Fig. 1).

Isolated phospholipid was analyzed on a silica gel plate in three different solvent systems. Its R_f values were different from common authentic phospholipids as shown in Table I. The relative migration of the vasoactive phospholipid to reference phospholipids was less in basic solvent system than in neutral and acidic ones. Pressor activity was recovered only from the zones containing the isolated phospholipid on the plates developed with above-mentioned solvent systems. The activity was not reduced by the treatment with triphenylphosphine (13), indicating that the vasopressor activity was not due to the possible minor contamination of peroxides.

The phosphorus/glycerol/fatty acid molar ratio of the active phospholipid (1:1.02:0.97) suggested that it was a lysophospholipid. This possibility was further supported by the following experiment. If the active phospholipid were a lyso-type, diacetylmonoglyceride could be produced by acetolysis with a mixture of acetic acid-acetic anhydride. Figure 2 shows the mass spectra of a representative peak in the gas chromatogram of acetolysis product of the phospholipid. On the basis of intense signals, which correspond to $[M\text{-}RCOO]^+$ and $[RCO]^+$ ions, the peak was identified as monopalmitoyldiacetyl-glycerol. Another more predominant peak appeared to be a mixture of diacetylmonoglyceride with C_{18}-fatty acids, mainly linoleic acid.

The isolated phospholipid was deacylated by a mild alkali hydrolysis, and the water-soluble product was analyzed on a cellulose plate (Table II). Its R_f values were the same as those of reference L-3-glycerophosphate in three different solvent systems. It was confirmed by the mass spectrum of the major peak in the gas chromatogram of TMS-deacylated phospholipid (Fig. 3). The spectrum was identical with that of authentic TMS-glycerophosphate. In the gas chromatogram, there was only one minor peak, and it was identified as trimethylsilyl-phosphate, which was possibly formed by decomposition of glycerophosphate deacylated from vasoactive phospholipid during mild alkali hydrolysis and subsequent silylation (7). These results strongly indicated that the vasoactive phospholipid may be lysophosphatidic acid, which was confirmed by the fair agreement of its R_f value with an authentic lysophosphatidic acid (Table I).

Fatty acid composition was as follows: palmitic acid 24.7%, stearic acid 7.5%, oleic acid 8.7%, linoleic acid 50.0%, and linolenic

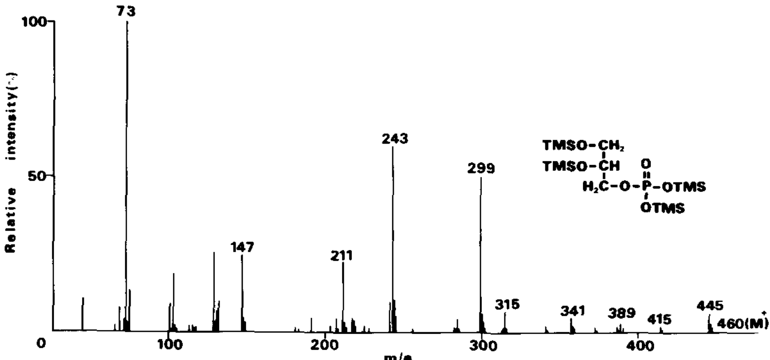

FIG. 3. The 70 eV mass spectrum of the TMS-deacylated product from the isolated vasopressor phospholipid. TMS = trimethylsilyl.

TABLE III

NMR Analysis of the Isolated Vasopressor Phospholipid from Crude Soybean Lecithin

Group	Chemical shift (τ) (ppm)	Number of protons	
		Calculated[a]	Found
CH=*CH*	4.71	2.63	2.50
CH$_2$O-COR *CH*-OH *CH*$_2$-O-P	5.88	5.00	5.10
=C-*CH*$_2$-C=	7.33	1.28	1.30
CH$_2$CO	7.66	2.02	2.09
CH$_2$C=	7.91	2.71	2.69
CH$_2$	8.76	19.32	19.26
CH$_3$	9.14	3.00	3.00[b]

[a]Values are calculated as 1-acyl-lysophosphatidic acid on the basis of its fatty acid composition.

[b]Three terminal methylene protons of fatty acid moiety taken as integral calibration standard.

acid 7.0%. These data agree quantitatively with the analysis for diacetylmonoglyceride derived from the vasoactive phospholipid.

The 100-MHz NMR spectra of the isolated phospholipid showed the expected signals for acyl group protons and glycerol-derived methylene and methine protons, as shown in Table III. Considering the data for fatty acid composition, assignment of proton signals and their integration favored the structure of 1-acylglycerophosphate rather than 2-acyl isomer. If the isolated phospholipid predominantly existed as a 2-isomer, the signal for proton on the secondary carbon of esterified glycerol would be expected at 4.8 ppm. There was an appreciable signal in this region in the NMR spectra, which would be ascribed to the resonance of ethylene protons, and it may be overlap of the proton mentioned above. It could be excluded by the calculated integration as 1-acyl isomer which agreed very closely with the found one. These results suggest that this preparation would be mainly 1-acyl-glycerophosphate.

For the evaluation of the complete structure of the vasoactive phospholipid, it is necessary to determine the location of the phosphate group and the configuration of the glycerol moiety. Deacylated product from the phospholipid was determined to be L-3-glycerophosphate by the experiment with L-3-glycerophosphate dehydrogenase.

Pharmacological Property of the Vasopressor Phospholipid

The isolated lysophosphatidic acid (lyso-PA) represented at most 0.1% of crude soybean lecithin. The dose-dependent transient rise of the systemic blood pressure was produced by its intravenous injection. The onset of the responses elicited by lyso-PA was observed within 5 sec after injection. Blood pressure reached the

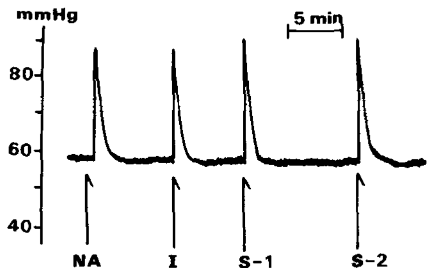

FIG. 4. Vasopressor responses of rat to the isolated and synthetic lysophosphatidic acid. I: isolated lysophosphatidic acid from soybean lecithin, 130 μg/kg. S_1: synthetic L-1-palmitoyl-lysophosphatidic acid, 620 μg/kg. S_2: synthetic L-1-linoleoyl-lysophosphatidic acid, 80 μg/kg. NA: noradrenalin, 0.4 μg/kg. Arrows indicate the times of injections.

maximum in 20-30 sec, followed by quick return to the control level within 1 min. The pattern of responses resembled that induced by noradrenalin (Fig. 4). The threshold dose (10-20 μg/kg) corresponded to that of prostaglandin $F_{2\alpha}$.

The short lasting vascular responses to the intravenous injection of relatively large amount of arachidonic acid (3) and slow reacting substance c (2) appeared to be induced by their unstable metabolites, intermediates in prostaglandin synthesis. The above-mentioned similar possibility for the hypertensive effect of lyso-PA is unlikely because of the following reasons: (a) The effective dose of lyso-PA was considerably lower than that of arachidonic acid or slow reacting substance c; (b) fatty acids derived from the isolated lyso-PA by mild alkali hydrolysis could not affect the blood pressure up to 500 μg/kg doses; and (c) none of phospholipids in crude soybean lecithin, such as lysolecithin and phosphatidic acid, has a vasoactive effect.

Previously, the treatment with a ganglion-blocking agent or α-adrenergic blocking agent and mechanical injury of the central nervous system in a rat could not reduce the rise of blood pressure elicited by lyso-PA (1). It was indicated that the hypertensive effect of lyso-PA appeared, like F-type prostaglandins, by directly acting on certain sites on the cell membrane of vascular smooth muscle before decomposition in the circulatory system.

It is interesting that our phospholipid is a lyso-type. Little has been known for phospholipids with a pharmacological effect except for some lysophospholipids. Although lysolecithin was reported to have a hypotensive effect on different animals (14,15), no detailed data can be seen in the literature. Lysophosphatidylethanolamine (16-18), renin inhibitor, and acetal-phosphatidic acid (19,20), smooth muscle stimulator for gut tissues, were found to provide no such acute effect as lyso-PA on the cardiovascular system in vivo.

Vasoactive effect of the isolated phospholipid was about five times more than that of synthetic palmitoyl-lyso-PA (Serdary), while significantly less than that of synthetic linoleoyl-lyso-PA prepared from corresponding lysolecithin with enzymic hydrolysis (21). The different effectiveness between them would be ascribed to their differences in fatty acid composition. The detailed study on this point is in progress.

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Lipogenesis in Iron-deficient Adult Rats

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ABSTRACT

Iron-deficient (5 ppm Fe) or control (307 ppm Fe) diets were fed ad libitum to female rats for 7 weeks, and then meal-fed for 4 weeks. Body weights, hemoglobin levels, and hematocrits were lower ($p < 0.01$) in deficient group (184 ± 7, 7.1 ± 0.4, 32.7 ± 0.6) than in the control group (220 ± 10, 16.9 ± 0.3, 51.8 ± 0.8) at the end of the 11-week experiment. Animals were killed 1 hr after meal feeding, and liver slices, mesenteric adipose tissue, and segments of mid-jejunum were incubated in vitro with [U-^{14}C] glucose or ^3H_2O. Adipose tissue from deficient rats had incorporation of [U-^{14}C] glucose into triglycerides two to three times greater than control rats ($p < 0.01$). Release of $^{14}CO_2$ from glucose was greater in adipose tisssue of deficient rats than controls ($p < 0.05$). Incorporation of ^3H_2O into triglycerides was also two to three times greater in deficient adipose tissue than in controls ($p < 0.02$). In liver slices, incorporation of glucose in polar lipids was slightly higher in deficient rats than in control rats ($p < 0.05$). No significant differences were found in incorporation of ^3H_2O or [U-^{14}C] glucose into lipids or CO_2 in jejunum. Thus, iron- deficient adult rats have greater lipid synthesis from ^3H_2O and glucose in adipose tissue than rats fed adequate levels of iron.

INTRODUCTION

Reports from this and other laboratories (1-5) have demonstrated that dietary iron restriction in several ages and strains of rats and chicks produces elevated serum lipid levels. In this laboratory, we have been studying the effects of iron restriction on serum lipid metabolism in suckling rats. Maternal iron deficiency during both pregnancy and lactation produces hyperlipidemia in 18-day-old offspring but not in maternal rats fed diets containing 5 ppm iron during pregnancy and lactation (1). Serum from pups whose mothers were deficient in iron during both pregnancy and lactation had significantly greater triglycerides, phospholipids, and total cholesterol levels than serum from pups whose dams were fed 307 ppm iron diet during either pregnancy or lactation.

In a subsequent study (2), we attempted to identify the mechanism involved in producing hyperlipidemia in 18-day-old offspring of iron-deficient rats. Three levels of iron (5, 29, 307 ppm iron) were fed to rats from conception through the 18th day of lactation. Three hypotheses were tested to explain the origins of the increased serum lipid levels observed in the pups in the 5 ppm iron group. Milk lipids were measured to determine if the primary site of interaction between iron deficiency and lipid metabolism was in the synthesis of milk by the dam. No differences were found in triglyceride or cholesterol content of milk produced by iron-deficient and control dams. The second hypothesis was that the clearance of exogenous lipids from the blood was impaired in iron deficiency. No differences were found in the lipid clearance as indicated in vitro post-heparin plasma lipoprotein lipase activity. The third hypothesis was that endogenous lipid synthesis was greater in the iron-deficient pups than in control pups. To test this, liver slices were incubated in vitro with [U-^{14}C] glucose, the lipid extracted, and extent of incorporation of label into triglycerides determined. Triglyceride and CO_2 production from [U-^{14}C] glucose were significantly greater in livers of iron-deficient pups (5 ppm iron) than in livers of control pups (307 ppm iron). Hyperlipidemia in 18-day-old iron-deficient rat pups thus appears to be related to increased endogenous production of triglycerides.

Serum lipid abnormalities associated with iron deficiency in more mature animals have been reported by others. Lewis and Iammarino (3) found that male weanling rats fed an iron-deficient diet for 5 weeks had blood triglyceride levels five times those in animals fed a diet containing 240 ppm iron. An observed decrease in serum and tissue lipoprotein lipase activity in anemic rats suggested that decreased clearing of triglycerides from serum might be the cause of increased triglyceride and chylomicron levels.

Amine and Hegsted (4) fed iron-deficient diets containing 10 ppm iron and a control (250 ppm iron) diet to male and female weanling rats for 5 weeks. All rats fed the deficient diets developed lipemia characterized by increased triglycerides and decreased cholesterol in serum taken following an overnight fast. Similarly, they showed that nonfasting blood of chicks fed casein diets containing 10 ppm iron or 50 ppm iron for 4 weeks had increased levels of plasma proteins, triglycerides, and phospholipids compared to those fed a diet with 250 ppm iron.

In a subsequently published report, Amine,

TABLE I

Composition of Diets

	307 ppm Iron (%)	5 ppm Iron (%)
Casein[a]	22.00	22.00
Sucrose	29.70	29.76
Cornstarch	29.70	29.76
Iron-free salt mix[b]	5.48	5.48
Vitamin Mix[c]	1.00	1.00
Corn oil[d]	10.00	10.00
Cellulose[e]	2.00	2.00

[a]Vitamin-free casein, Teklad, Chagrin Falls, OH.

[b]The levels of minerals used is at least 100% of the NRC recommendations (12). Composition of salt mixture (mg/100 g diet): $CaCO_3$, 1,680.0;$CoCl_2 \cdot H_2O$, 0.1; $CuSO_4 \cdot 5 H_2O$, 9.8; $MgSO_4 \cdot 7 H_2O$, 278.5; $MnSO_4 \cdot H_2O$, 19.1; KI, 0.02; NaCl, 1,000.0; K_2HPO_4, 2,483.6; $ZnCl_2$, 3.0. $FeSO_4 \cdot 7 H_2O$ was added to the supplemented diets in place of sucrose and cornstarch.

[c]Teklad, Chagrin Falls, OH, Cat. No. 40060. Supplies in mg per kg of diet when added at 1% of diet: P-Aminobenzoic acid, 110.1; ascorbic acid, 991.2; Biotin 0.4; Vitamin B_{12}, 0.03; Calcium pantothenate, 66.1; choline, 1433.7; Folic acid, 2.0; Inositol, 110.1; Menadione, 49.6; Niacin, 99.1; Pyridoxine HCl, 22.0; Riboflavin, 22.0; thiamine HCl, 22.0. Supplies in units per kg of diet when added at 1% of diet: Vitamin A palmitate, 19824; Vitamin D_2, 2203; Vitamin E acetate, 121. Cornstarch dilutent QS.

[d]Mazola corn oil, Best Foods, Englewood Cliffs, NJ.

[e]Alphacel non-nutritive cellulose, Teklad, Chagrin Falls, OH, with iron extracted by the method of Houk et al. (13).

et al. (5) studied the effects of dietary fats on lipogenesis in iron-deficient rats and chicks with elevated serum triglycerides. In vitro incorporation of ^{14}C-glucose into liver lipids was generally lower in the iron-deficient rats than in controls. Incorporation of ^{14}C-glucose into intestinal lipids was generally higher in iron-deficient than in control rats. Their lipid synthesis studies generally found that the iron-deficient rats made more lipid from glucose in the intestine when they were fed a diet high in fat, either saturated or unsaturated, than did controls.

Since iron deficiency anemia is a common nutritional problem, a role of iron in lipid metabolism may assume considerable importance in preventing elevated serum lipid levels generally recognized as risk factors in the development of atherosclerosis. The research reported here investigated the effects of iron nutriture on serum lipids and in vitro lipogenesis in adult female rats subjected to iron deficiency after weaning for 11 weeks.

MATERIALS AND METHODS

Lipogenesis was studied in vitro in liver, adipose, and jejunum tissues collected after rats were on iron-deficient or control diets for 11 weeks. Both glucose and water were used as precursors compounds for lipid synthesis. The utilization of glucose and incorporation into lipids may be dependent on the availability of other metabolities in the tissues. Use of water to estimate lipid synthesis avoids possible differences in endogenous pools of precursors for lipogenesis which may vary with the experimental treatment and are difficult to measure. To ensure that all rats were in a fed-state when lipogenesis was maximal, a meal-feeding regimen was implemented 4 weeks before the end of the enperiment. On the day of the in vitro experiment rats were fed, and 1 hr after the meal they were killed.

Female, albino, Sprague-Dawley rats (Charles River, Wilmington, MA) weighing an average of 68 g at the start of the experiment, were housed in individual stainless-steel cages. Eight rats were randomly assigned to each of the experimental diets and fed the diet and glass-distilled water ad libitum for 7 weeks. Table I shows the composition of the diets which contained 5 and 307 ppm iron. During the last 4 weeks of the experiment, the rats were meal-fed during 1200-1300 hours daily and glass-distilled water was available ad libitum. After 11 weeks on the diets, rats were fed and then fasted for 1 hr. Tail blood samples were taken for hemoglobin (6) and hematocrit determination (6) and then the rats were anesthetized with chloroform. Blood was collected by cardiac puncture, and serum was frozen for analysis of lipids.

Liver, mesenteric adipose tissue, and jejunum were quickly excised and prepared for the in vitro incubations. Liver slices, ca. 75 mg, were prepared with a Stadie-Riggs microtome. One hundred mg pieces of adipose tissue and

TABLE II

Body Weights, Hemoglobin, and Hematocrit Levels in Rats after 11 Weeks on Experimental Diets

Treatment	Body weight (g)	Hemoglobin (g/dl)	Hematocrit (%)
5 ppm Iron	184 ± 7[a]	7.1 ± 0.4	32.7 ± 0.6
307 ppm Iron	220 ± 10	16.9 ± 0.3	51.8 ± 0.8
Significance[b]	$p < 0.01$	$p < 0.001$	$p < 0.001$

[a]Mean ± SE, n = 8 rats per group.
[b]Statistical significance determined by Student's t-test.

TABLE III

Serum Lipid Concentrations (mg/dl)

Treatment	Triglycerides	Cholesterol	Cholesteryl ester	Phospho-lipids
5 ppm Iron	22.4[a]	20.9	146.9	37.3
	±5.8	±5.6	±35.8	±7.0
307 ppm Iron	87.8	23.3	104.4	12.2
	±12.4	±3.5	±18.8	±2.0
Significance[b]	$p < 0.001$	NS	NS	$p < 0.005$

[a]Mean ± SE, n = 8 rats per group.
[b]Statistical significance determined by Student's t-test.

150 mg sections of mid-jejunum were used. These tissues were incubated in 25 ml Erlenmeyer flasks containing 2 ml of Krebs-Ringer bicarbonate buffer (pH 7.4) fortified with 10 mM glucose (Sigma Chemical Co., St. Louis, MO) and 1 ml of 10% albumin (Sigma) and containing either 1 μ Ci [U-^{14}C] glucose (New England Nuclear, Boston, MA), or 2 mCi ^3H_2O (New England Nuclear). Adipose tissue incubation flasks contained 0.1 unit of insulin (Sigma), and jejunum was incubated with 1000 units of penicillin (Sigma). Tissues were oxygenated (in 95% O_2, 5% CO_2) for 15 sec and then incubated at 37 C in a shaking water bath at 60 cycles/min. The reactions were terminated and CO_2 liberated by adding 0.2 ml of 0.4 N H_2SO_4 directly into the flask through the serum stopper.

Carbon dioxide was collected during the incubations when [U-^{14}C] glucose was used as substrate. Plastic centerwells (Kontes Glass, Vineland, NJ) containing folded filter paper and 0.2 ml of hyamine hydroxide (Amersham-Searle Co., Arlington Heights, IL) were suspended over the incubation media. One hour after the reactions were terminated, the center wells were rinsed with water and placed in 10 ml of toluene containing 23% ethanol, 0.4% PPO, and 0.01% POPOP for liquid scintillation counting to determine the $^{14}CO_2$ production. Tissues were removed from the incubation media, rinsed in distilled water, and lipids were extracted by the method of Folch (7). The lipid fractions were separated on thin layer chromatography (TLC) plates (Merck Co., precoated Silica Gel 60 plates, 0.5 mm) developed in a solvent system of petroleum ether-ethyl ether-acetic acid (70:30:1, v/v/v). The triglyceride, fatty acid, and polar lipid bands were identified by comparison with triolein, palmitate, and phosphatidylcholine standards (Sigma). The lipid fractions were recovered from the plates and radioactivity determined by liquid scintillation counting *in* toluene *containing* 0.4% PPO and 0.005% POPOP.

Serum samples were analyzed for triglycerides, cholesterol, cholesteryl ester, and phospholipids. Lipids were extracted and separated as described above, and the following colorimetric assays were used to quantitate the lipid fractions: triglyceride concentration was determined by the method of Stern and Shapiro (8); cholesterol and cholesteryl ester were measured by the method of Searcy and Bergquist (9); and phospholipid concentration was determined by measuring phosphorus in the lipids remaining at the origin of the chromatographs using the method of Chen et al. (10). The statistical significance between means of experimental groups was determined by Student's t-test (11).

RESULTS

Body Weight, Hemoglobin, and Hematocrit

The iron-deficient rats in the 5 ppm iron

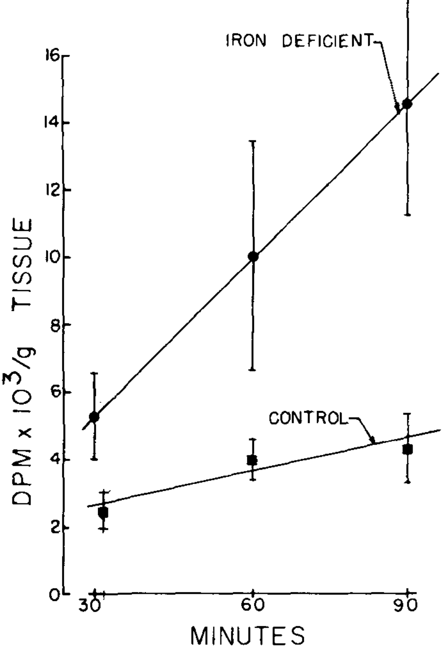

FIG. 1. In vitro incubation of adipose tissue. Incorporation of [U-^{14}C] glucose into ^{14}C-triglycerides. (Values are expressed as DPM/g tissue; mean ± SE; n = 8 rats per group.)

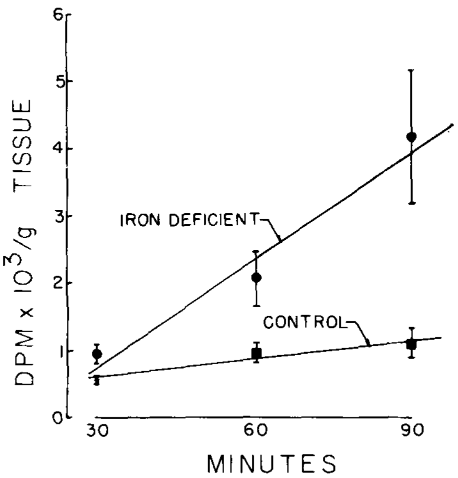

FIG. 2. In vitro incubation of adipose tissue. Incorporation of H_2O into ^{3}H-triglycerides. (Values are expressed as DPM/g tissue; mean ± SE; n = 8 rats per group.)

group had significantly lower body weights, hemoglobin, and hematocrit levels (Table II) than the rats fed adequate iron (307 ppm). No significant differences were found in food intake.

Serum

Significant differences were found in serum triglyceride and phospholipid concentrations (Table III). Rats in the group fed 307 ppm iron had higher serum triglyceride levels, and rats in the 5 ppm iron group had higher serum phospholipid levels. No significant differences were found in serum cholesterol or cholesteryl ester levels.

Adipose Tissue

Increased incorporation of [U-^{14}C] glucose into triglycerides in adipose tissue (Fig. 1) was observed in the iron-deficient rats when compared with control rats. The data, expressed as DPM/g tissue, show that triglyceride production in deficient rats is two to three times greater than in controls ($p < 0.01$). Similarly, triglyceride synthesis from ^3H_2O in adipose tissue (Fig. 2) of iron-deficient rats was two to four times greater than in control rats ($p < 0.02$).

No significant differences between groups were found in the incorporation of ^3H_2O into free fatty acids or polar lipids. Incorporation of [U-^{14}C] glucose into polar lipids was not different between groups. $^{14}CO_2$ production from [U-^{14}C] glucose (Fig. 3) was greater in the iron-deficient rats than in the controls ($p < 0.05$).

Liver Tissue

In liver slices, increased incorporation of [U-^{14}C] glucose into polar lipids was observed in the iron-deficient rats (Fig. 4) ($p < 0.05$). However, no significant differences were found between groups in the ^{14}C-triglyceride or $^{14}CO_2$ production from [U-^{14}C] glucose in liver. Similarly, no significant differences were found between groups in the production of triglycerides, free fatty acids, or polar lipids from ^3H_2O in the liver.

Intestine

No significant differences were found between groups in the production of CO_2, triglycerides, or polar lipids from [U-^{14}C] glucose in jejunum. No significant differences were found between groups in triglyceride, free fatty acid, or polar lipid synthesis from ^3H_2O.

DISCUSSION

Neither experimental group of adult rats had hyperlipidemia as we have reported in 18-day-old offspring of iron-deficient rats (1,2). Serum triglycerides during this experiment with older rats were higher in the control group than in the iron-deficient group. However, the trigly-

ceride concentrations for both control and deficients were within the normal range for rats. Serum phospholipid concentrations in the iron-deficient group were approximately three times those of the control group.

The concentrations of serum lipids reported here for 15-week-old rats are lower than the values we have previously reported in 18-day-old suckling rats (1,2). This may be related to the differences in metabolism of the suckling organism compared with the adult and to the degree of anemia. The 18-day-old pups in the previous papers, suckled by dams fed an iron-deficient diet during gestation and lactation, had hemoglobin and hematocrit levels which were considerably lower than the adult rats in this study.

Triglyceride synthesis from [U-^{14}C] glucose and ^3H_2O was elevated in adipose tissue of iron-deficient rats. Triglyceride production from both precursors was two to three times higher in deficient rats than in controls. The results of the experiments using ^3H_2O as a precursor demonstrate that the increased synthesis of triglyceride in adipose tissue of the iron-deficient rats is not dependent on an exogenous carbon source. Precursors to lipid synthesis must be found in the tissue.

The utilization of glucose by adipose tissue, as indicated by CO_2 production, was also greater in iron-deficient rats. This was not the case in liver where CO_2 production from glucose was similar in control and deficient tissue.

The differences in lipid synthesis in liver tissues were observed in production of polar lipids from [U-^{14}C] glucose. This was higher in the livers of iron-deficient rats than in controls. Possibly, this may be related to the increased levels of phospholipids found in sera of the iron-deficient rats. Again, there are differences in incorporation of [U-^{14}C] glucose into lipid fractions in the adult rats when compared to the results of our experiment on 18-day-old rat pups. Although incorporation of glucose into triglyceride and into CO_2 was greater in the iron-deficient suckling rats, adult rats fed both diets had similar glucose incorporation into triglycerides and CO_2. This may be reflective of a change in metabolism as the animal matures.

Unfortunately, insufficient adipose tissue was available from the suckling rats to do in vitro studies. It is not possible to examine the changes in adipose tissue lipid synthesis with maturation. In adult female rats, the anemia resulting from feeding an iron-deficient diet for 11 weeks is associated with a profound increase in lipogenesis in adipose tissue and to a lesser degree increase in polar lipid synthesis in liver. These differences in in vitro lipid synthesis are

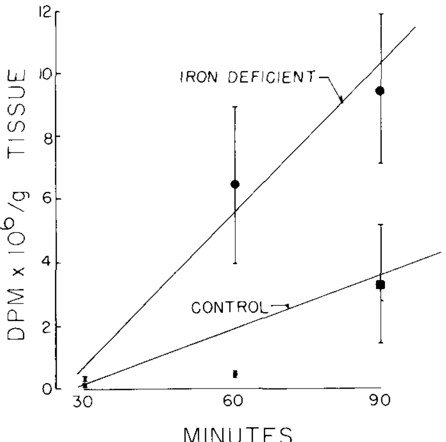

FIG. 3. In vitro incubation of adipose tissue. Incorporation of [U-^{14}] glucose into $^{14}CO_2$. (Values are expressed as DPM/g tissue; mean ± SE; n = 8 rats per group.)

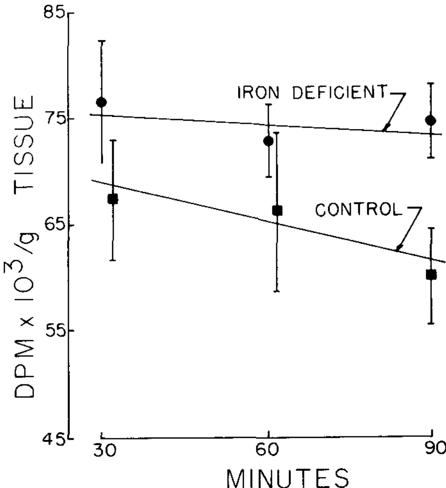

FIG. 4. In vitro incubation of liver slices. Incorporation of [U-^{14}C] glucose into polar lipids. (Values are expressed as DPM/g tissue; mean ± SE; n = 8 rats per group.)

not accompanied by elevated serum lipid levels which were observed in suckling rats. Perhaps the 18-day-old rats represent a greater degree of anemia and iron depletion during which the effects on lipid synthesis increase and normal control mechanisms cannot prevent the appearance of these lipids in the blood. The adult rats in this study were less anemic and perhaps this protected them from the elevation of serum lipids in spite of their increased rate of lipid synthesis in adipose tissue.

The results reported here and those of previous studies (1-5) indicate that iron may play an important role in the regulation of lipid metabolism in experimental animals. Should similar interrelationships between dietary iron and lipid metabolism be found in the human, the biomedical significance of iron in such disease processes as atherosclerosis and obesity will have to be considered.

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Bile Acid Sulfates: II.[1] Synthesis of 3-Monosulfates of Bile Acids and Their Conjugates[2]

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ABSTRACT

Bile acid 3-monosulfates were synthesized by the sulfation of 3-hydroxy formyloxy bile acids with sulfur trioxide-triethylamine in dimethylformamide at 25 C for 0.5 hr. The protecting formyl groups were then hydrolyzed under mild alkaline conditions, and the deformylated products were isolated as p-toluidinium salts. These p-toluidinium salts were converted easily to the corresponding disodium salts by methanolic sodium hydroxide. Disodium salts were then isolated by precipitation from methanol-ether. The corresponding glycine conjugates were similarly synthesized by the sulfation of ethyl esters of 3-hydroxy formyloxy bile acid glycine conjugates. However, the taurine-conjugated bile acid sulfates were obtained by conjugating bile acid 3-monosulfates, either as triethylammonium salt or as disodium salt, with taurine in dimethylformamide in the presence of N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline. These procedures produced the desired bile acid sulfates in high yield (typically above 90%) with minimum manipulation. No time-consuming and capacity-limited chromatographic purification was needed to isolate the pure sulfates. The thin layer chromatographic mobilities, the infrared spectra, and some of the preliminary studies of the properties of these synthesized 3-monosulfates are also discussed.

Sulfation has been established as an important metabolic pathway not only for monohydroxy(1), but also for dihydroxy (2) and trihydroxy (3) bile acids in man. This pathway is especially significant in hepatobiliary diseases. Over the past several years, a number of investigations devoted to the quantitation of these sulfates in biological fluids in various disease states have been reported (2-9). In these investigations, bile acid sulfates were isolated from biological fluids by using a nonionic macroreticular adsorbent such as Amberlite XAD-2 (2,4,7,8), or XAD-7 (9,10) or by using alcoholic sodium hydroxide (3,6,11). The isolated sulfates were deconjugated using cholic acid hydrolase or mild alkaline hydrolysis, followed by solvolysis (5,12), or these sulfates were solvolyzed first and then deconjugated (2,6). The free bile acids thus formed were derivatized and quantitated by gas chromatography.

The recovery of bile acid sulfates from biological fluid by using Amberlite resins needs to be critically evaluated. As a rule, the adsorption of organic compounds onto Amberlite resins decreases with increasing water solubility of the compounds (13). Whether all bile acid sulfates are adsorbed equally well and to the same extent as free and conjugated unsulfated bile acid is an unanswered question. Currently, there is an indication that diconjugates, which are more water soluble than monoconjugates, are not adsorbed onto Amberlite resin as well as monoconjugates using the conventional technique (14). The unequal recovery of sulfated and unsulfated bile acids will cause an incorrect ratio determination and some of the more water-soluble sulfates might escape detection completely. In addition, previous investigations have essentially left unstudied the best solvolytic condition for bile acid sulfates, the influence of conjugates on solvolysis, and the influence of the sulfate group on enzymatic hydrolysis (2-12).

Our interest in the application of stable isotope-labeled bile acids in bile acid metabo-

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[1] Part I of this series, see reference 18.

[2] *Abbreviations:* methanol, absolute methyl alchohol; ether, absolute diethyl ether; DMF, dimethylformamide; EEDQ, N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; TLC, thin layer chromatography; IR, infrared spectrum; 3-sulfocholic acid, 3α-sulfooxy-7α,12α-dihydroxy-5β-cholan-24-oic acid; 3-sulfoglycocholic acid, 3α-sulfooxy-7α,12α-dihydroxy-5β-cholan-24-oyl-glycine; 3-sulfotaurocholic acid, 3α-sulfooxy-7α,12α-dihydroxy-5β-cholan-24-oly-taurine; 3-sulfochenodeoxycholic acid, 3α-sulfooxy-7α-hydroxy-5β-cholan-24-oic acid; 3-sulfoglycochenodeoxycholic acid, 3α-sulfooxy-7α-hydroxy-5β-cholan-24-oyl-glycine; 3-sulfotaurochenodeoxycholic acid, 3α-sulfooxy-7α-hydroxy-5β-cholan-24-oyl-taurine; 3-sulfodeoxycholic acid, 3α-sulfooxy-12α-hydroxy-5β-cholan-24-oic acid; 3-sulfoglycodeoxycholic acid, 3α-sulfooxy-12α-hydroxy-5β-cholan-24-oyl-glycine; 3-sulfotaurodeoxycholic acid, 3α-sulfooxy-12α-hydroxy-5β-cholan-24-oyl-taurine; 7-formylchenodeoxycholic acid, 3α-hydroxy-7α-formyloxy-5β-cholan-24-oic acid; 12-formyldeoxycholic acid, 3α-hydroxy-12α-formyloxy-5β-cholan-24-oic acid; 7,12-diformylcholic acid, 3α-hydroxy-7α,12α-diformyloxy-5β-cholan-24-oic acid; ethyl 7-formylglycochenodeoxycholate, 3α-hydroxy-7α-formyloxy-5β-cholan-24-oyl-glycine ethyl ester; ethyl 12-formylglycodeoxycholate, 3α-hydroxy-12α-formyloxy-5β-cholan-24-oyl-glycine ethyl ester; ethyl 7,12-diformylglycocholate, 3α-hydroxy-7α,-12α-diformyloxy-5β-cholan-24-oylglycine ethyl ester.

lism (15) and inverse isotope dilution assay (16) using gas chromatography-mass spectrometry (GC-MS) prompted us to evaluate the existing methods of bile acid analysis. For this purpose, chemically pure labeled and unlabeled bile acid sulfates and their conjugates are needed as standards. The simple procedure of using sulfur trioxide-triethylamine (17) in preparing steroid sulfates has been successfully adopted to the synthesis of lithocholic acid sulfates in excellent yields (18). Here we report the synthesis of the specific 3-monosulfates of cholic acid, chenodeoxycholic acid, deoxycholic acid, and of their conjugates (glycine and taurine) using the same reagent. This procedure produces high yields of the desired compounds and thus makes them readily available for the evaluation of the solvolysis and adsorption behavior and of other fundamental properties of bile acid sulfates.

EXPERIMENTAL PROCEDURE

Methods and Materials

Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Chenodeoxycholic acid (Diamalt, Germany) was a gift of Dr. A.F. Hofmann of the Mayo Clinic, Rochester, MN. Deoxycholic acid (99$^+$%) and N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ, 99$^+$%) were purchased from Aldrich, Milwaukee, WI. Cholic acid (Kodak) was purified by crystallization from ethanol and drying overnight in vacuo at 150 C to constant weight. 7-Formylchenodeoxycholic acid, 12-formyldeoxycholic acid, and 7,12-diformylcholic acid were prepared by partial deformylation of the corresponding performylated bile acids (19). Ethyl glycinate hydrochloride was obtained from Pierce, Rockford, IL. Taurine was purchased from ICN Pharmaceuticals, Cleveland, OH, and Eastman Kodak, Rochester, NY.[3] p-Toluidine hydrochloride solution (1 M) was prepared by dissolving an appropriate amount of p-toluidine hydrochloride (Eastman Kodak) in water and was kept at 25 C for no more than 3 weeks. Sulfur trioxide-triethylamine was prepared as described before (18) and was stored at 4 C. This reagent is stable for at least one year under this storage condition. Dimethylformamide (DMF, reagent grade) was dried over Linde molecular sieves (1/16 in pellets, Linde Co.) and used directly. All other reagents were of commercial reagent grade and were used without further purification.

Thin layer chromatography (TLC) was carried out on precoated Silica Gel G plate (Prekote, 250 μ, Applied Science, State College, PA.). The plates were developed by solvent system EBAW, ethyl acetate-n-butanol-acetic acid-water, 40:30:15:15 (v/v); BAW, n-butanol-acetic acid-water, 10:1:1 (v/v) (20); and CMAW, chloroform-methanol-acetic acid-water, 65:24:15:9 (v/v) (21). Spots were detected by spraying the plates with 10% sulfuric acid in ethanol and heating at 120 C. Infrared (IR) spectra were determined on Perkin-Elmer 337 or 457 Infrared spectrometers.

Elemental analyses were performed by microtech Lab., Skokie, IL. Each sample was dried to constant weight at 100 C before analysis.

[3]Two different sources of commercial taurine were used in this investigation. Taurine from ICN pharmaceuticals was a puffy, microcrystalline form and was found to be more suitable for synthesis of taurine conjugates. It was used without further purification. The product from Kodak was in large prismic crystals and had to be ground to a fine powder and dried at 100 C, in vacuo, before use.

3-Sulfo-7-formylchenodeoxycholic Acid, Triethylammonium Salt, IIa

To a solution of 7-formylchenodeoxycholic acid, *Ia,* (1.68 g, 4 mmol) in 8 ml of DMF was added sulfur trioxide-triethylamine complex (740 mg, 4.04 mmol). After 0.5 hr at 25 C, the reaction mixture was then poured into 100 ml of diethyl ether. A precipitate formed immediately. The precipitate was collected, washed with ether, and air dried. The product weighed 2.408 g (quantitative); mp 200-203 C; IR (Nujol) 3610, 3540 ($\overset{+}{N}H$), 1715 (c=0), 1270, 1185 (ester), 1075, 1058, 1040, 990, 970, 820 cm^{-1}; elemental analysis: calcd, for $C_{31}H_{55}O_8NS$, C, 61.87, H, 9.21, S, 5.33; found, C, 61.60, H, 9.21, S, 5.58. Recrystallization could be effected from methylene chloride-ether.

3-Sulfochenodeoxycholic Acid, Disodium Salt, IIIa

A solution of 3-sulfo-7-formylchenodeoxycholic acid, triethylammonium salt, *IIa,* (601 mg, 1 mmol) in 10 ml of 0.5 N NaOH was heated on a steam bath for 10 min. After cooling to room temperature, the solution was acidified to pH ~ 5-6 with dil. HCl and then transferred to a 15 ml centrifuge tube. p-Toluidine hydrochloride (2 ml of 1 M solution) was added, and after vigorous mixing, the oily suspension so formed was centrifuged. The lower syrup-like layer was washed with ice-cold water (1 ml), centrifuged, and then dissolved in 10 ml of 0.2 N methanolic NaOH. The resulting solution was diluted with 40 ml of ether, and the precipitate so formed was collected, washed with methanol-ether (1:3), and ether, then dried in vacuo at room temperature. The disodium salt, *IIIa,* weighed 508 mg (98%); mp

210-212 C, lit. (22) mp 207-209 C; IR (Nujol) 3420 (broad, -OH), 1560 (C=O), 1410, 1220, 1068, 965 cm^{-1}; elemental analysis, calcd. for $C_{24}H_{38}O_7SNa_2$, C, 55.80, H, 7.41, S, 6.21; found, C, 55.24, H, 7.78, S, 6.44. Recrystallization could be effected from methanol (10 ml) and ether (30 ml).

3-Sulfotaurochenodeoxycholic Acid, Disodium Salt, VIIIa

Method A: To a solution of 3-sulfo-7-formylchenodeoxycholic acid, triethylammonium salt, *IIa* (601 mg, 1 mmole) and EEDQ (346 mg, 1.4 mmole) in 2 ml of DMF was added taurine[3] (138 mg, 1.1 mmole) and 0.2 ml of triethylamine. The resulting suspension was stirred in a 90 C bath for 0.5 hr, then at 25 C for 1 hr. The solution was then poured into 40 ml of ice-cold ether. After thorough trituration, the oil suspension changed to a precipitate, which was collected, washed thoroughly with ether, and then dissolved in 10 ml of 0.5N NaOH. This solution was heated briefly on a steam bath (5 min), then cooled to room temperature. After adjusting the pH to 7 with dil. HCl, the solution was transferred to a centrifuge tube and added 1 M p-toluidine hydrochloride (10 ml). The oil suspension was centrifuged, and the upper aqueous layer was pipetted off, and the last trace of aqueous phase was removed by adsorbing on filter paper strips. The syrupy lower layer was then dissolved in 10 ml of 0.2 N methanolic NaOH. This solution was diluted with 40 ml of ether, and the resulting precipitate was collected and washed with ether to yield a white amorphous powder (558 mg, 82%); mp 209-210 C; IR (Nujol) 3410 (OH), 1640, (C=0), 1550, 1210, 1060, 965 cm^{-1}; elemental analysis; calcd. for $C_{26}H_{43}O_9NS_2Na_2$, C, 50.07, H, 6.95, S, 10.28; found, C, 49.89, H, 7.20, S, 10.58.

Method B: A solution of 7-formylchenodeoxycholic acid, *Ia* (420 mg, 1 mmole) and sulfur trioxide-triethylamine (200 mg, 1.1 mmole) in 2 ml of DMF was kept at 25 C for 0.5 hr, then EEDQ (346 mg, 1.4 mmole), taurine (138 mg, 1.1 mmole), and triethylamine (o.2 ml) were added. The resulting suspension was heated at 90 C for 0.5 hr, then worked up as in *Method A* to give comparable yields of *VIIIa.*

This disodium salt can be recrystallized from methanol (10 ml)-ether (20 ml) or water (1 ml)-ethanol (30 ml). The sulfate crystallized as needles. The latter recrystallization procedure gave better crystals, but the recovery was lower.

Ethyl 7-formylglycochenodeoxycholate, IVa

A suspension of 7-formylchenodeoxycholic acid (4.2 g, 10 mmole), ethyl glycinate HCl (1.96 g, 14 mmole), EEDQ (3.46 g, 14 mmole), and triethylamine (2 ml) in 140 ml of ethyl acetate was refluxed for 4 hr. After cooling to room temperature, the reaction was washed successively with 0.5 N NaOH (50 ml), 0.5 N HCl (100 ml x 3), and H_20 (100 ml x 2), then dried over anhydrous $MgSO_4$. It was concentrated to about 20 ml and diluted with hexane (20 ml). After several hours at 5 C, the crystalline solid was collected, washed with ice-cold ethyl acetate, and then recrystallized from 25 ml of ethyl acetate to yield *IVa* (4 g, 80%), mp 158-159 C; IR (Nujol) 3260 (OH), 1750, 1715, 1650 (C=0), 1590, 1180 (ester) cm^{-1}; elemental analysis; calcd. for $C_{29}H_{47}O_6N$, C, 68.88, H, 9.37; found, C, 69.36, H, 9.23.

3-Sulfoglycochenodeoxycholic Acid, Disodium Salt, VIa

A solution of ethyl 7-formylglycochenodeoxycholate, *IVa,* (505 mg, 1 mmole) and sulfur trioxide-triethylamine (300 mg, 1.6 mmole) in 2 ml of DMF was kept at 25 C for 0.5 hr, then poured into 40 ml of ether. The flask was washed with small amounts of methylene chloride, and the washings were added to the ether solution. The resulting oily suspension was triturated, and the solid so formed was collected, washed with ether, and air dried to yield crude ethyl 3-sulfo-7-formyl glycochenodeoxycholate, *V*a. This crude product was hydrolyzed, precipitated as p-toluidinium salt (using 6 ml of 1 M p-toluidine HCl), and converted to disodium salt as described for *IIIa.* It yielded 578 mg (98%) of *VI*a; mp 209-210 C; IR (Nujol) 3400 (OH), 1600 (C=0), 1210, 1055, 955 cm^{-1}, identical to the published spectrum (2); elemental analysis: calcd. for $C_{26}H_{41}O_8NSNa_2$, C, 54.44, H, 7.20, S, 5.59; found, C, 54.17, H, 7.43, S, 5.56. This compound can be crystallized as described for *III*a.

3-Sulfodeoxycholic Acid, Disodium Salt, IIIb

12-Formyldeoxycholic acid, *I*b, (420 mg, 1 mmole) was sulfated as described for *III*a. The resulting crude 3-sulfo-12-formyldeoxycholic acid, triethylammonium salt, *II*b, was hydrolyzed, precipitated as p-toluidinium salt (using 2 ml of 1 M p-toluidine HCl), and converted to disodium salt as described before. The product weighed 492 mg (quantitative), mp 234-235 C, lit. (22) mp 225-239 C; IR (Nujol) 3400 (OH), 1560 (C=O), 1410, 1220, 1070, 980, 950 cm^{-1}; elemental analysis: calcd. for $C_{24}H_{38}O_7$ SNa_2, C, 55.80, H, 7.41, S, 6.21; found, C, 55.88, H, 7.70, S, 6.45.

3-Sulfotaurodeoxycholic Acid, Disodium Salt, VIIIb

A suspension of 3-sulfodeoxycholic acid, disodium salt, *III*b, (129 mg, 0.25 mmole), taurine (34.5 mg), and EEDQ (86.5 mg, 0.35 mmole) in 1 ml of DMF was stirred at 90 C for 0.5 hr, then another portion of EEDQ (86.5 mg) was added. The stirring was continued for another 0.5 hr. After cooling to 25 C, the solution was diluted with 2 ml of methanol and filtered to remove unreacted taurine. The filtrate and methanol washings (1 ml x 2) were combined and diluted with 20 ml of ether. The precipitate so formed was collected, dissolved in 3 ml of methanol, and filtered. This filtrate was diluted with 10 ml of ether to form a precipitate, which was collected, washed with methanol-ether (1:2) and ether, and dried to yield *VIII*b, (152 mg, 97%), mp 189-191 C; IR (Nujol) 3420 (OH), 1640, 1540 (C=O), 1215, 1055, 975, 950 cm^{-1}; elemental analysis: calcd. for $C_{26}H_{43}O_9NS_2Na_2$, C, 50.07, H, 6.95, S, 10.28; found, C, 49.90, H, 7.24, S, 10.19.

Ethyl 12-Formylglycodeoxycholate, IVb

12-Formyldeoxycholic acid, *I*b, (1.24 g, 0.3 mmole) was converted to *IV*b according to the procedure for *IV*a with proporitonally reduced amounts of other reagents. The reaction mixture, after washing, was evaporated to dryness to a syrup-like residue. It was used without further purification.

3-Sulfoglycodeoxycholic Acid, Disodium Salt, VIb

The sticky residue (*IV*b) obtained above was dissolved in 6 ml of DMF. Sulfur trioxide-triethylamine (800 mg) was added, and the solution was kept at 25 C for 0.5 hr. Ethyl 3-sulfo-7-formylglycodeoxycholate, triethylammonium salt, *V*b, was isolated as descrived for *V*a using a proportional amount of ether. The oily suspension was kept at 5 C until it settled. The clear ether supernatant was decanted, and the residue was triturated several times with ether, then dried with a stream of nitrogen. The residue was hydrolyzed (15 ml of 1N NaOH) at 100 C for 10 min. After cooling and acidifying with dil. HCl to pH ~ 5-6, the hydrolysate was added 1M p-toluidine HCl (6 ml). The turbid suspension was triturated to induce solidification, and the resulting crystalline p-toluidinium salt was collected, washed with ice-cold water, then converted to disodium salt as described before (using 35 ml of 0.2 N methanolic NaOH and 70 ml of ether). It was purified by redissolving in 40 ml of methanol with warming, then diluting with 60 ml of ether. The product weighed 1.451 g (84%, based on *I*b used), with mp 218-220 C, IR (Nujol) 3380 (OH), 1600 (C=O), 1220, 1070, 1040, 970, 945 cm^{-1}; elemental analysis: calcd. for $C_{26}H_{41}O_8NSNa_2$, C, 54.44, H, 7.20, S, 5.59; found, C, 54.21, H, 7.11, S, 5.56.

3-Sulfocholic Acid, Disodium Salt, IIIc

7,12-Diformylcholic acid, *I*c, (464 mg, 1 mmole) was sulfated as described for *III*a. The triethylammonium salt, *II*c, was hydrolyzed (with 12 ml of 0.5 N NaOH), precipitated as p-toluidinium salt (with 5 ml of 1 M p-toluidine HCl solution), and converted to disodium salt as described for *III*a (except that only 0.5 ml of ice-cold water was used for washing instead of 1 ml). The product weighed 450 mg (84%), mp 209-210 C, lit. mp 220-233 C (22), 189-192 C (20); IR (Nujol) 3400 (OH), 1560 (C=O), 1220, 1050, 960 cm^{-1}, identical with the published spectrum (20); elemental analysis. Calcd. for $C_{24}H_{38}O_8SNa_2$, C, 54.12, H, 7.19, S, 6.02; found, C, 54.32, H, 7.44, S, 6.03.

3-Sulfotaurocholic Acid, Disodium Salt, VIIIc

3-Sulfocholic acid, disodium salt, *III*c, (133 mg) was conjugated with taurine as described for *VIII*b to obtain 145 mg (90% yield) of *VIII*c, mp 182-183 C, IR (Nujol) 3400 (OH), 1635, (C=O), 1540, 1210, 1050, 970 cm^{-1}; elemental analysis: calcd. for $C_{26}H_{43}O_{10}NS_2$-Na_2, C, 48.82, H, 6.78, S, 10.02; found, C, 48.43, H, 7.24, S, 10.12.

Ethyl 7,12-Diformylglycocholate, IVc

7,12-Diformylcholic acid, *I*c (4.64 g, 10 mmole) was converted to *IV*c as descrived for *IV*a. The oily residue, after evaporating to dryness, was purified by passing through an aluminum column and eluting with benzene-acetone (70:30). The pure product was dried, in vacuo, to a crystalline powder weighing 5.23 g (95%), mp 79-81 C; IR (Nujol) 3300 (OH), 1715, 1660 (C=O), 1530, 1175 (ester) cm^{-1}; elemental analysis: calcd. for $C_{30}H_{47}O_8N$:1/6 C_6H_6, C, 66.17, H, 8.60; found, C, 66.30, H, 8.58.

3-Sulfoglycocholic Acid, Disodium Salt, VIc

Ethyl 7,12-diformylglycocholate, *IV*c (549 mg, 1 mmole) was sulfated as described for *VI*a. Water (1 drop) was added to the DMF solution, and this was heated to 70 C for 1 hr to destroy the excess sulfating agent. After dilution with 40 ml of ether, the supernatant was discarded, and the syrupy residue was dissolved in 25 ml of 9.4 N methanolic NaOH. The resulting mixture was refluxed for 0.5 hr. After cooling, the suspension was filtered and the crude product was precipitated from the filtrate by dilution with ether (50 ml). This crude product, *VI*c,

where in series,

a, $R_1 = R_3 = H$, $R_2 = OH$, $R_4 = OCHO$

b, $R_2 = R_4 = H$, $R_1 = OH$, $R_3 = OCHO$

c, $R_1 = R_2 = OH$, $R_3 = R_4 = OCHO$

FIG. 1. Synthesis of 3-monosulfates of unconjugated bile acids.

FIG. 2. Synthesis of 3-monosulfates of glycine-conjugated bile acids (for letter designations, see Fig. 1).

(590 mg) showed a single spot on TLC, and contained only sodium formate as impurity. Recrystallization twice (20 ml each) from absolute ethanol afforded 450 mg (76% yield) of pure *VI*c as needles, mp 205-207 C; IR (Nujol) 3360 (OH), 1600 (C=O), 1530, 1250, 1225, 1065, 970 cm^{-1}; elemental analysis: calcd. for $C_{26}H_{41}O_9NSNa_2$, C, 52.96, H, 7.01, S, 5.44; found, C, 52.89, H, 7.33, S, 5.45.

RESULTS AND DISCUSSION

Among the bile acid sulfates synthesized in this investigation, a number of them have been synthesized in other laboratories (2,3,20,22,23) with poor yields and tedious procedures. These were synthesized by the sulfation of unprotected bile acids with chlorosulfonic acid in pyridine (2,5,10,20) or by treatment of bile acids protected by acetyl groups with chlorosulfonic acid in diethyl ether (22,23). The former procedure produced a mixture of monosulfates and disulfates. The contaminating disulfates can be removed by column chromatography on Sephadex LH-20 (20), but the elimination of contaminating monosulfates was not as easy; hence, lower yields usually resulted. The use of partially acetylated bile acids as starting materials (22,23) has the advantage of directing the sulfate group entirely to the desired 3α-position and thus eliminating the task of separating unwanted isomers. However, the acetyl-protecting groups have the disadvantage of being difficult to be hydrolyzed. In fact, the 7- and 12-acetyl groups on a bile acid sulfate required more than 2 hr for complete removal (K.Y. Tserng, unpublished observation) using conditions similar to those of Haslewood and Haslewood (22). The long refluxing time required to remove protecting groups in acetylated bile acid sulfates will not have any deleterious effect on the synthesis of unconjugated bile acid sulfates. However, it is definitely not suitable for the synthesis of glycine- and taurine-conjugated bile acids, since this would result in partial deconjugation. In contrast, 3-hydroxy formyloxy bile acids, which can be easily synthesized in high yield by partial deformylation of performylated bile acids (19), are stable under the reaction conditions and are deformylated by refluxing for only a few minutes in mild alkaline medium. In our experience, 3-hydroxy formyloxy bile acids are the best

FIG. 3. Synthesis of 3-monosulfates of taurine-conjugated bile acids (for letter designation, see Fig. 1).

starting materials for the synthesis of bile acid sulfates.

The sulfations were carried out using sulfur trioxide-triethylamine in DMF at 25 C. This sulfating agent and reaction condition were found to be superior to other agents and conditions (18) employed by other investigators, such as chlorosulfonic acid in pyridine, sulfuric acid and dicyclohexylcarbodiimide in DMF, or chlorosulfonic acid in diethyl ether. Thus, sulfation was complete within 0.5 hr at 25 C with as little as 1.01 to 1.1 equivalent of sulfur trioxide-triethylamine. The products, formylated bile acid sulfates, were isolated simply by diluting the DMF solution with diethyl ether (see Fig. 1). The glycine-conjugated bile acid sulfates were similarly obtained by reacting ethyl esters of 3-hydroxy formyloxy bile acid glycine conjugates, *V*, with a slightly higher equivalent of sulfur trixoide-triethylamine (1.6 equivalent) (Fig. 2). The higher equivalent of sulfating agent required for the sunthesis of sulfates of glycine conjugates is due to the lower reactivity of glycine conjugates toward sulfation, possibly due to side chain interaction (18). The starting materials, ethyl esters of formylated glycine conjugates, *V*, were synthesized in high yield by conjugating 3-hydroxy formyloxy acids, *I*, with ethyl glycinate in a procedure described before (24). The conversions of unconjugated bile acids and glycine-conjugated bile acids into their corresponding sulfates were quantitative. No other side products could be detected by TLC examination of the reaction mixture. Due to side chain interaction, the sulfation of taurine-conjugated bile acids did not proceed as well as the unconjugated or glycine-conjugated bile acids, even with a large excess of sulfating agent (18). Thus the formyl group protected bile acids were sulfated first and then conjugated with taurine in DMF in the presence of EEDQ (24) (see Fig. 3). The sulfated formylated bile acids, *II*, used as starting materials can be generated in situ without isolation (see method B of compound *VIII*a in the experimental section). The "one pot" synthesis of sulfates of taurine conjugates shortened the reaction sequence considerably and produced the product in overall higher yields. This reaction sequence worked equally well for all taurine-conjugated bile acids. However, due to the difficulty encountered in isolation of deformylated taurine-conjugated sulfates (which will be discussed later), the sulfates of taurodeoxycholic acid and taurocholic acid were prepared by the conjugation of disodium salts of deoxycholic acid 3-sulfate and cholic acid 3-sulfate with taurine. Disodium salts are virtually insoluble compared with triethylammonium salts, in DMF solution; thus, the conjugation using the disodium salt required a longer reaction time and the addition of more EEDQ. In the synthesis of taurine conjugates, the ethyl ester of the unconjugated bile acid sulfate is formed (shown by TLC) as a minor side product, but is easily removed during workup.

After synthesis of the bile acid sulfates, the protecting formyl groups were hydrolyzed in a mild alkaline solution on a steam bath for several minutes to obtain the bile acid sulfates with free hydroxyl groups. The isolation of these sulfates free from any inorganic contaminants from their aqueous solutions represents a major problem in the yield of the products since these sulfates are all extremely water soluble. Prior methods (1-3,20,22) for sulfate isolation (e.g., Amberlite adsorption, solvent extraction, TLC) were not entirely satisfactory. Organic sulfates are known to form precipitable salts with aromatic amines (25). Furthermore, Hirschmann and Williams (26) used the p-toluidinium salt to isolate 5α-pregnane-3β,20β-diol 20-sulfate. These organic aromatic ammonium salts have the advantage of being easily converted to their alkali metal salts. Thus, p-toluidinium chloride forms precipitable salts with most of the sulfates synthesized in this investigation. The precipitability of bile acid sulfates and the approximate water solu-

TABLE I

Precipitability of Bile acid Sulfates as p-Toluidinium Salts and the Relative Solubility of the Precipitated Salt[a]

	Unconjugated	Glycine conjugate	Taurine conjugate
Lithocholic sulfates	++++	++++	++++[b]
Chenodeoxycholic 3-sulfates	+++	++	+
Deoxycholic 3-sulfates	+++	+++	—
Cholic acid 3-sulfates	++	—	—

[a]Degree of precipitability is represented by the following symbols: ++++, precipitable by the addition of 2 ml of 1 M p-toluidinium chloride to a solution of 1 mmole of bile acid sulfate in 10 ml of water at pH ~ 6, and the precipitates are practically insoluble in water; +++, precipitable by the addition of 2 ml of the reagent, but the precipitated salts are sparingly soluble in water; ++, precipitable by the addition of 6 ml of the reagent, and the precipitated salts are relatively soluble in water; +, precipitable by the addition of 6 ml of the reagent, and the precipitated salt is freely soluble in water; -, not precipitable by the reagent at all concentrations.

[b]Precipitated as gelatinous substance, see ref. 18.

TABLE II

Thin Layer Chromatographic Mobilities (R_f) of Bile Acid 3-Monosulfates

	Solvent system		
Bile salt	EBAW[a]	BAW[b]	CMAW[c]
Cholic acid	0.79	0.78	0.87
Glycocholic acid	0.66	0.58	0.66
Taurocholic acid	0.35	0.30	0.39
Cholic acid 3-sulfate	0.55	0.61	0.45
Glycocholic acid 3-sulfate[d]	0.39	0.37	0.36
Taurocholic acid 3-sulfate	0.12	0.14	0.15
Chenodeoxycholic acid	0.93	0.79	0.96
Glycochenodeoxycholic acid	0.80	0.70	0.80
Taurochenodeoxycholic acid	0.43	0.43	0.45
Chenodeoxycholic 3-sulfate	0.57	0.61	0.50
Glycochenodeoxycholic 3-sulfate[d]	0.46	0.48	0.40
Taurochenodeoxycholic 3-sulfate	0.17	0.23	0.18
Deoxycholic acid	0.96	0.81	0.96
Glycodeoxycholic acid	0.80	0.68	0.82
Taurodeoxycholic acid	0.43	0.43	0.47
Deoxycholic 3-sulfate	0.62	0.64	0.52
Glycodeoxycholic 3-sulfate[d]	0.43	0.49	0.45
Taurodeoxycholic 3-sulfate	0.20	0.22	0.22
Glycolithocholic 3-sulfate[d,e]	0.53	0.50	0.49

[a]Ethyl acetate-n-butanol-acetic acid-water, 40:30:15:15.

[b]n-Butanol-acetic acid-water, 10:1:1.

[c]Chlorodorm-methanol-acetic acid-water, 65:24:15:9.

[d]Tailing of all four glycine-conjugated sulfates.

[e]Included to serve as standard to calculate the relative mobilities of other lithocholic acid derivatives reported previously (18).

bility of the precipitated salts are summarized in Table I. Most of the sulfates were isolated via their p-toluidinium salts. 3-Sulfotaurodeoxycholic acid, 3-sulfoglycocholic acid, and 3-sulfotaurocholic acid were not precipitable by p-toluidinium chloride. In these instances, 3-sulfotaurodeoxycholic acid and 3-sulfotaurocholic acid were more conveniently synthesized by direct conjugation of their corresponding disodium salts of unconjugated sulfates with taurine, as described in the experimental section. Pure 3-sulfoglycocholic acid was obtained by repeated crystallization of the crude disodium salt, *VIc*, from ethanol to remove sodium formate formed from hydrolyzing the protecting formyl groups.

The identities of these sulfates were established by elemental analysis and their infrared (IR) spectra. All the sulfates show similar IR spectra, which are characterized by the typical strong absorption bands due to the sulfate group around 1200, 1060, and 965 cm^{-1} (18).

The only major difference among the IR spectra of unconjugated bile acid sulfates, glycine-conjugated bile acid sulfates, and taurine-conjugated bile acid sulfates is the absorption band due to carbonyl stretching. The unconjugated bile acid sulfates show a strong absorption band at 1560 cm^{-1} and a weaker band at 1420 cm^{-1}, a typical absorption pattern for the carboxylate anion. In addition to the absorption band at 1540 and 1410 cm^{-1} due to carboxylate anion, the IR spectra of glycine-conjugated bile acid sulfates also show a stronger amide carbonyl absorption band at 1600 cm^{-1}. The IR spectra of taurine-conjugated bile acid sulfates display typical amide I and II bands at 1640 and 1540 cm^{-1}.

All the sulfates synthesized are relatively nonhydroscopic (as the disodium salt) and can be kept at room temperature in an air-tight container for at least one year without any sign of decomposition. Their aqueous solutions are relatively stable at 25 C even at pH vlaues as low as 1. The solutions of the disodium salts in methanol or ethanol are also relatively stable at all temperature ranges. However, the acidification of these alcoholic solutions rapidly solvolyzes the sulfate group. The addition of water to these acidic alcoholic solutions slows down the solvolysis process.

The TLC of sulfated bile acids, using three different solvent systems is shown in Table II. All systems resulted in overlap with nonsulfated bile acids, making class separations impossible to achieve when all forms of each class were present. This experience was also confirmed by preliminary experiments with column chromatography on lipophilic Sephadex LH-20.

When the three variables of number of hydroxyl groups in a bile acid, its conjugation status, and the presence or absence of a 3-sulfate group are combined, the resulting physical properties cover a wide range of polarities. This makes the design and validation of analytical procedures, particularly for sulfates, exceptionally demanding, and requires that a spectrum of possible species be examined instead of a single form, such as the lithocholic acid sulfate. The procedures described in this paper now offer the possiblility to carry out such a complete analytical development using the full range of bile acid sulfates that have been synthesized.

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The Effects of Clofibrate Feeding on the Metabolism of Palmitate and Erucate in Isolated Hepatocytes

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ABSTRACT

The metabolism of palmitate and erucate has been investigated in hepatocytes isolated from control rats and from rats fed 0.3% clofibrate. Clofibrate increased the oxidation of [1-^{14}C]palmitate 1.5 to 2-fold while the esterification was decreased. At a high concentration of palmitate (1.5 mM), the total rate of fatty acid metabolism was stimulated. Clofibrate stimulated both the oxidation (3.5 to 5-fold) and the esterification (1.7-fold) of [14-^{14}C]erucate. Erucate undergoes chain-shortening in isolated liver cells. This chain-shortening was stimulated at least 2-fold by clofibrate feedings. The isolated mitochondrial fraction from clofibrate-fed rats showed an increased capacity for oxidation of short-chain acylcarnitines (including acetylcarnitine), while the oxidation of palmitoyl- and erucoylcarnitine showed little change. It is suggested that erucate is shortened by the recently detected β-oxidation system of peroxisomes.

INTRODUCTION

The partition of long chain fatty acids between oxidation and esterification in the liver is influenced by the nutritional and endocrine state of the animal. More fatty acids are oxidized and less incorporated into lipids in perfused livers and cells isolated from fasted rats than from carbohydrate-fed rats (1,2). In cells from carbohydrate-fed rats, the rate of oxidation is also strongly stimulated by glucagon (3). The shift in fatty acid metabolism may at least partly be explained by changes in the activities of the enzymes, carnitine palmitoyltransferase (EC 2.3.1.21) and glycerophosphate acyltransferase (EC 2.3.1.15). The former enzyme, which represents the first step in the oxidation pathway, is inhibited by malonyl-CoA (4), and its activity is increased in the liver of fasted rats (5,6).

Clofibrate (α-p-chlorophenoxyisobutyrate) is a drug used to lower the level of blood lipids. Feeding clofibrate to rats has been shown to increase the activity of the carnitine acyltransferases in the liver while the activity of the glycerophosphate acyltransferases is less affected (7). Clofibrate was also reported to reduce hepatic glycerolipid synthesis (8), and some evidence has been presented on its stimulatory effect upon fatty acids oxidation (9). The drug is also known to cause proliferation of mitochondria (10) and peroxisomes (11) in the liver. Peroxisomes have been reported recently to contain a β-oxidation system for fatty acids different from the β-oxidation system of the mitochondria (12).

Because of the reported effects of clofibrate, we have compared the partition of fatty acids between oxidation and esterification in hepatocytes isolated from control and clofibrate-fed rats. We have also tested the effect of clofibrate on the metabolism of very long chain fatty acids 22:1 such as erucic acid, since these fatty acids are poorly metabolized by isolated mitochondria (13).

MATERIAL AND METHODS

[1,3-^{14}C]Glycerol, [1-^{14}C] and [16-^{14}] palmitate were obtained from NEN Chemicals GmbH. [14-^{14}C]Erucic acid was from Centre d'Etudes Nucléaires de Saclay, Gif sur Yvette, France. [1,3-^{14}C]L-glycerol-3-phosphate was synthesized according to Smith and Hübscher (14) and [Me-^{3}H](-)carnitine according to Stokke and Bremer (15). Essentially, fatty acid-free bovine serum albumin, N-2-hydroxyethylpiperazine-N-2 ethanesulfonic acid (Hepes), and palmitoyl-CoA were purchased from Sigma Chemical Co., St. Louis, MO.

Male Wistar rats weighing 160-180 g were fed ad libitum either the usual laboratory pelleted diet or pelleted diet containing 0.3% (w/w) clofibrate (ICI Industrial Company, Macclesfield, England) for 8-12 days.

Isolated hepatocytes were prepared and purified according to Seglen (16) except that Ca^{+2}-free Krebs-Henseleit bicarbonate buffer was used as the suspension and incubation medium.

The hepatocytes were routinely preincubated at 37 C with 1 mM carnitine for 20 min. The incubation conditions were as described previously (3). Radioactive fatty acids were used with the specific activity of 800 cpm/nmole. The measurement of radioactive acid soluble products and the extraction of lipids were performed as described previously (2). Total lipid extracts were transmethylated (17) and analyzed by radio-gas chromatography using a Pye 104 gas chromatograph connected

TABLE I

The metabolism of [1-^{14}C] Palmitate and [14-^{14}C] Erucate in Hepatocytes Isolated from Control and Clofibrate-fed Rats[a]

Fatty acid	Conc. (mM)	Oxidation products		Esterification products	
		Control	Clofibrate	Control	Clofibrate
Palmitate	0.5	21.4 ± 2.0	33.4 ± 3.4[b]	34.7 ± 1.4	27.1 ± 1.7[b]
	1.5	55.6 ± 4.1	118.5 ± 6.0[b]	103.4 ± 3.5	74.0 ± 4.9[b]
Erucate	0.5	7.9 ± 0.9	41.3 ± 3.9[b]	11.2 ± 1.1	19.2 ± 3.0[c]
	1.5	14.5 ± 1.5	50.5 ± 4.1[b]	27.1 ± 1.4	47.2 ± 3.1[b]

[a]The results are expressed as nmoles/mg protein (mean ± SD from four cell preparations). 6-8 mg of cellular protein per sample were used, and the incubation time was 30 min. Oxidation products are the sum of radioactive acid soluble products, and $^{14}CO_2$, esterification products are the sum of mono, di, triacylglycerol and phospholipids.

[b]Different from the control with $p<0.001$.

[c]Different from the control with $0.001<p<0.005$.

to an ESI Nuclear radioactivity detector with a 1:1 outlet splitter. Fatty acid methyl esters were separated at 185 C using 10% SP 2340 on Supelcoport 100/120 (Supelco Inc., Bellefonte, PA). The peaks were identified on the basis of the retention time compared with the standards. The distribution of radioactivity between the peaks was calculated from counting data recorded on Printing Autoscaler 5680 (ESI Nuclear).

The same liver cell prepraration was used for the incubation experiments and for the assay of enzyme activities after the cells had been disrupted with a Dounce homogenizer in a medium containing 0.25 mM sucrose, 10 mM Hepes buffer pH 7.4, 2 mM EDTA, and 8 mM NaF. The cell homogenate was centrifuged at 500 g for 3 min, and the obtained supernatant was centrifuged at 100,000 g for 45 min. The resulting pellet was suspended in the homogenizing buffer to give ca. 5 mg protein/ml and used for the determination of carnitine palmitoyltransferase and glycerophosphate acyltransferase activities. The assay medium (1 ml) contained 25 mM Hepes, 3 mM KCN, 50 μM palmitoyl-CoA, 0.12 mM KCL, ca. 0.5 mg protein of the particulate fraction, and either 1 mM [1,3-^{14}C] L-glycerol 3-phosphate and 1% bovine serum albumin or 2.5 mM [Me-^{3}H] (-)carnitine. The incubation time was 1 min at 30 C. The radioactive acylglycerol and acylcarnitine were extracted with n-butanol as described previously (6).

The liver mitochondria were isolated according to Bremer and Davis (18). The measurements of the β-oxidation-dependent reduction of ferricyanide were performed as described previously (19) except that bovine serum albumin was included to a concentration of 2.5 mg/ml. The Student t-test was used for statistical treatment of the results.

RESULTS

The data presented in Table I compare the metabolism of [1-^{14}C] palmitate and [14-^{14}C] erucate in hepatocytes isolated from control and clofibrate-fed rats. In the control liver cells, erucic acid is more slowly metabolized than palmitate. Both the oxidation and esterification is only one-third to one-fourth of that of palmitate. This is strikingly different in cells from clofibrate-fed rats. In these cells, the rate of erucate oxidation had increased 3.5 to 5-fold, while palmitate oxidation increased only 1.5 to 2 times. With a low concentration of fatty acid in the medium (0.5 mM), erucate was now oxidized even faster than palmitate. The rate of erucate esterification was also increased in the cells from clofibrate-fed rats, while that of palmitate was decreased.

This different effect of clofibrate on palmitate and erucate metabolism is probably not explained by the difference in the position of fatty acid labeling. This was checked with [16-^{14}C] palmitate as substrate. The distribution of [16-^{14}C] palmitate between oxidation and esterification was identical with that of [1-^{14}C] palmitate. The only difference was a relatively higher labeling of CO_2 from the [1-^{14}C] palmitate, but the sum of radioactive oxidation products was identical with both [1-^{14}C] and [16-^{14}C] palmitate (not shown). The oxidation of both erucate and palmitate is stimulated by carnitine in liver cells (2 and R.Z. Christiansen, unpublished); differences in the carnitine content of the two types of cells cannot explain our results since the experiments of Table I were performed with an excess of intracellular carnitine (see Material and Methods).

It has been reported that erucate undergoes chain-shortening in the intact animals (20) and in isolated cells (21). We have, therefore,

TABLE II

The Chain-shortening of Erucic Acid in Hepatocytes Isolated from Control and Clofibrate-fed Rats[a]

Type of cells	Erucate (mM)	16:1	18:1	20:1	20:2	Σ[b]	C_2 units[c]
Control	0.5	0.7	4.5	1.4	0.6	7.2 ± 1.1	13.1 ± 2.1
	1.5	traces	6.1	2.2	5.3	13.6 ± 1.6	19.7 ± 1.5
Clofibrate	0.5	3.5	9.9	3.3	0.5	17.2 ± 1.2[d]	34.1 ± 1.8[d]
	1.5	0.6	13.5	6.6	7.4	28.1 ± 3.2[d]	42.8 ± 4.8[d]

[a]The experiments were performed as described in Material and Methods. 6-8 mg of cellular protein per sample were used. The incubation time was 30 min. The results are expressed as nmoles x mg protein^{-1}.

[b]Represents the sum of 16:1, 18:1, 20:1 and 20:2 (mean ± SD from four cell preparations).

[c]Acetyl units split off erucic acid were calculated as $[(C_{16} \times 3) + (C_{18} \times 2) + (C_{20} \times 1)]$ (mean ± SD from four cell preparations).

[d]Different from the control with $p<0.001$.

investigated the effect of clofibrate on the chain-shortening of [14-^{14}C]erucate by isolated hepatocytes. The data presented in Table II show that chain-shortening takes place both in control cells and in cells from clofibrate-fed rats. The identified products of the chain-shortening process of [14-^{14}C]erucate were 16:1, 18:1, 20:1, and 20:2. The incubation of [16-^{14}C]palmitate with the hepatocytes did not lead to the accumulation of any shorter or longer radioactive fatty acids either in control or in clofibrate cells. Since both [16-^{14}C] palmitate and [14^{14}C] erucate undergo oxidation to form [2-^{14}C]acetyl-CoA, it is rather unlikely that the radioactive fatty acids produced from erucic acid arose by the process of chain-elongation.

The pattern of chain-shortened fatty acids differs somewhat between hepatocytes from control and clofibrate-fed rats. Further, the pattern is also dependent on the concentration of erucic acid in the incubation medium (Table II). For these reasons, the results of Table II have been presented both as nmoles of erucic acid which have been chain-shortened and as nmoles of acetyl groups expected to have been split off erucic acid to form shorter fatty acids. In either case, there is a significant increase (2.1 to 2.6-fold) of chain-shortening with hepatocytes from clofibrate-fed rats. It should be pointed out that the measured chain-shortened products represent only the fraction of fatty acids which has been incorporated into acylglycerol and phospholipids. Only a negligible amount of chain-shortened fatty acids was found in the free fatty acid fraction (not shown). A complete estimate of the chain-shortening capacity would, however, require an estimate of the fraction of erucate which, following the chain-shortening, was completely oxidized, presumably in the mitochondria.

The amount of chain-shortened fatty acids in control cells is apparently not stimulated by carnitine (J. Norseth and B.O. Christophersen, unpublished). The chain-shortening reaction is, therefore, most likely independent of carnitine and the mitochondrial β-oxidation system.

We have also checked the effect of clofibrate on the activities of carnitine palmitoyltransferase and glycerophosphate acyltransferase in the particulate fraction of disrupted cells (Table III). The activity of the former enzyme increased by a factor of 3 ($p < 0.001$). The increase in the activity of glycerophosphate acyltransferase was not significant ($0.1 < p < 0.2$). These results would support the idea that these acyltransferases have a vectorial effect on the metabolism of fatty acids.

The fatty acid oxidation capacity of the isolated mitochondrial fraction from control and clofibrate-fed rats has been compared by using a method recently developed in our laboratory (19). Under the conditions used, the contaminating peroxisomes most likely will not contribute to the oxidation of acylcarnitines since the peroxisomal β-oxidation system requires added NAD and acyl-CoA (12).

Figure 1 shows that, on a protein basis, the mitochondrial fraction from clofibrate-fed rats exhibits only a small increase in capacity to oxidize long chain fatty acids and no change in relative capacities to oxidize erucoyl- and palmitoylcarnitine. The increased mitochondrial content in the liver of clofibrate-fed rats (10) should, therefore, increase the capacity to oxidize these fatty acids about equally.

An unexpected finding was that the mitochondrial fraction from the clofibrate-fed rats showed a distinct relative increase in the capacity to oxidize short chain acylcarnitines. The oxidation of butyroylcarnitine increased about 2.5-fold on a protein basis, while the oxidation of palmitoylcarnitine increased by only about 10%. A 3-fold increase in the rate of oxidation

TABLE III

The Activities of Carnitine Palmitoyltransferase and Glycerophosphate Acyltransferase in the Particulate Fraction from Hepatocytes Isolated from Control and Clofibrate-fed Rats[a]

Enzyme	Control	Clofibrate
Carnitine palmitoyltransferase	3.92 ± 0.45 (3)	12.7 ± 1.52[b] (4)
Glycerophosphate acyltransferase	0.85 ± 0.22 (4)	1.20 ± 0.40[c] (4)

[a]The enzyme activities are expressed as nmoles of acylglycerol or acylcarnitine formed x mg protein^{-1} x min^{-1} (mean ± SD). Number of observations is given in parentheses.
[b]Different from the control with $p<0.001$.
[c]Different from the control with $0.1<p<0.2$.

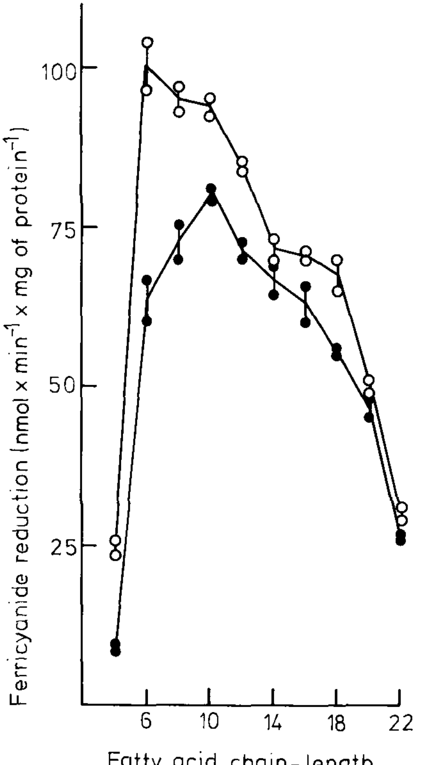

FIG. 1. The effect of clofibrate feeding on the rates of β-oxidation with acylcarnitines of different chain lengths. The rates of β-oxidation were measured by spectrophotometric recording of acylcarnitines-dependent reduction of ferricyanide in the presence of 10 mM oxaloacetate using liver mitochondria isolated from a control rat (•) and clofibrate-fed rat (○). The details of the assay have otherwise been described in Material and Methods. The C_{22} acylcarnitine represents results obtained with erucoylcarnitine.

of acetylcarnitine was also demonstrated in polarographic experiments with the isolated mitochondrial fraction in the presence of malate (not shown). These results can be explained by the increased activity of the short chain acyltransferase in mitochondria (22).

DISCUSSION

The present results give some support to the idea that the relative activities of carnitine palmitoyltransferase and glycerophosphate acyltransferase influence the fate of fatty acids in the liver. The cells from clofibrate-fed rats showed a significant increase in the activity of the carnitine palmitoyltransferase, and concomitantly more palmitate was oxidized and less esterified than in the cells from control rats. This is in accordance with the observed increase in the mitochondrial protein in the liver of clofibrate-fed rats (10) and with the slightly increased capacity (on a protein basis) of the mitochondiral β-oxidation system for long chain fatty acids reported here.

The much stronger effect of clofibrate on the metabolism of erucate requires an additional explanation. We have found that clofibrate stimulates the capacity for chain-shortening of erucate in the liver. This process is apparently not dependent on carnitine. The stimulation of chain-shortening may even be more pronounced than is apparent from our experiments since the shortened fatty acids will be oxidized by the mitochondria in preference to erucate (13). It seems likely, therefore, that the chain-shortening of erucate is an extramitochondrial process which may be rate limiting for the complete oxidation of very long chain fatty acids.

Erucic acid is also a poor substrate for acylation of glycerophosphate (Table I). It is likely that the chain-shortening can be rate limiting also for the incorporation of erucate into triacylglycerol and phospholipids. This is supported by the finding that more erucate was incorporated into lipids in cells from clofibrate-fed rats and that relatively more of the in-

corporated radioactivity was recovered in shortened fatty acids (Tables I and II). Recently, it has been reported that clofibrate stimulates β-oxidation of fatty acids in peroxisomes (12). The function of this previously unknown β-oxidation system is not clear. Its chain-length specificity is also unknown. Our results strongly suggest that one function of this system is to shorten very long chain fatty acids (C_{22} and longer), thus, making these fatty acids generally easier to metabolize further in the liver cell. In this connection, it is noteworthy that the mitochondria from clofibrate-fed rats show an increased rate of oxidation of short chain fatty acids which may be reaction products from the extramitochondrial β-oxidation system of peroxisomes. It has been reported that acetyl-CoA is a reaction product of this extramitochondrial β-oxidation (12,23). The activity of acetylcarnitine transferase increases up to 10-fold in the mitochondria and ca. 2-fold in the peroxisomes of the liver of clofibrate-fed rats (22). Thus, one function of this enzyme may be to facilitate the transfer of acetyl groups formed extramitochondrially in the peroxisomes into the mitochondria for oxidation in the citric acid cycle or for the conversion to ketone bodies.

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Uropygial Gland Alkane Diol Diesters in the K^n Mutation of the Domestic Chicken[1,2]

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ABSTRACT

Uropygial glands from chickens which are know to have the K^n gene secrete qualitatively the same types of alkane diol diesters as the normal domestic chicken. However, there are many significant differences in the mole percent of individual fatty acids and diols within the diester between the two genetic types of chickens. In general, the mutant chickens have lesser amounts of the shorter chain fatty acids and diols and greater amounts of the longer chain fatty acids and diols. There is also a gene dosage effect with two mutant genes having a greater effect in many cases than a single mutant gene.

INTRODUCTION

A delayed feathering mutant in the domestic chicken has been described (1). The sex-linked gene designated K^n that is responsible for this trait also causes the affected chickens to develop a greatly enlarged uropygial gland. The total amount of lipid within the uropygial glands of the mutant chickens is increased over that of normal chickens. Somes (2) showed that this increase in lipids was the direct result of the K^n gene. The major lipid class in the chicken's uropygial gland is alkane diol diesters (3). This paper presents comparisons of the composition of fatty acids and diols comprising the diesters of male and female K^n mutants with that of nonmutant chickens.

[1]Scientific contribution no. 705, Agricultural Experiment Station, University of Connecticut Storrs, CT 06268.

[2]Presented at the AOCS Meeting, New York City, May 1977.

MATERIALS AND METHODS

A cross was made between heterozygote mutant male chickens and both hemizygous mutant and normal female chickens to produce the five possible genotypes under study. Nine male chickens and eight female chickens of each genotype were raised intermingled in battery cages under similar environmental conditions. They were fed standard rations until 14 weeks of age when they were sacrificed. The uropygial glands were removed at the time of death and frozen to facilitate lipid removal. Following extraction of the uropygial lipids (4), the alkane diol diesters were purified by thin layer chromatography (TLC) and subsequently methanolyzed in BF_3-methanol according to the method of Hansen et al. (5). The resulting methyl esters and diols were separated by TLC in a solvent system of petroleum ether-ethyl ether-acetic acid (80:20:1). Prior to examination by gas liquid chromatography (GLC), acetonide derivatives were made from the diols (3).

The methyl esters and acetonides were

TABLE I

Genotype Comparisons between Uropygial Gland and Lipid Weight Relationships

Sex	Phenotype	Genotype	Total gland wt (g)	Total gland wt/body wt x 100 (%)	Lipid wt (g)	Lipid wt/total gland wt x 100 (%)
Male	Mutant	K^nK^n	3.86 (1.00)	0.28[a] (0.10)	0.64 (0.31)	16.6[a] (6.8)
Male	Mutant	K^nk	2.04 (0.51)	0.11[b] (0.00)	0.12 (0.06)	5.9[b] (3.8)
Male	Normal	kk	1.31 (0.17)	0.07[c] (0.00)	0.08 (0.00)	6.1[b] (2.5)
Female	Mutant	K^n-	2.55 (0.89)	0.27[a] (0.15)	0.38 (0.32)	14.9[c] (9.6)
Female	Normal	k-	0.89 (0.20)	0.07[c] (0.00)	0.13 (0.04)	14.6[c] (5.4)

[abc]Means in each column with different superscripts are significantly different (P<.05). ( ) Represents the standard deviation.

analyzed by an F and M 810 research gas chromatograph using columns containing Supelco 10% SP2330 on 100/120 mesh Supelcoport at 190 C and 3% SP2100 on 100/120 mesh Supelcoport at 250 C, respectively. A recorder with an integrator was attached to the gas chromatograph and used to quantitate the areas under the peaks. Carbon number plots were made from the retention times of appropriate standards in order to tentatively identify the unknown peaks. An aldehyde derivative was made from the diols for their identification (5).

The mole percent of each carbon chain length of the fatty acids and diols was analyzed between each genotype using analysis of variance. Differences between means which were found significantly different at the 5% level were tested using Newman-Keuls test (6).

RESULTS

Genotype comparisons between uropygial gland and lipid weight relationships are shown in Table I. The homozygous mutant males and hemizygous mutant females had significantly larger uropygial glands than the other chicken types. The gene in the heterozygous mutant males acts as an incomplete dominant as their gland size is in between both homozygous male types and significantly different from both. However, the heterozygous mutant males have relatively the same amount of gland lipid as the normal males. Although the mutant female chickens had uropygial glands relative to body weights four times as large as did the normal female chickens, no difference was found between the two female types in the total lipid relative to the gland size.

In all five genotypes, the fatty acids were found to be saturated and straight chain ranging from 10 to 22 carbons. However, the mole percent of the individual uropygial gland fatty acids were in many cases significantly different between the K^n mutants and the normal chickens. These results are reported in Table II. The normal male and female chickens had greater amounts of the shorter chain fatty acids, 11:0, 12:0, and 14:0, and lesser amounts of the longer chain fatty acids, 17:0, 18:0, 19:0, and 20:0 than the mutant type chickens. The fatty acid compositions of the normal male and female chickens were for the most part similar. The mutant female chickens and the heterozygous mutant male chickens both having one K^n gene were found to have similar fatty acid compositions but differed somewhat from the homozygous mutant males having two K^n genes. The homozygous mutant males were significantly different from the other genotypes

TABLE II

The Fatty Acid Species in the K^n Mutant and in the Normal Chicken

Genotype	Mole percent of total uropygial fatty acids												
	10:0	11:0	12:0	13:0	14:0	15:0	16:0	17:0	18:0	19:0	20:0	21:0	22:0
K^nK^n	3.3^a (1.3)	0.4^a (0.5)	10.4^a (5.0)	0.9^a (0.5)	20.1^a (6.3)	1.2^a (0.6)	14.1^a (3.3)	3.9^a (1.2)	22.2^a (4.8)	14.6^a (3.9)	7.7^a (4.0)	1.3^a (0.9)	trace
K^nk	5.1^a (4.8)	0.5^{ab} (0.4)	14.8^a (4.1)	1.6^a (1.2)	28.6^b (3.5)	1.4^a (0.7)	13.0^a (2.4)	2.1^b (0.4)	15.1^b (3.0)	9.2^b (2.7)	6.9^a (3.3)	1.5^a (1.3)	trace
kk	6.7^{ab} (4.6)	1.0^b (0.6)	22.3^b (5.2)	1.8^a (0.6)	35.5^c (4.9)	1.2^a (0.5)	12.6^a (3.1)	1.3^c (0.3)	9.7^c (3.2)	4.6^c (2.1)	2.7^b (1.2)	0.5^a (0.4)	0.0
K^n-	6.7^{ab} (4.0)	0.3^a (0.4)	13.6^a (6.2)	1.5^a (1.0)	24.8^b (5.3)	1.4^a (1.0)	15.1^a (3.7)	2.8^b (1.2)	16.2^b (5.0)	9.2^b (2.7)	7.4^a (3.5)	0.9^a (0.5)	trace
k-	10.2^b (3.6)	0.8^b (0.4)	25.2^b (4.6)	1.5^a (0.4)	37.2^c (4.1)	0.9^a (0.4)	10.9^a (4.0)	0.9^c (0.3)	6.0^d (2.7)	2.8^c (1.3)	2.9^b (1.3)	0.5^a (0.3)	0.0

[abc]Means of individual fatty acids with different superscripts are significantly different ($P<.05$). ( ) Represents the standard deviation.

TABLE III

The Diol Species in the K^n Mutant and in the Normal Chicken

Genotype	Mole percent of diols											
	20:0	Threo*	21:0	Threo	22:0	Threo	23:0	Threo	24:0	Threo	25:0	Threo
K^nK^n	1.2[a] (0.6)	18.9	8.6[a] (3.5)	16.1	30.8[a] (3.9)	24.4	31.7[ab] (4.1)	29.2	26.0[a] (5.8)	36.8	1.5[a] (1.4)	61.3
K^nk	0.5[b] (0.4)	50.1	4.1[b] (2.2)	31.6	19.2[b] (3.1)	24.4	29.1[b] (1.7)	29.5	43.4[b] (4.4)	40.9	3.4[b] (0.8)	44.6
kk	0.4[bc] (0.4)	56.4	2.2[c] (1.0)	28.4	20.5[b] (3.9)	37.1	24.4[c] (3.5)	42.5	50.6[c] (7.6)	49.9	1.7[a] (0.6)	56.4
K^n-	0.5[b] (0.3)	48.4	4.0[b] (1.8)	10.6	25.1[c] (4.3)	21.0	33.0[a] (2.8)	24.3	35.6[d] (6.1)	30.3	1.8[a] (0.8)	35.4
k-	0.2[c] (0.2)	66.7	1.2[d] (0.3)	61.5	15.0[d] (3.5)	39.3	23.7[c] (3.5)	43.5	57.8[e] (4.6)	52.5	2.2[a] (1.1)	60.4

[abc]Means of individual diols with different superscripts are significantly different ($P<.05$). ( ) Represents the standard deviation. *Relative amount of threo isomer in each mole percent of diol with the remainder being the erythro isomer.

in several of the fatty acids. They had the least amount of 14:0 and greater amounts of 17:0, 18:0, and 19:0. There were no differences between the five genotypes for fatty acids of the 13:0, 15:0, 16:0, 21:0, and 22:0 chain length.

Both erythro and threo diol isomers occurred in all five genotypes. The diols were saturated, straight chain, and ranged from 20 to 25 carbons. Significant differences were found in the mole percent of the individual uropygiols between the different genotypes. These data are shown in Table III. In general, the K^n mutant chickens had greater amounts of the shorter chain diols, 20:0, 21:0, 22:0, and 23:0 and lesser amounts of 24:0 than did the normal chickens. The homozygous mutant males were found to have the greatest amounts of 21:0 and 22:0 and the least amount of 24:0 than the other genotypes.

The percentages of the threo and erythro isomers were also analyzed. There was considerable variation in the amounts of each isomer, but no significant differences were found between the five genotypes.

DISCUSSION

The sex-linked K^n gene has several pleiotropic effects in the domestic chicken including enlargement of the uropygial gland with a corresponding increase in the quantity of lipid within the gland. Total gland enlargement, including tissue and lipid, relative to genotype, appears to respond to a gene effect. The K^nK^n males and K^n- females have equal and maximum gland weights relative to body weights, while the kk males and k- females have equal but much smaller gland weights. The K^nk males, carrying one of each allele, have intermediate gland sizes and ones nearer in size to those of the normal males. Lipid content of these glands, however, do not follow the same pattern. The K^nK^n males, K^n- females, and the k- females all show large lipid contents relative to gland size. The kk and K^nk males, however, have lipid contents which are 2.5 to 3 times less than the other three genotypes. These latter differences do not follow the genotype pattern, but they suggest that testosterone may have influence on the uropygial glands lipid production.

Although the effect of testosterone on the uropygial gland has been debated, both Selye (7) and Kar (8) found that the size of the uropygial gland was depressed following testosterone treatment. No one, however, has observed the effect of testosterone on lipid production in the uropygial gland. Comb size in chickens is a good indication of relative testo-

sterone levels. Somes (2) has shown that at 14 weeks of age the combs of K^nK^n, K^n-, and k-birds are 0.1% or less of body weight, wheras the comb sizes of the K^nk and kk birds are 0.4% or greater of body weight indicating a difference in testosterone production in these two groups of birds.

It would appear from the data presented in Table I that the K^n gene's effect is more directly related to the gland's hypertrophied tissue growth and that lipid production of the gland, regardless of its size, may be directly under hormonal control. If this is so, then both types of females produce relatively large amounts of lipid because they have low levels of testosterone regardless of the allele at the k locus. The effect of the K^n gene on homozygous males is to suppress testosterone production, and at the age of 14 weeks it is only slightly higher than that of the females (2). The heterozygous males (K^nk) with a single K^n gene, on the other hand, have testosterone levels and uropygial gland sizes which are only slightly different than those of the normal males (kk) (2). These testosterone level differences, as judged by comb growth, tend to match the lipid production date in Table I and are offered here as a possible explaination for the differences observed in the data.

Although the diesters in the uropygial gland lipid of birds with the K^n gene are qualitatively the same as those in the normal chicken's gland, there are significant differences in the distribution of some of the individual fatty acids and diols. There appears to be very little difference due to sex among the fatty acids of the normal males and females. Of the 13 fatty acids measured, the only difference was in the 18:0 fatty acid. There was a 50% increase in this fatty acid in males as compared with the females. Within the diol fraction there were significant differences in the 21:0. 22:0, and 24:0 diols between the normal males and females.

The effect of the K^n gene on the fatty acid distributions was such that there was a decrease in the shorter chain fatty acids (11:0-14:0) and an increase in the longer chain (17:0-20:0) acids. There were no signifcant differences between any of the fatty acids of the K^n- and K^nk birds. The K^nK^n males were also similar to the K^nk and K^n- birds in the distribution of nine of the fatty acids but showed significant decreases in 14:0 and significant increases in 17:0, 18:0, and 19:0. Thus, it appears that the K^n gene has a definite dosage effect on some of the fatty acids. One mutant gene significantly effects some of the fatty acids while two mutant genes further influence four of these.

The effect of the K^n gene on the diols was to significantly decrease the 24:0 and significantly increase the 20:0, 21:0, 22:0, and 23:0 diols. The K^nk and K^n- birds showed equal amounts of the 20:0 and 21:0 diols, but the K^n-females had significantly more 22:0 and 23:0 and significantly less 24:0 and 25:0 than did the K^nk males. The K^nk^n males were similar to both the K^nk and K^n- birds in the 23:0 diols but had significantly more of the 20:0, 21:0, and 22:0 and significantly less of the 24:0. Thus, it would appear that for the diol fraction one K^n gene significantly influenced five of the six diols and that two doses of the gene effected four of these to a significantly greater extent.

The normal chicken's fatty acids and diols are similar to the values reported in the literature (9). The mutant chicken's fatty acids and diols are also similar to values in the literature, but they more closely resemble those of older chickens (9). Birds with the K^n mutant gene lack feathers in early life, and as they mature they never obtain the same amount of feather growth as do normal chickens. It has been shown that chickens with less feather cover have an increased basal metabolic rate over that of chickens with normal feather cover (10). According to Brody (11), there is a theory which says that "any factor which accelerates the metabolic processes beyond a relative low level should accelerate the rate of aging and shortens the life span." When chickens with the K^n gene are raised at a suboptimal temperature, their continuously elevated metabolic rate may cause them to age faster. If this is true, it could be hypothesized that the changes that occurred in the uropygial lipid may be due to the chicken's increased rate of aging.

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Generation of Phospholipid Artefacts during Extraction of Developing Soybean Seeds with Methanolic Solvents

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ABSTRACT

The major phospholipids of soybean cotyledons during development were phosphatidylcholine (45-55%), phosphatidylethanolamine (24-28%), and phosphatidylinositol (15-18%) when the tissue was steam-killed prior to extraction of the lipids. The only other phospholipids of any significance (4-6%) was identified as phosphatidylglycerol. Phosphatidic acid was a minor constituent (<1%), and neither N-acyl phosphatidylethanolamine nor *bis*-phosphatidic acid were detected in appreciable (> 0.1% of the total lipid phosphorus) quantities. When fresh cotyledons were rapidly homogenized in mixtures of chloroform and methanol or in methanol alone, phosphatidylmethanol was formed in variable amounts (0-20% of the total phospholipid), and when cotyledons were soaked in methanol prior to homogenizing, phosphatidylmethanol became the major phospholipid, accounting for up to 75% of the total lipid phosphorus. Phosphatidylmethanol was formed by the phospholipase D-catalyzed transphosphatidylation of phosphatidylcholine and phosphatidylethanolamine during extraction.

INTRODUCTION

There is some confusion over the phospholipid composition of developing soybean seeds; phosphatidic acid (1,2), N-acyl phosphatidylethanolamine (3), *bis*-phosphatidic acid (4), and phosphatidylcholine (5) have, in turn, been identified as quantitatively the major phospholipid in such tissue. In contrast, there has been general agreement that phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) account for 85-90% of the total phospholipids of mature soybeans (2,3). Phosphatidic acid (PA) is now considered to be a minor constituent of fresh, developing seeds but is generated in vivo upon freezing and thawing the tissue prior to analysis (6). Soybeans which were analyzed without prior freezing and thawing apparently contained N-acyl phosphatidylethanolamine (NAPE) at levels as high as 65% of the total phospholipid (3). More recently, it has been suggested that the identification of NAPE may have been erroneous and that *bis*-phosphatidic acid (BPA) instead accounts for 30-40% of the total phospholipid in very immature soybean seeds (4). The latter, it was reported, has chromatographic mobilities similar to those of NAPE. Both NAPE (7) and BPA (4) exhibited a high rate of fatty acid turnover relative to other glycerolipids.

Recent extensions of earlier studies on polyunsaturated fatty acid biosynthesis in leaf tissues (8,9) to developing seeds of safflower, linseed, and soybean (5,10) have shown that in each species PC accounted for more than 50% and PE for about 20% of the total, extractable phospholipid. In these studies, and in accordance with recommendations (11,12) for preventing the phospholipase D-catalyzed transphosphatidylation of PC and PE during extraction, the cotyledons were steam-killed before analysis. Preliminary evidence was presented (5) that the compound previously identified as the major phospholipid in developing soybean cotyledons (3) was not NAPE but was probably phosphatidylmethanol (PM) formed from the breakdown of PC and PE during extraction of the fresh tissue with methanol (11). Since neither NAPE nor BPA were apparently of any quantitative significance in extracts of steam-killed soybean cotyledons, it seemd possible that these compounds could have been artefacts arising from the isolation procedures employed.

In an attempt to resolve this uncertainty, we have now studied the effects of different extraction techniques on the recoveries of individual phospholipids of immature soybean seeds and have undertaken a rigorous identification of the most variable constituent, PM.

MATERIALS AND METHODS

Phosphatidylcholine was isolated from egg yolks by alumina column chromatography (13) followed by preparative thin layer chromatography (TLC) or was purchased from Sigma Chemical Co., St. Louis, MO. Phosphatidylmethanol and PA were prepared from egg Yolk PC by an enzymatic procedure. The lipid (200 μmole) in 10 ml of methanol was added to 80 ml of 62.5 mM sodium acetate, pH 5.5, containing 50 mM $CaCl_2$ to produce a slightly turbid solution. Three ml of a phospholipase D preparation (4.5-5.0 mg protein) was mixed in, water was added to bring the final volume to

100 ml, and the mixture was incubated at 20-22 C for ca. 90 min. Increasing turbidity indicated that the reaction was proceeding, and after about 30 min the calcium salts of the lipid reaction products began accumulating at the surface of the liquid. The reaction was terminated by the addition of 90 ml of methanol, the phospholipids were extracted into 100 ml of chloroform, and the chloroform was washed once with 190 ml of 53% (v/v) methanol containing 5 mmole of HCl to convert the phospholipids to the free acids. This procedure yielded about 160 μmole PM, 30 μmole PA, and 5 μmole unchanged PC, all of which were readily separated by DEAE-cellulose column chromatography (14). Phospholipase D was prepared by homogenizing fresh cauliflower florets in 2 volumes of water, filtering the homogenate through Miracloth, and centrifuging the filtrate for 10 min at 13,000 g. The supernatant solution contained the enzyme. Having eluted from DEAE-cellulose as ammonium salts, PM and PA in chloroform were reconverted to the free acid form by partitioning against aqueous methanol containing 1-2 mmole of HCl and in this form were methylated using diazomethane (15). The appearance of the doublet at 3.6 and 3.8 ppm, characteristic of the CH_3OP- group (15), in the NMR spectrum of both PM and of the phosphatidyldimethanol (PMM) synthesized either from PM or from PA, confirmed the identifies of these lipids, which were then used as standards.

Phosphatidylglycerol was purified from white clover leaves using a combination of silicic acid (16) and DEAE-cellulose (14) column chromatography, while phosphatidylglycerol-^{14}C was isolated from 1-^{14}C-acetate-fed, mature maize leaves (8) by silicic acid column chromatography and preparative TLC.

Soybean plants (cv Amsoy) were grown in a glasshouse in soil-less media and were irrigated with nutrient solution. Pods at different stages of maturity were removed as required, the seeds dissected out, and normally separated into cotyledons and seed coats. For quantitative analyses of the constituent phospholipids, weighed amounts of cotyledons or seed coats were steamed for 15 min prior to comminution in 20 vol of chloroform-methanol (C/M; 2:1, v/v) using a Polytron PT 20 homogenizer (Kinematica, Lucerne, Switzerland). Filtrates from these homogenates were partitioned against 0.2 vol of 0.9% (w/v) NaCl. When testing the effect of different extraction techniques on the types and amounts of phospholipid recovered, the fresh or steamed cotyledons were pretreated and homogenized as described in the text and in the tables. Filtered homogenates plus washings were brought to 2:1 (v/v) or 1:1 (v/v) with respect to chloroform and methanol and were washed according to Folch et al. (17) and Bligh and Dyer (18), respectively. Washed lipid extracts were dried on a rotary evaporator and the dry lipid redissolved in chloroform so that 1 ml of lipid solution was equivalent to 1 g fresh weight of cotyledons. These lipid extracts were initially analyzed by two-dimensional TLC; 100 μl ($\equiv$ 100 mg fresh tissue) was streaked across 1 cm near one corner of a 20 x 20 cm layer of Silica Gel G, and the chromatogram was developed using $C/M/NH_3$ (65:25:2, by vol) in the first direction and $C/M/HAc/H_2O$ (85:15:10:3, by vol) in the second direction. The adsorbent was reactivated between the two developments during 30 min under reduced pressure (0.5 mm Hg). Chromatograms were sprayed with 50% (v/v) H_2SO_4 and were heated to 120 C so that steroids, glycolipids, and phospholipids could be recognized by their characteristic colors (19). In some cases, the separated lipids were lightly stained with iodine vapor, the iodine removed under reduced pressure, and the phospholipids transmethylated using $NaOCH_3$ in methanol (8). Total lipid extracts were also fractionated into nonpolar lipids, glycolipids, and phospholipids by silicic acid column chromatography (16) and into nonpolar lipids, neutral polar lipids, and acidic lipids by DEAE-cellulose column chromatography (14). The constituents of these various fractions were resolved by unidirectional TLC in either of the solvents used for two-dimensional TLC. Fatty acid methyl esters were analyzed by gas liquid chromatography using columns of 15% ethylene glycol succinate on Chromosorb W.

Phospholipids were deacylated (20), and the water-soluble products were separated on Whatman 3 MM paper using the solvent system phenol-water-ethanol-acetic acid (50:22:3:3, by vol). Phosphorus-containing spots were located by spraying the dried chromatograms with the $FeCl_3$-sulphosalicylic acid reagent (21). Glycerol:phosphorus:acyl group ratios for purified lipids were determined essentially according to the method of Townsend et al. (22), NMR spectra were recorded using 20% (w/v solutions of the purified lipids in CCl_4 (TMS as external standard), and mass spectra were obtained from samples introduced into the mass spectrometer via the solid probe and subsequently heated to 200 C. Phospholipase D activity was measured in situ and in vitro by previously published methods (23,24).

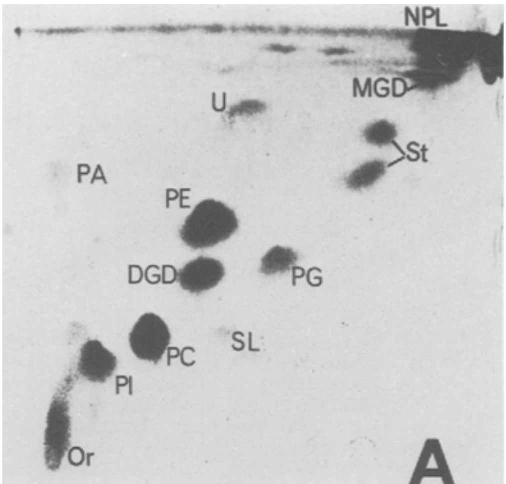
RESULTS

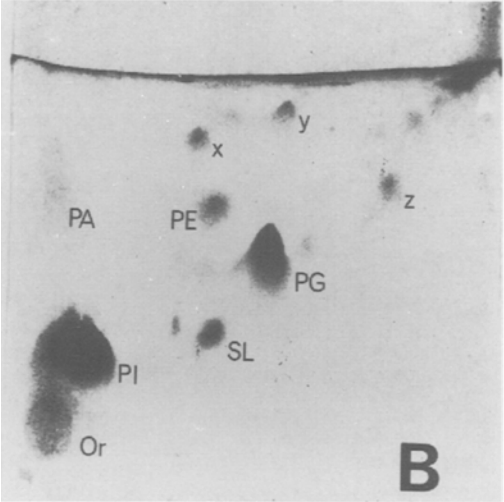
Extraction Method and Phospholipid Composition

The major phospholipids of soybean seeds at all stages of development were PC, PE, and PI when the tissue was killed by steaming prior to lipid extraction (Fig. 1A). Extracts of fresh seeds or cotyledons, homogenized in methanol or in mixtures of chloroform and methanol, contained varying amounts of another phospholipid with higher chromatographic mobilities (Fig. 1C) than the above phospholipids in both of the solvents used in the two-dimensional TLC. When fresh tissue was homogenized rapidly in 10-20 vol of solvent, this lipid accounted for 0-20% of the total phospholipids, but when sliced seeds or cotyledon halves were soaked in methanol before homogenizing or when the killing technique of Wilson and Rinne (3) was used, the less polar lipid became a major constituent accounting for 25-75% of the total phospholipids of the extract (Table I). This lipid was absent from extracts of steam-killed tissues irrespective of how the extracts were prepared. Since the amount of total phospholipid extracted was similar for all of the methods used (Table I), the variable phospholipid was probably formed during homogenization by the breakdown of PC and PE in a phospholipase D-catalyzed transphosphatidylation (11,12). The conditions under which the lipid was formed in greatest amounts were similar to those proposed for an in situ assay of phospholipase D in plants (23); the fatty acid composition of the lipid (Table II) was consistent with its formation from PC and PE and so the fluctuating phospholipid was tentatively identified as phosphatidylmethanol.

Characterization of Phosphatidylmethanol

Five grams (fresh weight) of soybean seeds





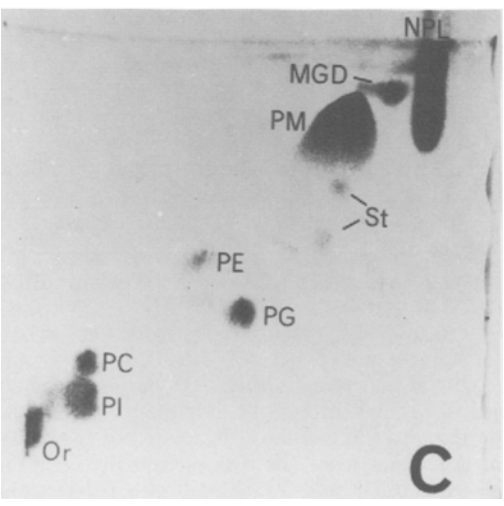


FIG. 1. Two-dimensional TLC separations of polar lipids in extracts of immature soybean cotyledons. A. Total lipids in chloroform-methanol extracts of steam-killed cotyledons. B. Acidic lipids in chloroform-methanol extracts of steam-killed cotyledons. C. Total lipids in chloroform-methanol extracts of unsteamed cotyledons which had been soaked in 10 vol of methanol before being homogenized in the methanol. Chloroform was added to the homogenate. Amounts of lipid chromatographed were equivalent to 100 mg fresh weight of tissue in A and C and 500 mg fresh weight of tissue in B. Developed chromatograms were sprayed with 50% (v/v) H_2SO_4 and heated to 120 C. Or = origin; PI = phosphatidylinositol; PA = phosphatidic acid; PC = phosphatidylcholine; DGD = digalactosyl diacylglycerol; SL = sulfolipid or sulfoquinovosyl diacylglycerol; PE = phosphatidylethanolamine; U = unknown; PG = phosphatidylglycerol; St = sterols; MGD = monogalactosyl diacylglycerol; NPL = nonpolar lipids; x, y, and z were unidentified acidic lipids.

TABLE I

Effect of Different Extraction Procedures on the Phospholipid Composition of Soybean Cotyledons[a]

Extraction procedure	μmole Lipid per gram fresh wt cotyledons					
	PC	PE	PI	PG	PA	PM
1	5.95	2.50	1.79	0.35	0	0
2	6.00	2.60	1.80	0.45	0	0
3	6.15	2.65	1.75	0.48	0	0
4	5.52	2.40	1.84	0.45	0	0.86
5	4.55	1.23	1.84	0.40	0.11	2.59
6	0.54	0.30	1.70	0.30	0.36	7.54

[a]Cotyledons from seeds 30-40 days after flowering were randomized into 1-g lots and (*1*) steam-killed prior to extraction into 20 ml of chloroform-methanol (2:1, v/v); (*2*) steam-killed prior to suspension in 3 ml of tap water and extraction by the method of Wilson and Rinne (3); (*3*) extracted fresh into 20 ml of chloroform-methanol (2:1, v/v); (*4*) extracted fresh into methanol-chloroform-water (26:8:6, by vol) (6); (*5*) treated as for (*2*) except that tissue was not steam-killed; (*6*) soaked in 10 ml of methanol for 5 min then homogenized in the methanol prior to adding 20 ml of chloroform. After filtering and washing, all extracts were concentrated to 1 ml in chloroform and 100 μl was analyzed by two-dimensional TLC.

TABLE II

Fatty Acid Composition of Phospholipids of Developing Soybean Cotyledons Extracted with Methanol[a]

Phospholipid	% Of total fatty acids				
	16:0	18:0	18:1	18:2	18:3
PC	14	6	14	49	18
PE	20	2	7	44	26
PM	16	4	10	44	26
PI	31	8	13	32	16

[a] Cotyledons (1 g) from developing seeds were homogenized in 10 ml of methanol before 20 ml chloroform was mixed in and the slurry filtered. The extract contained 4.4 μmole PC, 2.2 μmole PC, 2.6 μmole PM and 1.6 μmole PI. An amount of extract equivalent to 100 mg of tissue was chromatographed in the two-dimensional TLC system, the separated lipids were detected by very light staining with iodine vapor and were transesterified in $NaOCH_3$ in methanol (8). Fatty acid methyl esters were analyzed by gas liquid chromatography.

were sliced and soaked in 50 ml of methanol for 5 min and then homogenized in the methanol. Chloroform (50 ml) was mixed in, the slurry filtered, and the filtrate washed with 0.45 vol of 0.9% (w/v) NaCl. The extract contained 32.1 μmole of PM which was isolated by DEAE-cellulose column chromatography and preparative TLC. The lipid from soybean (a) was a nitrogen-free phospholipid which behaved as an acidic lipid on DEAE-cellulose; (b) showed high mobility on silica gel in both alkaline and acidic developing solvents and cochromatographed with standard PM in the two-dimensional TLC system; (c) had a glycerol: phosphorus:acyl ratio of 1.00:1.15:2.04; (d) produced a water-soluble deacylation product which cochromatographed on paper with that of standard PM (R_f 0.61) but which was readily distinguished from the deacylation products of PG (R_f, 0.51) and PA (R_f, 0.40); (e) reacted with diazomethane to form a derivative which cochromatographed with phosphatidyldimethanol (PMM) synthesized from standard PM (15).

The PMM synthesized from soybean PM was a mixture of molecular species and when analyzed in the mass spectrometer produced just discernible molecular ions at m/e 720, 722, 724, and 726. These correspond to molecular species of PMM containing two 18-carbon fatty acids having a total of 6, 5, 4, and 3 double bonds, respectively. Stronger peaks at m/e 594, 596, 598, 600, and 602 represented the diacylglycerol portions of molecules containing two 18-carbon acyl chains, while another group of peaks at 572, 574, and 576 represented diacylglycerols from molecules containing one 18-carbon and one 16-carbon acyl chain. The strongest peak occurred at m/e 127 and corresponded to the dimethyl-phosphate ion (25)

(Fig. 2). Relative abundances of the most prominent fragments in a single scan were m/e 127 (100), 574 (36), 596 (22), and 722 (0.4).

From the combined data, we conclude that the variable phospholipid was indeed PM.

The Acidic Lipids of Developing Soybean Seeds

Column chromatography using DEAE-cellulose (acetate) provided a convenient means of separating the acidic lipids from the neutral and bipolar lipids, hence concentrating into a single fraction any PA, NAPE, and BPA present in extracts of soybean seeds. In fact, the acidic lipids of developing soybeans contained in significant quantities only PI and another phospholipid (Fig. 1B) tentatively identified as phosphatidylglycerol (PG). The identification of this acidic, soybean phospholipid was based on (a) its cochromatography in two dimensions with PG from white clover leaves; (b) its precise coincidence in two-dimensional TLC and radioautography with ^{14}C-labeled PG from maize leaves; (c) the measured glycerol:phosphate: acyl ratio of 2.00:1.02:1.94; (d) the cochromatography on paper of its deacylation product with that of PG from white clover leaves; and (e) the high proportion (> 50% by weight of the total fatty acids) of palmitate in its constituent fatty acids – a property shared with PG of etiolated pea and bean leaves (26). Other constituents on the chromatogram of acidic lipids (Fig. 1C) either contained no phosphorus or contained phosphorus in amounts which were < 3% of that in PI. A spot giving a characteristic glycolipid stain chromatographed with mobilities similar to those of the plant sulfolipid.

$$HO-\overset{\overset{OH^+}{\|}}{\underset{\underset{OCH_3}{|}}{P}}-OCH_3$$

FIG. 2. The dimethylphosphate ion; a fragment produced in the mass spectrometric analysis of phosphatidylmethanol.

Phospholipase D in Developing Soybeans

Confirming the assumption that the formation of PM resulted from the degradation of PC and PE, choline was detected in the aqueous methanol phase of washed lipid extracts (17) only when significant amounts of PM had been formed during extraction. Both cotyledons and seed coats exhibited high phospholipase D activity in an in situ assay with about 75% of the endogenous PC and PE being converted to PM in the assay. However, only seed coats showed high activity in the in vitro assay (24); either something in the aqueous extract of cotyledons prevented the enzyme from reacting with exogenous PC or the enzyme was pelleted with the cellular debris. At 12-15 μmole choline released/min/mg protein (which was equivalent to 0.24-0.30 μmole choline released/min/mg fresh weight of seed coats), the specific activity of the enzyme in seed coat extracts was higher than that previously reported for any tissue with the possible exception of cauliflower floret (24,27). Phospholipase D was readily detectable in situ in cotyledons from very large seeds (0.4-0.5 g fresh weight) immediately prior to maturation but could not be detected in whole mature seeds.

TABLE III

Relationship of Seed Coat to Total Seed Fresh Weight at Different Maturities[a]

Seed fresh weight (g/10 seeds)	Proportion of seed coat (% of total seed fresh wt)
0.17 g	61
0.58 g	39
2.50 g	25
4.50 g	14
2.67 g (mature)	6

[a]Ten to twenty freshly harvested seeds were weighed, then the seed coats were removed and both coats and cotyledons weighed separately to get a measure of the proportion of seed coat. All tissue was kept at 0-4 C.

Since the seed coat was such a potent source of phospholipase D activity, it was of interest to compare the proportions of seed coat to toal seed fresh weight at different stages of development. In very immature seeds, the coat weighed more than the cotyledons, but by maturity the coat contributed only 5-6% of the fresh weight of the whole seed (Table III).

The Phospholipids from Seed Coats of Developing Soybeans

Highest levels of PA (2), NAPE (3), and BPA (4) have been associated with very immature seeds in which the seed coat represented a high proportion of the seed weight (above) and it was, therefore, possible that these phospholipids were concentrated in the seed coat. However, when separated seed coats were steam-killed prior to extraction or were homogenized quickly in 20 vol of chloroform-methanol (2:1, (v/v), the tissue was found to contain the same phospholipids in the same proportions as those in the cotyledons, but only 65-80% of the total phospholipid of cotyledons on a fresh weight basis, depending upon the stage of seed development.

DISCUSSION

The results presented here indicate that developing soybean seeds, like developing flax seeds and developing safflower seeds (5), contain PC, PE, and PI as their major phospholipids. Of these, PC accounts for about one-half of the total phospholipid, whereas PA, NAPE, and BPA, if present, represent together something less than 1% of the total phospholipid of developing soybeans. In our view, the identification of NAPE as the major phospholipid in developing soybeans (3) was erroneous and that lipid was in fact PM, and artefact produced from the degradation of PC and PE in the extraction process. An inability to find high concentrations of "NAPE" in mature seeds (3) may now be explained by the absence of detectable phospholipase D in that tissue. The compound X, or PX, of Stearns and Morton (28) may also have been PM since it did not cochromatograph with synthetic NAPE (28), and it had a fatty acid composition which was almost exactly midway between that for PC and PE (28). More recently, however, PX has been identified as BPA (4), a lipid which exhibited a high turnover rate with respect to its fatty acids and which could account for 30-40% of the total phospholipids in developing soybeans (4). We used the chromatographic techniques described by Stearns and Morton (4) to resolve further a phospholipid fraction (from silicic acid columns) and an acidic lipid fraction (from DEAE-cellulose columns) from developing soybeans and concluded that the zone identified as predominantly BPA by those authors was in our preparations predominantly PG. Since PG accounted for 3-5% of the total phospholipid in our preparations and the recovery of pure BPA suggested a similar proportion for that lipid in the work of Stearns and Morton (4), this could be another case of mistaken identity. One piece of circumstantial evidence favoring this possibility is that BPA was undetectable in mature seeds (4). If the lipid was really PG, then this would be consistent with the virtual disappearance of the other plastid membrane lipids, monogalactosyl and digalactosyl diacylglycerols, at ripening (2). But, while consistent with the remainder of their analytical data, the recasting of BPA as PG would contradict the glycerol:phosphorus:acyl ratio (2:1:4) reported for BPA by Stearns and Morton (4). We have no explanation for the apparent anomaly.

We could find no evidence for large oscillations in the phospholipid composition of soybean cotyledons during their development when the tissue was steam-killed prior to extraction. However, when fresh tissue was homogenized in chloroform-methanol (2:1, v/v), there were slight differences between replicated extractions of samples from the same bulked material. In some of these extracts there was no PM, whereas in others PM could account for 8% of the total phospholipid. Extracts from cotyledons initially homogenized in methanol alone showed a much greater variation with PM constituting 5-25% of the total phospholipid. We suggest that these variations are likely to be a result of slight differences in the homogenizing procedure; variations in the time in which the fresh tissue is exposed to solvents, the actual speed and efficiency with which the tissue is comminuted, and the relative volumes of solvent to tissue are all likely to influence the period available for endogenous phospholipase D to act upon PC and PE. Thus, in our experience, extracts prepared from the same batch of fresh cotyledons using the Polytron PT20 homogenizer contained less PM than extracts prepared using a Ten Broeck tissue grinder. When using a rapid and efficient homogenizer, the only way to ensure very high proportions of PM, such as those found by Wilson and Rinne (3,6) in lipid extracts, was to soak cotyledon halves in methanol for 2-5 min before beginning homogenization. This implies that the methanol enters the cells and stimulates the transphosphatidylation reaction before the cells are disrupted and the membrane lipids assimilated into the solvent.

Providing the tissue was homogenized rapidly in 20 vol of chloroform-methanol (2:1, v/v) so that no PM was formed, the phospholipid composition of steamed and unsteamed cotyledons was identical. Steam-killing, therefore, provides a convenient means of ensuring that no artefacts are formed during extraction and would probably also prevent PC and PE breakdown during storage of the seeds at low temperatures.

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Incorporation of [1-^{14}C] Acetate into Lipids of Soybean Cell Suspensions[1]

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ABSTRACT

Suspension cultures of soybean cells incorporated [1-^{14}C]acetate very rapidly into the fatty acid moieties of phospholipids and glycolipids when incubated at 26 C for up to 22 hr. The most rapidly labeled lipid was 3-*sn*-phosphatidylcholine, which contained 58% of the total fatty acid radioactivity after 16 min; more than 75% of this label was found to be in the oleic acid of the phosphatidylcholine. After longer periods of incubation, the proportion of ^{14}C label decreased exponentially in phosphatidylcholine and increased markedly in an unidentified phospholipid (tentatively, *bis*-phosphatidic acid), di- and triacylglycerols, and glycolipids. The proportion of radioactivity in oleic acid also decreased exponentially, accompanied by increases in linoleic acid first and then in linolenic acid. Most of the labeled linolenic acid at 22 hr was found in the unidentified phospholipid, di- and triacylglycerols, and the glycolipid fraction.

INTRODUCTION

Increased chilling resistance in seedlings has been associated with increased contents of unsaturated fatty acids, e.g., linoleic and linolenic acids, in membrane lipids (1,2). Studies in this laboratory on biochemical parameters selecting for increased cold tolerance in soybeans require a knowledge of the biosynthetic pathways for these polyunsaturated fatty acids. Use of plant cell culture techniques is of great potential value in such studies (3-5).

Previous investigations of lipid metabolism in soybean suspension cultures have been mainly concerned with incorporation of [^{14}C] acetate into fatty acids of total lipids (6,7) or incorporation of ^{14}C-labeled fatty acids into lipid classes (7).

The present communication reports on the kinetics of incorporation of [1-^{14}C]acetate into individual lipids of soybean cell suspension cultures and the distribution of ^{14}C among the fatty acids in the individual lipids.

EXPERIMENTAL PROCEDURES

Materials

[1-^{14}C]Acetate (49 mCi/mmole), [1-^{14}C] linoleic acid, and [1-^{14}C]linolenic acid were purchased from New England Nuclear, Lachine, Que. Radioactivity was determined in Aquasol liquid scintillation cocktail obtained from the same source. Gas chromatographic column packings were obtained from Supelco, Bellefonte, PA. All solvents were of reagent grade and were distilled before use.

Cell Culture

Suspension cultures of soybean (*Glycine max* (L) Merr. strain PI 189-950) were grown in the mineral salt medium described by Murashige and Skoog (8) using the vitamin and hormone supplements described for the B5 medium by Gamborg (9). The cultures (40 ml) were maintained at 26 C with shaking at 170 rpm in 125 ml Erlenmeyer flasks under fluorescent light (3000 lux) and were subcultured every 5-6 days.

[1-^{14}C] Acetate Incorporation

For long term labeling of cells, a 40-ml culture of 4-day-old cells was added to 500 ml of fresh medium and incubated for 4 days in a 2.8 liter Fernbach flask. One mCi of [1-^{14}C] acetate was added and incubation continued for 66 hr. Cells were harvested by vacuum filtration, and lipids were extracted as described below.

For kinetic studies, 50 μCi of [1-^{14}C] acetate was added to a 40-ml culture of 4-day-old cells; 5-ml aliquots were removed after 10, 16, 45, 85 and 155 min, and after 22 hr incubation at 26 C, and were extracted with 5 ml of boiling isopropanol (10,11); the mixture was centrifuged and the residue extracted twice with 5 ml of methanol-chloroform (2:1). The combined extracts were brought to dryness under reduced pressure and total lipids were extracted from the residue by the method of Bligh and Dyer as described elsewhere (10,11). The total lipids were dissolved in chloroform

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and aliquots were taken for thin layer chromatography (TLC) and determination of radioactivity.

Chromatographic Separations

Separation of the lipid classes was achieved by chromatography on a column of silicic acid which was eluted with chloroform (neutral lipids), acetone (glycolipids), and methanol (phospholipids) as described elsewhere (10). Each fraction was further separated by TLC on Silica Gel H with chloroform-methanol-28% ammonia (65:35:5, v/v) for phospholipids, chloroform-methanol (90:10, v/v) for glycolipids, and petroleum ether (bp 60-70 C)-ethyl ether-acetic acid (80:20:1, v/v) for neutral lipids. The components were identified by comparison of their mobilities with those of authentic lipid standards and by the use of specific spray reagents (10). Two-dimensional TLC was performed with chloroform-methanol-28% ammonia (65:35:5, v/v) in the first dimension and chloroform-acetone-methanol-acetic acid-water (10:4:2:2:1, v/v) in the second dimension. Spots were located by spraying the plates with a 0.02% solution of 2′,7′-dichlorofluorescein and viewing under ultraviolet light. Lipids were eluted from the silica gel with chloroform-methanol (1:1), the eluates were taken to dryness under N_2, and the residues were dissolved in chloroform. Aliquots were taken for deacylation and ^{14}C-counting.

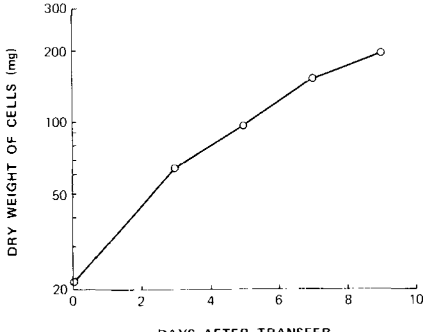

FIG. 1. Growth curve of soybean cells in suspension culture after transfer to fresh medium. Cells from each culture flask were harvested by filtration, washed, and dry weight was determined after 2 days freeze-drying followed by 1 hr at 110 C.

Analysis of ^{14}C-Labeled Fatty Acids

Total labeled lipids were saponified by refluxing with 90%-methanolic NaOH (0.3N) for 1 hr; sterols and other nonsaponifiables were extracted with petroleum ether (bp 40-60 C); the alcoholic phase was acidified with 6N HCl,

TABLE I

Lipid Analysis and Fatty Acid Composition of Individual Lipids
(5-Day-Old Soybean Suspension)

Lipid	% by weight	Fatty acids (% by weight)[a]					
		14:0	16:0	18:0	18:1	18:2	18:3
Total lipid	100	tr	22.0	3.1	5.5	18.9	50.9
Phospholipids							
Unidentified phospholipid[b]	0.2	0.9	26.9	6.5	18.2	23.8	22.5
Phosphatidylglycerol	6.8	---	35.0	4.0	10.0	22.5	28.2
Phosphatidylserine	trace	---	---	---	---	---	---
Phosphatidylethanolamine	24.1	---	24.1	2.9	14.4	30.7	28.0
Phosphatidylcholine	23.3	---	18.4	4.1	16.0	26.5	35.1
Phosphatidylinositol	4.3	---	31.4	5.5	10.3	22.9	30.0
Phosphatidic acid	0.2	---	26.4	7.6	13.6	23.9	28.5
Glycolipids							
Monogalactosyl diacylglycerol	3.8	---	3.6	1.5	5.0	7.0	82.2
Digalactosyl diacylglycerol	7.6	---	12.3	3.2	6.0	8.5	70.0
Esterified steryl glycoside	3.6	---	37.3	8.3	16.1	11.7	26.6
Steryl glycoside	2.8	---	---	---	---	---	---
Cerebroside	1.0	---	---	---	---	---	---
Neutral Lipids							
Triacylglycerol	13.1	---	2.9	1.0	11.3	24.6	60.2
Free fatty acid	0.1	3.6	21.6	5.7	14.8	17.5	36.8
Diacylglycerol	0.4	2.4	25.1	6.8	23.8	19.3	22.8
Sterols	7.1	---	---	---	---	---	---
Hydrocarbons	0.6	---	---	---	---	---	---
Unidentified alcohols	1.0	---	---	---	---	---	---

[a]Trace amounts of 15:0, 16:1, and a long chain fatty acid were also detected, but not 16:1-Δ^3-*trans* or 16:3.
[b]Tentatively, *bis*-phosphatidic acid (see ref. 17).

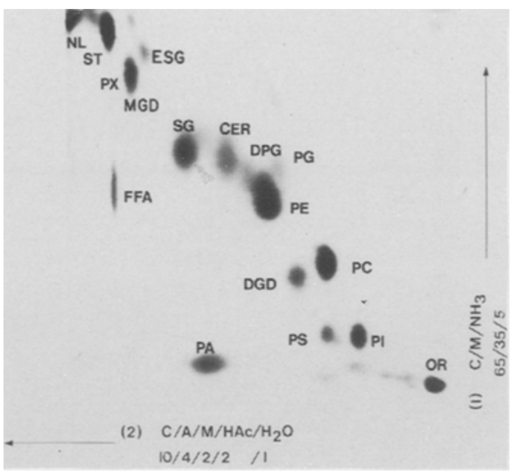

FIG. 2. Autoradiogram of a two-dimensional thin layer chromatogram of ^{14}C-labeled total lipids of soybean suspension cells (4-day-old cells labeled with [1-^{14}C]acetate for 66 hr at 26 C). First dimension: chloroform-methanol-18% ammonia (65:35:5, v/v); second dimension: chloroform-acetone-methanol-acetic acid-water (10:4:2:2:1, v/v). Abbreviations: NL: neutral lipid; SG, sterylglycoside; ESG, esterified sterylglycoside; CER, cerebroside; DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PA, phosphatidic acid; MGD, monogalactosyldiacylglycerol; DGD, digalactosyldiacylglycerol; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; OR, origin.

and the fatty acids were extracted with petroleum ether (10). Fatty acid methyl esters were prepared by treatment of the free acids with a freshly prepared solution of diazomethane (10).

Fatty acid methyl esters from individual lipids separated by TLC were prepared directly by adding 2 ml of 2.5% methanolic-HCl to the silica gel scraped from the plate and heating under reflux for 1 hr (10). Water (0.2 ml) was added, and the methyl esters were extracted with petroleum ether. Fatty acid methyl esters were analyzed by gas chromatography on a column of 5 % SP-2310 on 100/120 Chromosorb W (AW) (6 ft x 4 mm) at 190 C, using a flame ionization detector. The column was fitted with an effluent stream splitter, and radioactive methyl esters were either collected on cellulose filters (12) and counted in the scintillation cocktail described above, or were monitored continuously by a gas radioactivity counting system (Perkin-Elmer). Radioactive fatty acid methyl esters were also separated according to their degree of unsaturation by TLC on 10% $AgNO_3$-Silica Gel H as described elsewhere (13). Radioactive areas were detected by a plate scanner (Varian) and scraped into scintillation vials for counting ^{14}C. Fatty acid methyl esters were identified by comparison of retention times and mobilities to those of standards (e.g., [^{14}C]linoleate, [^{14}C] linoleate, etc.).

Deacylation of Lipids

Individual lipids separated by TLC were deacylated as described elsewhere (10), and water-soluble phosphate esters and other products were identified by paper chromatography in phenol-water (100:38, w/v).

RESULTS AND DISCUSSION

Soybean Cell Cultures

The growth rate of the soybean cells grown in suspension culture, as assessed by increase in dry weight (Fig. 1), was much greater (doubling time 48 hr) in the revised Murashige-Skoog medium (8) than that previously reported for cells grown in the B5 medium (6).

The total lipid content of the cells averaged about 6% on a dry weight basis in agreement with previously reported values (3). The fatty acid composition (Table I) of the total lipids was characterized by a high concentration of linolenic acid and resembled most closely that of soybean leaves, as noted before by Tattrie and Veliky (14).

The total lipids of 4-day-old soybean cell cultures contained about 59% phospholipids, 19% glycolipids, and 22% neutral lipids (Table I) in general agreement with the values reported by Stearns and Morton (6). A typical separation of these components is shown in Figure 2, and their quantitative analysis and fatty acid composition are given in Table I. The present results are in marked contrast to the low triacylglycerol, phospholipid, galactolipid, and sulfolipid contents and elevated sterol, sterol ester, sterol glycoside, and esterified sterol glycoside contents reported earlier for soybean suspension cultures (3). The differences may well be due to lipolysis occurring during extraction at room temperature with solvents such as chloroform-methanol or ether (3), but not with hot isopropanol as in the procedure used here (10,11), nor with boiling chloroform-methanol (6). Such lipolytic activity would result in preferential hydrolysis of the glycerolipids producing high contents of free fatty acids and resulting in artificially elevated contents of sterols and sterol glycosides. The fact that relatively low contents of free fatty acids, phosphatidic acid, and lyso-compounds were found in our culture extracts (Fig. 2, Table I) confirms the absence of significant phospholipase activity during the extraction procedure.

TABLE II

Incorporation of [^{14}C] Acetate into the Lipids of Soybean Cell Suspension Cultures[a]

Incubation time (min)	Total radioactivity in lipids 10^{-3} x dpm per ml culture	% Distribution of radioactivity in each class[c]					
				Total NL			
		Total PL	Total GL	TG+DG	ST	FA	H+A
10	167 (6.0)[b]	60	6	5	1	2	26
16	649 (23.4)	73	4	4	1	3	15
45	698 (25.2)	63	4	5	3	12	13
85	716 (25.8)	66	4	6	8	5	11
155	709 (25.5)	67	5	10	9	3	6
22.0 hr	611 (22.0)	56	21	8	13	1	1

[a]Suspensions (40 ml) of 4-day-old cells were incubated with 50 μCi of [1-^{14}C]acetate; lipids were extracted and counted as described in the text.

[b]Values in parentheses are lipid ^{14}C-activities expressed as percentages of added [^{14}C]acetate.

[c]Neutral lipids (NL), glycolipids (GL), and phospholipids (PL) were separated by chromatography on a column of silicic acid eluted with chloroform, acetone, and methanol, respectively (10). Neutral lipids were further separated by preparative TLC into tri- and diacylglycerols (TG + DG), sterols (ST), free fatty acids (FA), hydrocarbons (H), and long chain alcohols (A). Values are expressed as % of radioactivity in total lipids.

The lipid composition observed here (Table I) is similar to that reported for immature soybean seeds (15) or for nonphotosynthetic plant tissues generally (16). The major lipids were the phospholipids phosphatidylcholine (PC) and phosphatidylethanolamine (PE) followed by triacylglycerol, sterols, and digalactosyldiacylglycerol (DGD). Smaller amounts of monogalactosyldiacylglycerol (MGD), phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), phosphatidylinositol (PI), and esterified and unesterified steryl glycosides were also present. Our cultures also contained a small amount of an unidentified phospholipid with TLC mobilities similar to those of *bis*-phosphatidic acid (*bis*-PA) previously identified in developing soybeans and suspension cultures of soybean cells (17). In contrast to earlier reports (3), sterol esters were not detected in the cultures examined here.

[1-^{14}C] Acetate Incorporation

Incorporation of [1-^{14}C]acetate into total lipids of soybean cell suspensions was rapid up to 16 min and remained essentially constant at a level of about 25% of the added ^{14}C up to 22 hr (Table II). Phospholipids accounted for most of the radioactivity (56-73%) in the lipids at all times. Of these, PC showed the most rapid labeling, a maximum value being reached at 16 min followed by a rapid decrease in ^{14}C content reaching very low values at 22 hr (Fig. 3). PE, PI, and PG were labeled slowly to maxima at 1.5 to 2.6 hr, thereafter showing decreases in radioactivity. In contrast the unidentified phospholipid (*bis*-PA) showed little labeling up to 2.6 hr but at 22 hr was found to contain most of the radioactivity present in the total lipids (Fig. 3).

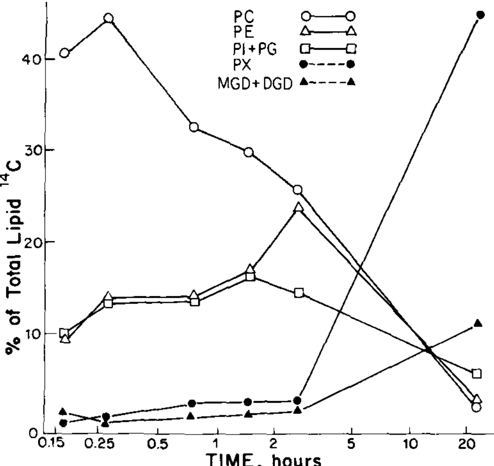

FIG. 3. Time course of incorporation of [1-^{14}] acetate into major lipids of 4-day-old soybean suspension culture cells. Soybean cells were incubated with [^{14}C]acetate and sampled as described in the Experimental section of each of the times shown. Abbreviations: see legend to Figure 2. ○ PC; △, PE; ●, PX; □, PI + PG; ▲, MGD + DGD. For convenience, time is represented on a logarithmic scale.

Glycolipids contained relatively low proportions (<5%) of ^{14}C up to 2.6 hr but then showed a considerable increase to 21% at 22 hr (Table II). Galactolipids (MGD + DGD) accounted for most of the activity in the glycolipid fraction (Fig. 3, Table II), and neutral lipids accounted for 23-33% of the total lipid radioactivity at all times (Table II). Labeling of di- and triacylglycerol was rapid and remained relatively constant at about 10% up to 22 hr (Table II). Sterols, however, were labeled slowly at first and reached a maximum of 13%

TABLE III

Incorporation of [^{14}C]Acetate into Fatty Acids of Major Lipids of Soybean Suspension Cells[a]

Lipid[b]	Time after addition of [^{14}C]acetate (min)	Total ^{14}C in fatty acids 10^{-3} x dpm per ml culture	Distribution of [^{14}C]fatty acids in individual lipid component (%)			
			16:0	18:1	18:2	18:3
Total	16	490	29.1	60.4	8.9	1.6
	45	535	28.5	52.6	16.7	2.2
	85	546	25.9	45.8	22.8	5.5
	155	548	26.3	34.1	28.2	11.4
	22.0 hr	522	14.4	8.2	24.6	52.8
PC	16	289	19.8	76.2	3.0	1.0
	45	227	21.5	60.7	16.0	1.8
	85	215	21.9	51.4	23.4	3.2
	155	182	23.1	42.9	28.2	5.8
	22.0 hr	13	19.6	11.9	18.0	50.5
PE	16	89	36.8	57.1	5.2	1.0
	45	97	37.1	52.2	8.9	1.8
	85	121	38.4	44.9	13.7	3.0
	155	169	36.5	38.5	19.4	5.6
	22.0 hr	16	30.6	15.4	24.0	29.9
PX	16	5	53.8	40.9	3.9	1.3
	45	16	42.4	48.3	8.0	1.2
	85	18	40.1	43.7	12.9	3.3
	155	17	35.7	45.1	15.0	4.2
	22.0 hr	277	27.6	8.0	24.5	40.0
TG+DG	16	59	26.4	60.5	10.0	3.1
	45	105	23.1	55.7	17.6	3.6
	85	92	18.8	48.2	26.1	6.9
	155	95	15.5	36.5	33.9	14.1
	22.0 hr	76	15.1	6.4	18.8	59.7
MDG+	16	18	37.7	38.2	11.3	12.9
DGD+	45	21	36.6	39.0	16.7	8.7
ESG	85	25	27.4	34.2	23.9	14.4
	155	31	21.9	26.8	26.9	24.4
	22.0 hr	109	26.7	10.6	15.3	47.4

[a]The percentage distribution of fatty acid radioactivity was obtained by gas liquid chromatography (GLC) and thin layer chromatography (TLC) analysis of methyl esters prepared from the isolated lipids. Total fatty acid radioactivity in each lipid component was determined after saponification as described in the text.

[b]Abbreviations: PC = phosphatidylcholine, PE = phosphatidylethanolamine, PX = unidentified phospholipid, TG = triacylglycerol, DG = diacylglycerol, MGD = monogalactosyldiacylglycerol, DGD = digalactosyldiacylglycerol, ESG = esterified sterolglycoside.

of the total lipid radioactivity at 22 hr, while hydrocarbons and long chain alcohols contained 15-20% of the total lipid ^{14}C at early times, this proportion then decreasing to 1% at 22 hr (Table II).

The pattern of [^{14}C]acetate labeling of glycerolipids observed in the present studies is similar to that reported previously for leaves (18-20). The effect of darkness and low temperature (16 C) on the pattern of labeling of the lipid classes was examined, but no significant differences were noticed, apart from the expected depression in the rate of labeling at the lower temperature.

Labeling of Fatty Acids

At all incubation times radioactivity was present mostly in palmitic (16:0), oleic (18:1), linoleic (18:2), and linolenic (18:3) acids, no significant ^{14}C being detected in myristic (14:0, palmitoleic (16:1), *trans*-3-hexadecenoic (16:1), hexadecatrienoic (16:3), or stearic (18:0) acids. In the total lipids, 18:1 was labeled preferentially (>50%) at early incubation times while relatively low percentages of ^{14}C were found in 18:2 and 18:3; 16:0 contained about 30% of the ^{14}C and this proportion decreased only slightly after longer incubation times (Table III). ^{14}C-Labeling of 18:1 decreased during the remaining incubation period while that of 18:2 increased reciprocally up to 2.6 hr and thereafter began to decrease. ^{14}C-Labeling of 18:3 increased after 2.6 hr at a rate which appeared comparable to the combined rates of decrease of labeling in 18:1 and 18:2.

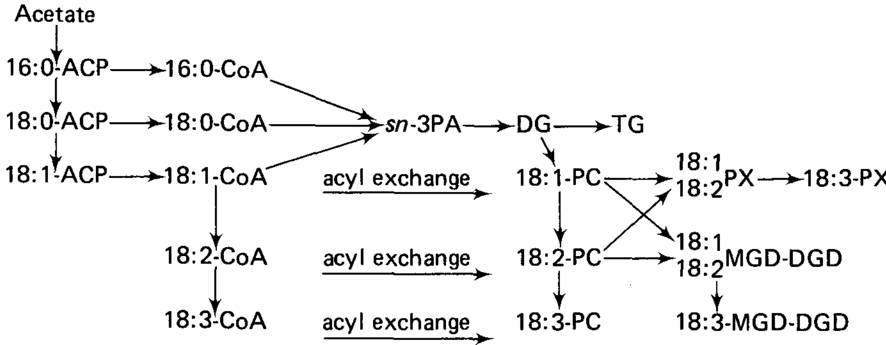

FIG. 4. Scheme for proposed mechanism of desaturation sequence of fatty acids in soybean cell cultures [adapted in part from Stumpf and Weber (5)].

Oleic acid of PC accounted for about 75% of the radioactivity in PC (Table III) corresponding to 44% of the total lipid fatty acid radioactivity at 16 min, thereafter decreasing to values of 12% and 0.3%, respectively, at 22 hr. The proportion of ^{14}C in the 18:2 component of PC was very low at 16 min but increased tenfold to a maximum at 2.6 hr, thereafter decreasing up to 22 hr (Table III). Only 1% of the ^{14}C in PC was present in the 18:3 component at 16 min, this value increasing slowly to 6.0% at 2.6 hr, and then to 50% at 22 hr (Table III); as a percentage of the total fatty acid ^{14}C, the proportion at 22 hr was, however, only about 1%. The decrease in proportions of ^{14}C in 18:1 and 18:2 of PC during the period from 2.6 hr to 22 hr was compensated for by increases in these acids in the unidentified phospholipid (*bis*-PA) and in the glycolipid fraction; the di- and triacylglycerol fraction, however, showed an increase only in the 18:3 component (Table III).

Stearns and Morton (6) reported a low labeling rate of fatty acids from [^{14}C]acetate in 21-day-old soybean cell cultures, but after a lag period of 30 min to 1 hr, the percentage distribution of ^{14}C among the fatty acids, with the exception of saturated acids, was very similar to that found here. Using 5-day-old cells, Stumpf and Weber (7) reported rapid incorporation of [^{14}C]acetate in 18:1 and 18:2 acids but not into the 18:3 acid. However, conversion of [^{14}C]oleate into 18:2 acid and [^{14}C]linoleate into 18:3 was also observed. These results (7) as well as those of Morton and Stearns (6) were suggestive of sequential desaturation of 18:1 → 18:2 → 18:3 acid.

While the present results on labeling of the total fatty acids (Table III) are also consistent with such a sequential mechanism, the pattern of labeling of the fatty acids in the individual lipids we observed suggests that a more complex desaturation sequence may be proposed (Fig. 4).

In this sequence, labeled 18:1-PC rapidly accumulates by synthesis de novo from DG or by "acyl exchange" of unlabeled PC with labeled 18:1-CoA (21). The 18:1 of PC is then rapidly converted to 18:2 of PC during the first 2 hr of incubation, either by direct desaturation of 18:1-PC, as demonstrated previously with *Chlorella* chloroplasts (22) and pea-leaf microsomes (23) or by "acyl exchange" with 18:2-CoA formed by desaturation of 18:1-CoA (7,24,25). Subsequently, both 18:1 and 18:2 of PC are then transferred to other glycerolipids, in particular the phospholipid PX (*bis*-PA) and the glycolipids (MGD + DGD).

Linolenic acid accumulates in these lipids during the same period, and also to a limited extent in PC and PE (Table III). The 18:3-PC could be formed by desaturation of 18:2-PC or by "acyl exchange" with 18:3-CoA, but the low amounts of labeled 18:3 detected in PC would tend to eliminate PC as a significant source of 18:3 for transfer to PX and MGD + DGD. The accumulation of 18:3 in PC and the galactolipids may perhaps be explained by desaturation of 18:2 → 18:3 in situ in these components or by "acyl exchange" with 18:3-CoA formed by desaturation of 18:2-CoA (7). Pulse-chase experiments using [^{3}H]glycerol and [^{14}C]acetate in developing maize leaves (26) have confirmed that the 18:1 of PC is a major precursor of the 18:3 of galactolipids.

The conversion of 18:1 → 18:2 has been associated with the endoplasmic reticulum of maize and pea-leaves, whereas desaturation of 18:2 → 18:3 has been proposed to occur in the chloroplasts (18,27). The mechanism of these desaturations and of the interorganelle lipid transfer still remains to be elucidated. Soybean

suspension cultures may prove to be very useful systems in which to pursue these problems since advantage can be taken of existing methodology to produce protoplasts, allowing gentle cell breakage without damage to the intracellular organelles and minimizing cross-contamination and release of lytic enzymes. Furthermore, manipulation of the growth medium composition to promote differentiation might allow expression of cultivar differences in response to cold temperature stress.

ACKNOWLEDGMENTS

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COMMUNICATIONS

Thermal Decomposition of Methyl Oleate Hydroperoxides and Identification of Volatile Components by Gas Chromatography-Mass Spectrometry

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ABSTRACT

The role of methyl oleate hydroperoxides as precursors of volatile compounds was investigated by thermal decomposition in the injector port of a gas chromatograph attached to a computerized mass spectrometer. The major volatile compounds identified correspond to those formed from triolein heated in air at 192 C.

INTRODUCTION

In a previous investigation on the volatile compounds formed from triolein heated in air at 192 C, the major components identified were those expected from the decomposition of the four isomeric 8-, 9-, 10-, and 11-oleic hydroperoxides (1). Although hydroperoxides are the first major product of autoxidized oleate and related compounds, they are rapidly decomposed at temperatures exceeding 100 C (2), producing 90% polymeric and 10% volatile materials (3). Some nonperoxidic secondary products in heated fats such as dimers and polymers are known to be rich sources of volatile carbonyl compounds (4) and to decrease flavor and oxidative stability of soybean oil (5). Therefore, there is a question as to whether hydroperoxides are even formed at cooking temperatures of 190 C and above. If hydroperoxides are formed as intermediates at high temperatures, there is no information about their finite existence and about the kinetics that control their decomposition.

In this report direct evidence for the precursors of major volatiles was obtained by thermally decomposing pure methyl oleate hydroperoxides directly in the injector port of a gas chromatograph attached to a computerized mass spectrometer (GC-MS). With this technique (1) volatiles were separated, identified, and their relative proportion estimated by direct analysis of micro samples.

EXPERIMENTAL METHODS

The hydroperoxides from methyl oleate autoxidized in O_2 at 40 C to a peroxide value

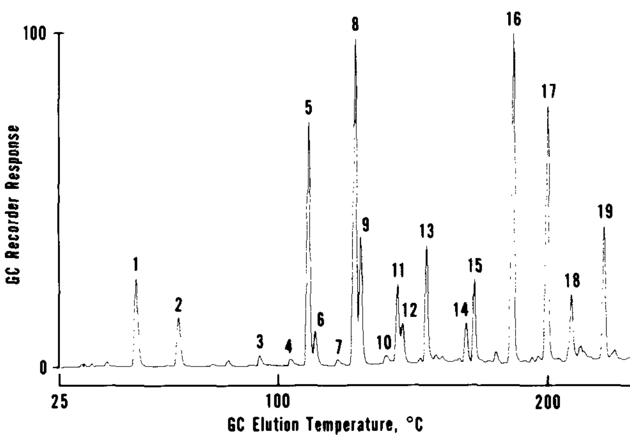

FIG. 1. Gas chromatogram of volatile compounds from thermally decomposed methyl oleate hydroperoxides.

TABLE I

Comparison of Volatiles from Decomposed Oleate Hydroperoxides and Heated Triolein

Peak no.[a]	Compound	Oleate-hydroperoxides		Triolein (1)	
		Rel %	Normalized	Rel %	Normalized
1	Heptane	4.4	9.6	8.6	9.5
2	Octane	2.7	5.9	9.7	10.8
3	Heptanal	0.5	1.1	5.1	5.6
4	1-Heptanol	0.4	0.9	1.6	1.8
5	Octanal	11	23.9	8.5	9.4
7	1-Octanol	0.4	0.9	2.5	2.4
8	Nonanal	15	32.6	22	24.3
10	2-Nonenal	0.5	1.1	2.0	2.2
11	Decanal	3.9	8.5	2.8	3.1
13	*c/t*-2-Decenal	5.4	11.8	17	18.8
14	*t*-2-Undecenal	1.7	3.7	11	12.1
		45.9	100	90.8	100
6	Me heptanoate	1.5			
9	Me octanoate	5.0			
12	Me nonanoate	1.5			
15	Me 8-oxooctanoate	3.5			
16	Me 9-oxononanoate	15			
17	Me 10-oxodecanoate	12			
18	Me 10-oxo-8-decenoate[b]	3.4			
19	Me 11-oxo-9-undecenoate[b]	5.8			
		47.7			
	Other minor peaks	6.4			

[a]See Figure 1 for numbered peaks.
[b]Tentative identification based on GC elution and MS without reference compound.

of 1051 were purified by column partition chromatography (6). The hydroperoxides (checked for purity by thin layer chromatography) were analyzed for isomeric composition by GC-MS (7): 27% 8-, 23% 9-, 23% 10-, and 27% 11-OOH isomers. A neat sample of oleate hydroperoxides (3.8 μl) was injectd into the same GC-MS system used previously (1). The GC parameters were similar except the injector port temperature was 210 C, and temperature programming was initiated at 25 C instead of -60 C. Identifications of volatile compounds were based on mass spectra matched manually and by computer with those of reference compounds and were confirmed by GC-retention data.

RESULTS AND DISCUSSION

The GC-MS computer-generated chromatogram depicts 19 peaks of interest (Fig. 1). Eleven of these peaks are due to the same compounds previously identified from heated triolein (Table I). If the relative concentrations of only the nonester compounds are normalized, the relevant volatiles from oleate hydroperoxides correspond to those of heated triolein. These compounds, together with the methyl ester fragments, represent 93.6% of the relative total peak area of Figure 1. The remaining peaks are due to minor components which were too small to identify reliably. These results clearly indicate that oleate hydroperoxides are the major precursors of volatiles produced from triolein even at 192 C. However, these data are insufficient to prove that hydroperoxidation is the only route by which such products form.

The well-recognized mechanism of carbon-carbon scission on either side of the alkoxy radical intermediate produced from hydroperoxides (8) was checked by matching the concentration of cleavage products expected from each part of the oleate hydroperoxide isomers (Table II). We assumed further that 1-enols are produced from the reaction of hydroxy radicals with 1-olefins to form the corresponding saturated aldehydes by tautomerism. For example, decanal would be formed as follows:

$$CH_3(CH_2)_7CH{=}CH\cdot + \cdot OH \rightarrow$$

$$CH_3(CH_2)_7CH{=}CH\text{-}OH \rightleftharpoons CH_3(CH_2)_7CH_2CHO$$

The yields of cleavage products arising from each side of the hydroperoxide isomers were in remarkably good agreement.

This mechanism accounts for all the volatiles listed in Table I except for heptanal, 2-nonenal, and methyl nonanoate. Although the isomeric composition of the oleate hydroperoxides was

TABLE II

Decomposition of Methyl Oleate Hydroperoxide Isomers

	Cleavage products	Yield %
	$CH_3(CH_2)_7$-CH=CH-[B]-CH(O·)(8)-[A]-$(CH_2)_6COOMe$	
A:	2-Undecenal (1.7%) + Me heptanoate (1.5%):	3.2
B:	Decanal (3.9%) + Me 8-oxooctanoate (3.5%):	7.4
		10.6
	$CH_3(CH_2)_6$-CH=CH-CH(O·)(9)-$(CH_2)_7COOMe$	
A:	2-Decenal (5.4%) + Me octanoate (5.0%):	10.4
B:	Nonanal (4.0%)[a] + Me 9-oxononanoate (4.0%)[a]:	8.0
		18.4
	$CH_3(CH_2)_7$-CH(O·)(10)-CH=CH-$(CH_2)_6COOMe$	
A:	Nonanal (11%)[a] + Me 9-oxononanoate (11%)[a]:	22.0
B:	Octane (2.7%) + Me 10-oxo-8-decenoate (3.4%) + 1-octanol (0.4%):	6.5
		28.5
	$CH_3(CH_2)_6$-CH(O·)(11)-CH=CH-$(CH_2)_7COOMe$	
A:	Octanal (11%) + Me 10-oxodecanoate (12%):	23.0
B:	Heptane (4.4%) + Me 11-oxo-9-undecenoate (5.8%) + 1-heptanol (0.4%):	10.6
		33.6

[a]Because nonanal and 9-oxononanoate arise from both the 9- and 10-hydroperoxides, the concentrations of these compounds were divided by assuming that the same amount of saturated aldehydes would be produced from the 9- and from the 8-hydroperoxides.

symmetrical (e.g., 8- = 11-OOH, 9- = 10-OOH), the total relative concentration of volatiles from the 10- and 11-hydroperoxides was 61% and that from the 8- and 9-hydroperoxides was 29%. This divergence is apparently related to the position of the hydroperoxide group and allylic unsaturation. Cleavages yielding octanal, nonanal, and decanal seemed favored kinetically in the oleate hydroperoxides. The implication of these results will be discussed in the full paper that will deal also with the decomposition of linoleate and linolenate hydroperoxides.

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^{13}C Nuclear Magnetic Resonance Spectroscopy of Cyanolipids and Cyanolipid-Containing Seed Oils

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ABSTRACT

^{13}C nuclear magnetic resonance (NMR) signals of all carbon atoms of the hydroxynitrile moieties on which cyanolipids are based have been assigned. The four known types of cyanolipids can be conveniently distinguished and identified by ^{13}C-NMR whether they are in pure form or in unfractionated seed oils from which they are derived.

INTRODUCTION

Cyanolipids are a relatively new class of lipids thought to be found only in seed oils of the Sapindaceae; four distinct but structurally related types (I-IV, Fig. 1) have been isolated and characterized by conventional chemical methods (1). However, the hydroxynitrile moieties which are the alcohol portions of these esters tend to be unstable when not esterified, and this makes the chemical identification lengthy and tedious. Nuclear magnetic resonance (NMR) techniques have proven more appropriate for detection of cyanolipids than other spectral or chromatographic analyses (1). A ^{1}H-NMR approach to their detection in seed oils has been reported (1,2).

In view of expanding applicability of ^{13}C-NMR techniques to lipid structure determination (3-6), we wish to report results of our ^{13}C-NMR analyses, both of isolated cyanolipids and also of unfractionated seed oils containing the four types of cyanolipids. This procedure complements the ^{1}H method mentioned and provides an equally suitable alternative approach to this problem.

EXPERIMENTAL PROCEDURES

Seed oils were extracted from ground meal by conventional methods. Cyanolipids were isolated from appropriate seed oils and were purified by previously described techniques (1). The ^{13}C-NMR spectra were determined on a Bruker WH-90 Fourier Transform NMR spectrometer operating at 22.63 MHz with proton noise decoupling. The computer data memory size used was 4k. A 5 μsec (ca. 30°) pulse width was used, and there was no delay between pulses. No attempt was made to determine the range of T_1 values. The spectra (900-15000 accumulations) were obtained from solutions in $CDCl_3$ which also served as an internal deuterium lock. Chemical shifts are given as δ values in ppm downfield from the internal TMS-^{13}C signal and are accurate to within ± 0.1 ppm.

```
     H2C=C——CH—CN                 H2C——C=CH—CN
         |   |                         |  |
R—C—O—CH2 O—C—R'              R—C—O  H2C—O—C—R'
  ||        ||                  ||          ||
  O         O                   O           O
        I                              II

     H3C—C=CH—CN                 H2C=C——CH—CN
         |                           |   |
R—C—O—CH2                          CH3 O—C—R
  ||                                     ||
  O                                      O
       III                             IV
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FIG. 1. Cyanolipid structures.

RESULTS AND DISCUSSION

Differences in substitution patterns of the hydroxynitrile moieties' five carbon atoms produce significant variations in their chemical shifts. The principal structural features involved are the α,β-unsaturated nitrile grouping of II and III (shown in Fig. 1), the β,γ-unsaturated nitrile and terminal methylene groupings of I and IV, and the diester nature of I and II as opposed to the monoester character of III and IV. This last characteristic contributes only different types of carbon atoms and produces no major chemical shift variations in itself.

Effects of these structural variations are most pronounced in chemical shifts of unsaturated carbon atoms with no protons attached and of those in cyano groups (Table I). In spectra of cyanolipids II and III, the unsaturated carbons bearing no protons give signals at 155.0 and 158.1 ppm, respectively, while signals for the corresponding atoms in I and IV appear at 135.6 and 136.1 ppm. The larger downfield shift observed for II and III is due to the effect of conjugation of the double bond with the nitrile grouping. A pronounced shielding effect experienced by olefinic carbons α- to nitrile carbons in cyanolipids II and III may

TABLE I

^{13}C-NMR Assignments for Selected Carbon Atoms of Cyanolipids[a]

Carbon atom	Cyanolipid analyzed			
	I	II	III	IV
CH_3-C=	---	---	20.6	18.2
-CH_2-O-[b]	62.7(t)	61.7(t),62.7(t)	64.3(t)	---
-CH-O-[b]	60.9(d)	---	---	64.1(d)
-C= (no H)	135.6	155.0	158.1	136.1
CH_2=	129.9[c]	---	---	130.0[c]
-HC=[d]	---	98.7	97.6	---
-C≡N	120.8	129.7[c]	129.9[c]	118.0
-C=O[e]	171.4,172.9	172.6,172.9	173.1	171.6

[a]In ppm downfield from TMS; signals of all acyl group carbon atoms other than -C=O are omitted.

[b]Triglycerides from *Koelreuteria paniculata* seed oil gave CH_2-O- and -CH-O-signals at 62.1(t) and 69.0(d) ppm, respectively. All assignments for these carbon atoms were made by partial decoupling experiments.

[c]Single peak, but either broadened or having a shoulder due to overlap with acyl group olefinic carbon signals.

[d]Triglycerides from *Koelreuteria paniculata* seed oil (as well as cyanolipid acyl groups) gave -HC= signals in the range 129.7 to 130.2 ppm.

[e]Triglycerides from *Koelreuteria paniculata* seed oil gave -C=O signals at 172.7 and 173.2 ppm.

also be the result of conjugation. The signals for these carbons are shifted upfield from their normal position to 98.7 and 97.6 ppm. α-Carbons of other α,β-unsaturated nitriles exhibit a similar shift (7).

Signals of -C≡N carbon atoms in cyanolipids are also shifted downfield when these atoms are in a conjugated system, although the shifts are considerably less than those of the carbon atoms bearing no protons. In the spectra of I and IV, these signals appear at 120.8 and 118.0 ppm, respectively, as opposed to 129.7 and 129.9 ppm for the corresponding carbons of the conjugated cyanolipids, II and III. The overall range of -C≡N chemical shifts observed here, 11.9 ppm, is comparable to that given in other reports (8), but the conjugated -C≡N signals of II and III are nearly 5 ppm downfield from any nitrile listed in this compilation (8).

Many of the ^{13}C signals due to hydroxynitrile moiety carbons have distinctive chemical shifts and are readily assignable to cyanolipids I, II, III, or IV whether they occur singly, in combination, or in an oil along with triglycerides. Unfractionated seed oils that were analyzed were those of *Paullinia meliaefolia*, *Koelreuteria paniculata*, and *Ungnadia speciosa*. Signals that are especially valuable for identification purposes are those of acylated carbons (-CH_2-O-,-CH-O-), of two (in I and IV) of the four nitrile carbons, and of the internal olefinic carbons. The vinylic methyl signals of III and IV are also outstanding since they appear in an area between the acyl group terminal methyl signal at 14.1 ppm and the ω-2 carbon atom signal at 22.7 ppm that is normally blank in spectra of triglycerides or fatty acids.

Conversely, the use of terminal methylene signals, of carbonyl signals, and of the nitrile signals of II and III for identification of a particular cyanolipid in a seed oil is not feasible. These signals are extensively overlapped with those of certain triglyceride carbon atoms as shown by footnotes b, c, d, and e of Table I.

Triglycerides isolated from *Koelreuteria paniculata* seed oil give a ^{13}C spectrum in which the C-2 acyl group carbons (not listed in Table I) appear as two signals at 34.1 and 34.3 ppm. Presumably, C-2 of the acyl group on the glyceryl 2-position gives a different signal than those on the other positions. This same type of discrimination appears in spectra of isolated diester cyanolipids I and II. Chemical shifts of the two C-2 acyl carbon signals for I are 33.6 and 34.1 ppm and for II are 33.9 and 34.0. This is probably due to the fact that one group is *cis* and one *trans* to the nitrile. Also, in the spectrum of I (but not of II), there are two signals for the acyl C-3 carbon atoms at 24.6 and 24.8 ppm; the more unsymmetrical environment of I provides a distinction between the C-3 atoms that is not seen in cyanolipid II.

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Autoxidation of Fatty Acid Monolayers Adsorbed on Silica Gel: III. Effects of Saturated Fatty Acids and Cholesterol

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ABSTRACT

Autoxidation of fatty acid monolayers on silica consisting of multiple components to simulate biomembranes has been studied by the rate of fatty acid disappearance and the products formed. When palmitic acid was incorporated into linoleic acid monolayers, the decrease in rate was proportional to the amounts of plamitic acid present. The protective effect of the saturated fatty acid diminished rapidly as the chain length of the saturated fatty acid decreased below C_{12}. With acids of medium chain length, C_{12} was more effective than C_{16}. In pure linoleic acid monolayers, when the surface coverage was reduced to only 5% of the available adsorption sites, and in the case of palmitic acid-linoleic acid monolayers, the rate dropped drastically and the major identified product formed was hydroxyepoxyoctadecenoic acid. On the contrary, the major product formed in the case of saturated monolayers of pure linoleic acid was a mixture of unsubstituted epoxy acids. The inclusion of cholesterol in linoleic acid monolayers increased the rate of disappearance of linoleic acid slightly, whereas cholesteryl acetate decreased the rate. The protective effect exerted by cholesteryl acetate appeared to be similar to that of palmitic acid.

INTRODUCTION

Model membrane systems, though similar to cell membranes only in some parameters, have been widely used in studying autoxidation and other membrane-related phenomena. Using simple model systems, such as mono- and bilayers, investigators have been able to observe the effect of a single parameter in a particular process; the result is often useful in understanding processes occurring in more complex living systems.

Several recent studies of autoxidation of membrane lipids using adsorbed unsaturated fatty acid monolayers have been reported. Porter and co-workers (1-3) have examined several aspects of autoxidation using a linoleic acid-silica system. For example, it was found that metals, especially iron, promote autoxidation, whereas tocopherol and ascorbic acid-type synergists inhibit it. α-Tocopherol apparently forms a one-to-one adduct with linoleic acid in the silica monolayer system. We have recently reported results of two studies also using linoleic acid monolayers adsorbed on silica gel (4,5). The effective adsorption sites were shown to be the isolated, nonhydrogen-bonded hydroxyl groups on the surface. The basic rate of autoxidation of linoleic acid monolayers without additives was measured, and the considerable differences between monolayers and bulk phase were noted. For example, the major products formed in the monolayer system were identified as isomeric unsaturated epoxides, whereas for the bulk phase, hydroperoxides are the principal initial products.

In this paper, we report a further study on autoxidation of adsorbed acid monolayers concentrating on the effects of other important membrane constituents, cholesterol and saturated fatty acids. The effect of inclusion of cholesterol in lipid monolayers spread on an air-water interface has been studied extensively in the past. The purpose of such studies, however, has been primarily from the viewpoint of physical changes, such as transition temperatures (6), lateral diffusion (7), and surface areas under compression (8,9). Saturated fatty acids, as single constituents of air-water interface monolayers, have also been studied in detail with regard to surface areas and phase transformations (10,11). We undertook this study on autoxidation rates and products of monolayers with multiple components, a step closer to biomembranes, hoping to gain further insight into membrane autoxidation.

EXPERIMENTAL PROCEDURES

Materials and Methods

Linoleic acid, palmitic acid, lauric acid, caprylic acid, caproic acid, and cholesteryl acetate purchased from Applied Science Laboratories, Inc. (State College, PA) and cholesterol from Mann/Schwartz Laboratories (Orangeburg, NY) were 99+ % pure and were used directly after a purity check with thin layer chromatography (TLC) and gas liquid chromatography (GLC). The important physical characteristics of Silica Gel G have been reported (4).

GLC was carried out using a Varian Aerograph Model 2100 equipped with flame ionization detectors and an electronic integrator (Infotronics Corp., Austin, TX, Model CRS-11 HSB). The GLC column used was an 0.20 x 183 cm glass U-tube containing 3% OV-101 on 100/120 mesh Gas Chrom Q. Column chromatography was carried out using a 3.4 x 8.0 cm silicic acid column (J.T. Baker Chemical Co., Phillipsburg, NJ) prewashed with acetone, ether, and n-pentane, and was developed with pentane with increasing amounts of ether. Two column volumes of each solvent mixture were used with collection of half column-volumes of eluate for each fraction. Mass spectrometry was performed on a Finnigan Model 300 quadrupole spectrometer coupled with a Varian Aerograph Series 1400 gas chromatograph with an 0.20 x 150 cm coiled column packed with 3% OV-1 on 60/80 mesh Gas Chrom Q. The trimethylsilyloxy derivatives were obtained by use of a silylating reagent, TRI-SIL (Pierce Chemical Co., Rockford, IL) containing Me_3-SiCl, $(Me_3Si)_2NH$, and pyridine. Infrared spectra were obtained with an Infracord Model 137 instrument (Perkin-Elmer Corporation, Norwalk, CT), using CCl_4 as solvent. TLC was carried out using precoated Silica Gel G plates (0.25 mm thick, Analtech, Inc. Wilmington, DE) with the solvent system: petroleum ether-diethyl ether-acetic acid, 80:20:1. Visualization of spots was accomplished by spraying with 3% cupric acetate solution in 8.5% phosphoric acid with subsequent charring at 140 C. Quantitation of TLC spots was carried out using a Kontes densitometer (Model K-495000) equipped with a calculating integrator.

Preparation and Autoxidation of Saturated Fatty Acid-Linoleic Acid Monolayers

A detailed description of the preparation and autoxidation of monolayers has been published (5). Various combinations of initial concentrations of saturated fatty acid and linoleic acid were used to produce monolayers with the desired mole ratios of saturated fatty acid to linoleic acid. In palmitic acid-linoleic acid monolayers, this ratio varied from 0.3 to 3. In monolayers including lauric acid, caprylic acid, and caproic acid, the mole ratios were all close to 1.

In a typical preparation, a solution of 0.258 g (0.919 mmole) of linoleic acid and 0.236 g (0.920 mmole) of palmitic acid in 44 ml of hexane was stirred with 2.002 g of Silica Gel G for 1 hr. After the adsorption, the supernatant was withdrawn and was freed from solvent to give 0.154 g of acid. The weight of the total acid adsorbed was thus 0.340 g. The actual mole ratio of two acids adsorbed was usually obtained by GLC of the extracted material before incubation, using methyl stearate as internal standard. The dry, coated silica, about 0.220 g portions, was incubated at 60 C for the desired length of time and extracted with methanol. The unchanged acid was methylated and quantitated by GLC.

Preparation and Autoxidation of Cholesterol-Linoleic Acid Monolayers

A solution of 0.398 g (1.42 mmole) of linoleic acid and 0.093 g (0.24 mmole) of cholesterol in 95 ml of 3% ether in hexane was poured on 1.756 g of Silica Gel G. The mixture was stirred for 1 hr and the supernatant was removed. Evaporation of solvent left 0.297 g of residue (cholesterol and linoleic acid) from the supernatant. The total weight of cholesterol and linoleic acid adsorbed was 0.194 g, which amounts to about 40% of a theoretical saturated monolayer, and the mole ratio of linoleic acid to cholesterol was determined by GLC to be 1.58.

About 0.220 g portions of dried, coated silica gel were oxidized at 60 C, extracted, methylated, and quantitated by GLC as previously described (5). Methyl stearate was also used as an internal standard to determine the unchanged substrates by first chromatographing isothermally at 175 C to determine the amounts of linoleate and then, on a subsequent run, immediately following the injection of a sample, the column temperature was programmed from 175 C to 260 C at a rate of 2°/min to determine the amount of cholesterol in relation to methyl stearate. The relative GLC responses of cholesterol and methyl stearate were established by chromatographing an artificial mixture containing known weights of methyl stearate and cholesterol under identical conditions.

Preparation and Autoxidation of Cholesteryl Acetate-Linoleic Acid Monolayers

To 0.702 g of Silica Gel G, a solution of 0.142 g (0.332 mmole) of cholesteryl acetate and 0.093 g (0.332 mmole) of linoleic acid in 15 ml of hexane was added. After stirring for 1 hr, the supernatant was removed. The amount of substrate left in the equilibrium solution was found to be 0.069 g. The mole ratio of linoleic acid to cholesteryl acetate actually adsorbed was 1.22. The autoxidation of cholesteryl acetate-linoleic acid monolayers and the determination of the extent of disappearance of substrate were carried out as described for cholesterol-linoleic acid monolayers.

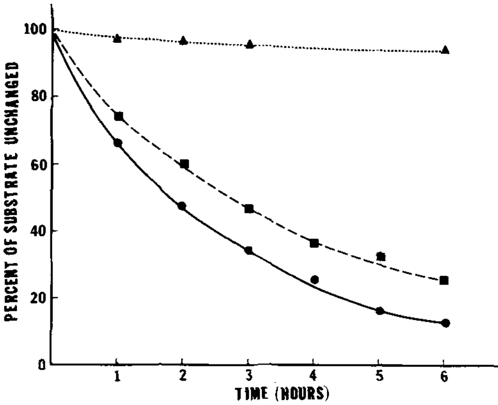

FIG. 1. Rate of autoxidation of palmitic acid-linoleic acid monolayers in comparison with that of pure linoleic acid monolayers at 60 C. —●—●—, Disappearance of linoleic acid in pure linoleic acid monolayers; —■— —■—, disappearance of linoleic acid in palmitic acid-linoleic acid monolayers; ···▲······▲···, disappearance of palmitic acid in palmitic acid-linoleic acid monolayers.

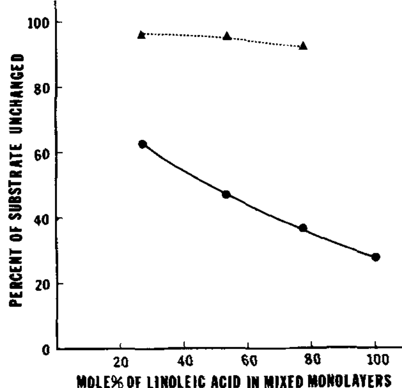

FIG. 2. Extent of autoxidation at 3 hr, 60 C of mixed monolayers of different proportions of palmitic and linoleic acids. —●—●—, Disappearance of linoleic acid; ···▲······▲···, disappearance of palmitic acid.

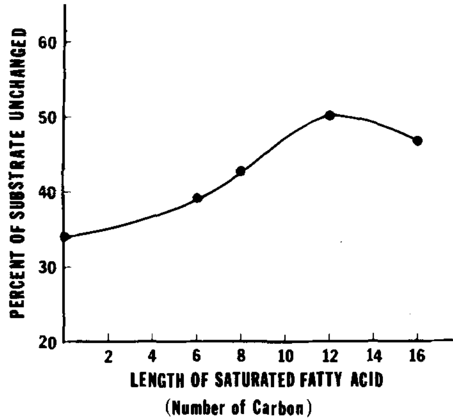

FIG. 3. Extent of autoxidation of linoleic acid in mixed monolayers of linoleic acid and saturated fatty acids of various chain lengths.

Preparation of Linoleic Acid Hydroperoxide and Its Reaction on Silica Gel

Linoleic acid hydroperoxide was isolated from a bulk-phase autoxidation of linoleic acid. Purification was achieved by chromatography twice through silica gel columns, the hydroperoxide being eluted by 25% ether in pentane. Sample purity was monitored by silica gel TLC as the methyl esters after reaction with diazomethane. Purified chromatographic fractions were combined and evaporated in an ice bath to about 25 ml. This solution, which amounted to ca. 100 mg hydroperoxide is an unknown ratio of ether-pentane, was diluted to 100 ml with hexane. A 30 ml aliquot (30 mg hydroperoxide) was mixed with 20 ml hexane and slurried with 100 mg silica gel. After removing the bulk of the solvent, the hydroperoxide-coated silica gel was dried under a stream of nitrogen and then in vacuo. A 50 mg portion of the coated silica gel was incubated at 60 C for 4 hr, extracted with methanol, and the isolated product reacted with diazomethane. GC-MS was performed after reacting with TRI-SIL.

RESULTS

For preparing saturated fatty acid-linoleic acid mixed monolayers, palmitic acid was chosen as the major saturated constituent. Palmitic acid is known to be the most abundant saturated fatty acid in mammalian membranes. For example, in erythrocytes, the amount of palmitic acid far exceeds the next most abundant saturated fatty acid, stearic acid (12). The rate of autoxidation of palmitic acid-linoleic acid monolayers (with a mole ratio of 1:1, representing a frequently occurring ratio in membrane phospholipids in which the acyl moiety in the α-position is generally saturated and that in the β-position, unsaturated) is shown in Figure 1 in comparison with the rate of autoxidation of pure linoleic acid monolayers. It can be seen that in palmitic acid-linoleic acid monolayers, the decrease in rate of disappearance of linoleic acid is appreciable, and rate studies revealed that, as in the case of pure linoleic acid, the rate follows apparent first order kinetics. As expected, the oxidation of palmitic acid, even after 6 hr of incubation, is very small.

In palmitic acid-linoleic acid monolayers, the relationship between autoxidation rate and the

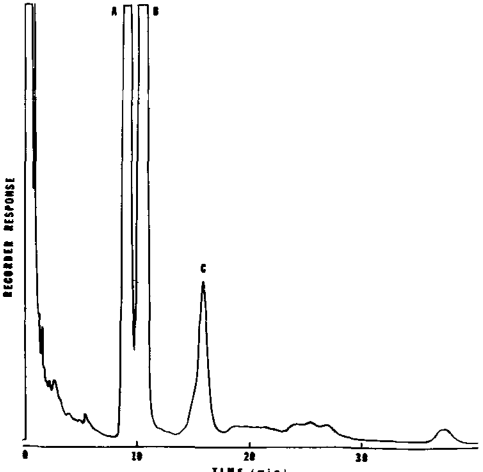

FIG. 4a. Gas chromatography of autoxidized products from linoleic acid monolayers. A, unchanged methyl linoleate; B, methyl stearate added as internal standard; C, methyl esters of I and II.

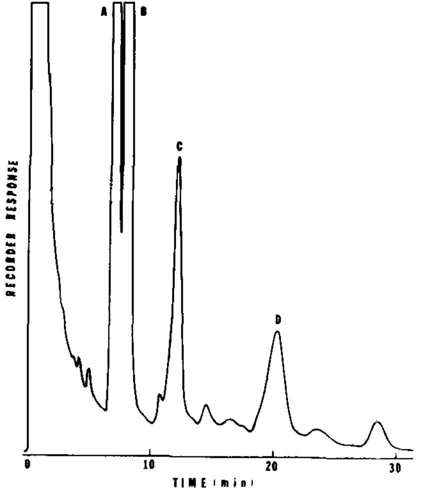

FIG. 4b. Gas chromatography of silylated autoxidized products from linoleic acid monolayers. A, unreacted methyl linoleate; B, methyl stearate added as internal standard; C, methyl esters of I and II; D, methylated trimethylsilyl ether derivatives of V and VI.

amount of palmitic acid incorporated is shown in Figure 2. With a 3 hr incubation, the amount of linoleic acid unchanged is roughly proportional to the mole percent of palmitic acid in mixed monolayers. The remaining palmitic acid is also shown to be a function of the composition of the mixed monolayers. However, the overall change is only about 4%. With saturated acid-linoleic acid monolayers, the variation in autoxidation rate with respect to the chain length of saturated fatty acid was also examined. The results are summarized in Figure 3. The protective effect of the saturated hydrocarbon chains apparently diminishes rapidly as the chain length decreases below C_{12}. With acids of medium chain length, C_{12} is more effective than C_{16}.

Pure linoleic acid monolayers, when exposed to air at 60 C for 3 hr, produce only traces of conjugated hydroperoxide and the major identifiable products are epoxy acids I and II in roughly the same quantities (5). The other minor products from autoxidation appear to be more polar than epoxide. On TLC, methyl esters of these products spread in the region between the origin and an R_f value of 0.08 and give four separate densitometer counts, 119, 416, 123, and 779. The most intense spot, presumably polymeric material, is located on the origin. The second most intense spot, with a count of 416, was later identified as methyl esters of hydroxy epoxides V and VI by the procedure described below.

$$CH_3(CH_2)_4CH{=}CHCH_2\overset{O}{CH\text{-}CH}(CH_2)_7COOH \quad (I)$$
$$CH_3(CH_2)_4\overset{O}{CH\text{-}CH}CH_2CH{=}CH(CH_2)_7COOH \quad (II)$$
$$CH_3(CH_2)_4CH{=}CHCH{=}CH\overset{OOH}{C}H(CH_2)_7COOH \quad (III)$$
$$CH_3(CH_2)_4\overset{OOH}{C}HCH{=}CHCH{=}CH(CH_2)_7COOH \quad (IV)$$
$$CH_3(CH_2)_4CH{=}CH\overset{OH}{C}H\overset{O}{CH\text{-}CH}(CH_2)_7COOH \quad (V)$$
$$CH_3(CH_2)_4\overset{O}{CH\text{-}CH}\overset{OH}{C}HCH{=}CH(CH_2)_7COOH \quad (VI)$$

GLC of methylated total autoxidation product from linoleic acid monolayers incubated at 60 C for 3 hr, without derivatization, gave a series of complex, low intensity peaks after emergence of epoxides I and II (peak C, Fig 4a). After the addition of silylation reagent, trimethylsilyloxyepoxyoctadecenoate emerged as a distinct peak of fair intensity (peak D, Fig. 4b). The ratio of peak areas of V and VI to I and II as indicated in Table I is close to 1.

Using silicic acid column chromatography, methyl esters of V and VI could be conveniently isolated from the total crude autoxidized material. The fraction eluted with 40% ether in pentane was found by GLC to be at least 70% pure (Fig. 5). The infrared spectrum of this fraction reveals a broad strong absorption at 2.82 μ characteristic of hydroxyl groups (13). The known absorption for the epoxide ring at 8.00 μ, which often appears with variable intensity, is not discernible from a broad absorption in this region.

The structures of hydroxy epoxides V and VI were confirmed by gas chromatography-

TABLE I

Yields of 9,10- and 12,13-Epoxy-11-hydroxyoctadecenoic Acid Compared to 9,10- and 12,13-Epoxyoctadecenoic Acid in Monolayers with Different Constituents

Monolayers	Extent of conversion %	Yield of hydroxy epoxide/epoxide[a]
Pure linoleic acid	34	0.47
Pure linoleic acid	66	0.95
Palmitic acid-linoleic acid (1:1)	53	1.06
Palmitic acid-linoleic acid (3:1)	37	1.50
Sparsely coated monolayers[b]	50	55.0

[a]Relative GLC peak areas of hydroxy epoxide/epoxide.
[b]The loading amounts to 5% of saturated monolayers.

mass spectrometry. Mass spectra of several positional isomers of hydroxyepoxyoctadecenoate have been reported recently (14-16). The fragmentation of trimethylsilyl derivatives of hydroxyepoxy compounds occurs predominately at both sides of trimethylsilyl ether to give simple spectra with two major ions. When an epoxide ring is located immediately adjacent to a trimethylsilyloxy group, the scission between the two functional groups preponderates over the other, so that only one major peak is observed. This is the case in our hydroxyepoxide, the major ions being m/e 199, $CH_3(CH_2)_4CH{=}CHCHOTMS$ and m/e 285, $CH(OTMS)CH{=}CH(CH_2)_7COOCH_3$, characteristic of 11-hydroxy-9,10-epoxy-12-octadecenoate and 11-hydroxy-12,13-epoxy-9-octadecenoate, respectively. Other major but less intense ions, besides m/e 398 (M) and m/e 383 (M - CH_3), to support the above two structures are for V, m/e 241, $CH_3(CH_2)_4CH{=}CHCH(OTMS)CH\overset{O}{-}CH$ and for VI, m/e 327, $CH\overset{O}{-}CHCH(OTMS)CH{=}CH(CH_2)_7COOCH_3$. The intensities of two peaks, m/e 285 and m/e 199, are strikingly similar, indicating the presence of equal quantities of two isomers. Mass spectra taken from sampling different portions of the GLC peak revealed that no resolution of the two isomers, V and VI, was attained by the OV-101 column and no other isomeric hydroxy epoxide was present in autoxidized linoleic acid.

Experiments were performed to show that linoleic acid hydroperoxides, III and IV, can be the precursors of hydroxyepoxy compounds V and VI. The major identified product obtained after exposure of the hydroperoxy-coated silica gel to our autoxidation conditions was a mixture of hydroxyepoxy compounds V and VI, identified as the trimethylsilyl derivatives on gas chromatography-mass spectrometry.

Palmitic acid-linoleic acid monolayers, when incubated for 3 hr at 60 C, gave major products similar to those from pure linoleic acid monolayers, namely epoxides I and II and hydroxy epoxides V and VI. In Table I, the ratios of yields of V and VI to I and II in different types of monolayers, including palmitic acid-linoleic acid monolayers, are summarized. GLC of silylated total autoxidized mixtures from palmitic acid-linoleic acid monolayers with mole ratio of 3:1 is also shown in Figure 6.

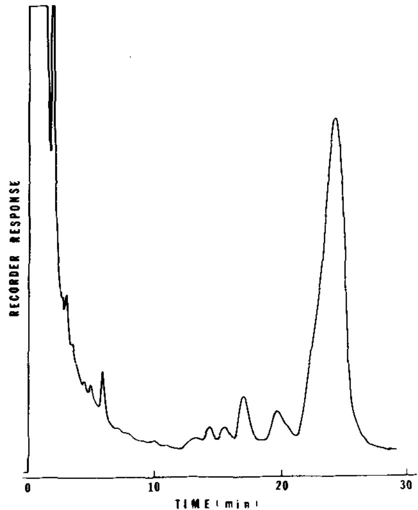

FIG. 5. Gas chromatography of relatively pure V and VI, methyl ester-trimethylsiloxy derivative.

Previously we investigated the rate of autoxidation of linoleic acid monolayers with respect to the ratios of fatty acid to adsorbent (17). The rate remains essentially constant throughout a wide range of ratios, then decreases rather sharply below loadings of 5% of saturated monolayers. GLC analysis of silylated total autoxidized product from monolayers with this particular low coverage is shown in Figure 7. It is evident from Figure 7 and Table I, that with a conversion of near 50%, the production of epoxides I and II is reduced to an extremely

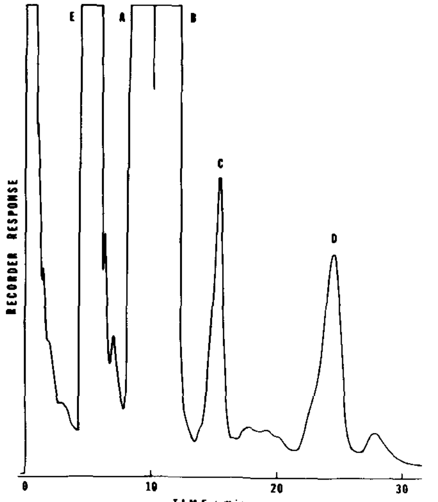

FIG. 6. Gas chromatograph of silylated autoxidized products from palmitic acid-linoleic acid monolayers. Peaks. A, B, C, and D represent the same compounds as in Figure 4b; peak E, unchanged palmitic acid.

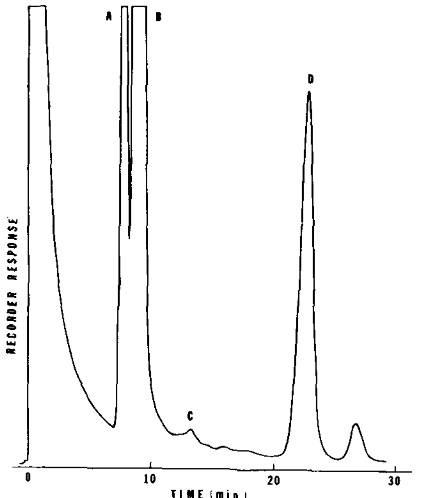

FIG. 7. Gas chromatography of silylated autoxidized products from sparsely coated linoleic acid monolayers. Peaks A, B, C, and D represent the same compounds as in Figure 4b.

small proportion and that the product is almost exclusively V and VI.

The identity of hydroxy epoxides V and VI from all three different types of monolayers, including pure linoleic acid, palmitic acid-linoleic acid, and sparsely loaded linoleic acid monolayers was established by their retention times on GLC and fragmentation patterns on

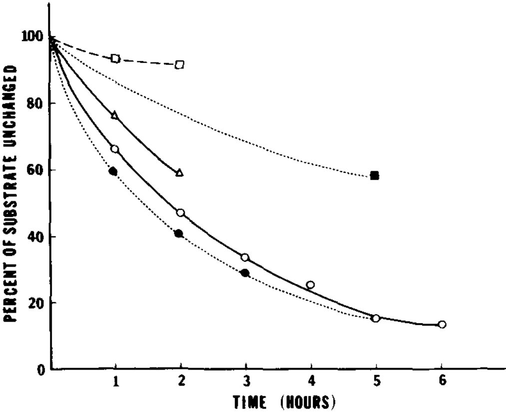

FIG. 8. Rate of autoxidation of cholesterol-linoleic acid and cholesteryl acetate-linoleic acid monolayers in comparison with that of pure linoleic acid monolayers at 60 C. —o—o—, Disappearance of linoleic acid in pure linoleic acid monolayers; ···•·······•···, disappearance of linoleic acid in cholesterol-linoleic acid monolayers; ···■·······■···, disappearance of cholesterol in cholesterol-linoleic acid monolayers; —△—△—, disappearance of linoleic acid in cholesteryl acetate-linoleic acid monolayers; —□— —□—, disappearance of cholesteryl acetate in cholesteryl acetate-linoleic acid monolayers.

gas chromatography-mass spectrometry. Hydroxyepoxide fractions from all sources were found to contain only V and VI in roughly equal quantities.

To incorporate cholesterol in linoleic acid monolayers, it was necessary to increase the polarity of the solvent to 3% ether in hexane, because of an extremely low solubility of cholesterol in the pure hexane used routinely in depositing monolayers on the silica gel surface. The slight increase in polarity of the solvent, however, drastically alters the partition coefficient between adsorbent and solvent, so that with an initial adsorbate concentration to give 100% coverage of adsorption sites (4) the more polar solvent resulted in coverage of only 40%. Autoxidation was attempted with monolayers of the reduced coverage, since we previously demonstrated that the overall rate is not affected by the extent of coverage in this range (17). Inclusion of cholesterol in linoleic acid monolayers, as shown in Figure 8, increases the rate of autoxidation slightly as measured by the disappearance of linoleic acid. The rate of autoxidation of cholesterol, although slower, is also considerable; the disappearance of the starting cholesterol is ca. 40% at the end of a 5 hr incubation.

Investigation of the products of cholesterol oxidation under these conditions is currently underway and will be described in a subsequent

publication.

Cholesteryl acetate dissolves readily in pure hexane, and the usual procedure of depositing the monolayer with hexane could be used. In preparation of cholesterol-linoleic acid monolayers, cholesterol showed preference over linoleic acid in adsorption from hexane, whereas in cholesteryl acetate-linoleic acid monolayers, slightly more linoleic acid was adsorbed from an initially equimolar mixture of the two substrates. As shown in Figure 8, with the inclusion of cholesteryl acetate in the monolayers, the autoxidation rate is somewhat lowered. Unlike cholesterol, cholesteryl acetate is autoxidized only to a small extent after a 2 hr incubation.

DISCUSSION

A possible process for the formation of epoxides reported in Part II of this series (5) is the reaction of peroxy radical with an olefin (Equation A) (18).

$$C{=}C + ROO\cdot \rightarrow ROOC\text{-}C\cdot \rightarrow \overset{O}{C\text{-}C} + RO\cdot \quad (A)$$

Further, it is possible that the hydroxyepoxy compounds, V and VI, are formed by way of an intramolecular rearrangement of the diene hydroperoxides, III and IV (Equation B) (15).

H O O → H O O (B)

When linoleic acid molecules on the silica gel surface are separated by interspersed saturated fatty acids, which are relatively inert to the oxidizing conditions, it is expected that the apparent rate of disappearance of linoleic acid, which will include the rate of the bimolecular reaction Equation A, will decrease. Experiments described above show that the rate of autoxidation in palmitic acid-linoleic acid monolayers is inversely roughly proportional to the mole fraction of palmitic acid in the mixed monolayers. As the concentration of linoleic acid in the monolayer decreases, the relative amount of the bimolecular reaction product, the epoxides, decreases. At the same time, there is an increase in the relative amount of the presumed unimolecular product, the hydroxyepoxides.

The apparent rate of disappearance of linoleic acid is also influenced by the chain length of the intervening fatty acid in the mixed monolayers. At the same molar ratio of linoleic to saturated acid, the autoxidation rate decreases as the saturated acid chain length is increased from caproic to lauric acid. In the C_6 - C_{12} acid range, it appears that lengthening the hydrocarbon chain of the saturated acid into the vicinity of the double bonds of linoleic acid blocks the radical chain process of the autoxidation. Increasing the chain lenth from C_{12} - C_{16} results in a reversal of this trend. Perhaps this reversal is the result of removal of the methyl end of the saturated acid farther from the reaction area of the linoleic acid thereby moving the area of greatest disorder caused by the rapid rotation at the terminal C-C bond (19,20).

An alternative for the above explanation of the effects of saturated fatty acids larger than lauric derives from consideration of a model in which the saturated and linoleic acids tend to form areas on the surface that are homogenous in a particular fatty acid. One would expect this tendency to increase as the saturated acids become larger. If the areas covered by linoleic acid were large, the kinetics and products would be the same as for the complete monolayer.

Further insight into the distribution of fatty acids can be garnered from the experiments described here. We are unaware of any experimental evidence to either confirm or deny the mobility of fatty acids (or for that matter, any molecule) on silica gel. Following our desire to keep the model for this system as simple as possible, and to be faithful to our intuition, we are discounting the possibility that the fatty acids migrate on the surface of the prepared monolayers. When linoleic acid-silica gel monolayers are varied from 5 to 100% surface coverage, the autoxidation rate and product distribution remain the same. This implies that as the average coverage of the silica gel is decreased, there is sufficient van der Waals attraction between the fatty acid chains so that they are arranged in patches of monolayer distribution over the surface. With coverage of less than 5% of the surface, the arrangement seems to tend toward smaller patches or isolated molecules. At this low surface coverage, the apparent rate of linoleic acid oxidation decreases. The product distribution changes so that the relative amount of epoxide, I and II, is greatly reduced and hydroxyepoxide, V and VI, becomes the major product.

In regard to the changes in rate of linoleic acid autoxidation in monolayer containing cholesterol and cholesteryl acetate, it appears that cholesteryl acetate is behaving in the same manner as a saturated fatty acid in a mixed monolayer in that it acts as a spacer to keep the linoleic acid molecules farther apart while being

only slowly oxidized itself.

The rate of disappearance of linoleic acid in the cholesterol mixed monolayer is similar to that in the simple monolayer suggesting that there are patches of linoleic acid and cholesterol on the surface and that in each patch, autoxidation is proceeding at a rate characteristic of each pure compound.

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Long Chain Phenols: Part XI. Composition of Natural Cashew Nutshell Liquid *(Anacardium occidentale)* from Various Sources

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ABSTRACT

The composition of cashew nuts *Anacardium occidentale* from different terrestrial sources has been studied. Samples from Brazil, Ceylon, Kenya, Mozambique, Nigeria, and Tanzania have been solvent extracted to recover the phenolic shell liquid (natural CNSL) separate from the kernel oil. The recovered materials from the different sources were present from 23.6% to 27.7%. After hydrogenation of the side chains and methylation of the acidic groups, the component phenols anacardic acid (74.1% to 77.4%), cardol (15.0% to 20.1%), 2-methyl cardol (1.7% to 2.6%), and cardanol (1.2% to 9.2%) were determined by gas liquid chromatography on polyethyleneglycol adipate. The component phenols have been separated by adsorption thin layer chromatography, and their triene (AN-15:3, 36.3% to 50.4%), diene (AN-15:2, 17.8% to 32.1%), monoene (AN-15:1, 25.0% to 33.3%), and saturated (AN-15:0, 2.2% to 3.0%) constituents determined by mass spectroscopy. The results of mass spectroscopic analysis have been confirmed by methylation of the separated component phenols and gas liquid chromatography. It is apparent that the largest variation is in the % cardanol (1.2% to 9.2%). While the total percentage of unsaturated constituents is quite similar, the distribution of triene, diene, and monoene varies widely.

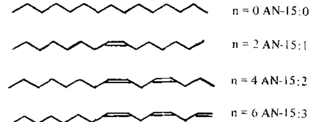
INTRODUCTION

The cashew tree *Anacardium occidentale* of Brazilian origin is the most abundant member of the Anacardiacae family. Probably as a result of Portuguese influence, it is now grown in many other tropical countries, notably in Mozambique, Tanzania, India, Kenya, Nigeria, Malagasy Republic, Ceylon, Indonesia, and other areas. The first three countries were, in order, the principal producers and Brazil the fourth (1); however, since January 1978, Brazil is first.

Although the kernel is the primary objective of cultivation, a major by-product of considerable industrial importance in polymer applications, chiefly in friction dusts, is the liquid phenolic mixture, cashew nutshell liquid (technical CNSL, sometimes termed industrial CNSL) derived from the intact shell by a roasting process (2) which converts the principal component phenol, anacardic acid (I) in the natural product, to cardanol (II). Natural CNSL is best isolated by solvent extraction and, like technical CNSL, contains cardol (III) and 2-methyl cardol (IV) as well as anacardic acid.

This work concerns the quantitative composition of natural CNSL derived from different regions with respect to the main (C_{15}) component phenols present, (I, II, III, and IV) and to the four constituents present in each (n = 0, 2, 4, 6).

$C_{15}H_{31-n}$, CO_2H, OH (I); $C_{15}H_{31-n}$, OH (II); HO, $C_{15}H_{31-n}$, OH (III); HO, $C_{15}H_{31}$, CH_3, OH (IV)



Extensive early structural work served as a basis for various analytical procedures, and a gas liquid chromatographic (GLC) method on hydrogenated and methylated natural and technical CNSL (3) was useful for determination of the component phenols. A thin layer chromatographic (TLC) separation of the component phenols followed by methylation and GLC analysis (4) enabled the unsaturated constituents to be determined. More recently a TLC/mass spectroscopic (MS) method (5,6) has been found invaluable in the analysis of large numbers of samples. Compositional studies on technical CNSL from different sources have also been made (7). The significance of the present results with references to the technical CNSL derivable from the particular regional source and to biosynthetic aspects is discussed. The work is the first approach to comparative chromatographic analysis of natural CNSL.

EXPERIMENTAL PROCEDURES

Materials

Samples of cashew nuts from Brazil, Kenya, Mozambique, and Tanzania were obtained in 1971-1972 from J.O. Duce, (Wigglesworth and Co. Ltd., London). Samples of Tanzanian and Mozambique cashew nuts were also obtained from British Coco Mills, Hull, Yorks. Nigerian

TABLE I

Retention Times (Uncorrected) in the Gas Liquid Chromatography Analysis of the C_{15} Component Phenols (and Their Methyl Ether) in Natural CNSL

(CNSL) Country of origin	Treatment[a]	Retention time (min, RT) and relative retention (RR): AN-15:0 cardanol RT	RR	AN-15:0 cardol RT	RR	AN-15:0 2-methyl cardol RT	RR	AN-15:0 methyl anacardate RT	RR	Column
Brazil	X	19.2	(1)	48.6	(2.53)	---	---	36.0	(1.87)	A
Ceylon	X	19.4	(1)	48.6	(2.51)	---	---	36.4	(1.87)	A
Kenya	X	18.8	(1)	46.4	(2.47)	---	---	34.8	(1.85)	A
Mozambique	X	19.8	(1)	48.4	(2.44)	---	---	35.8	(1.81)	A
Nigeria	X	18.8	(1)	47.2	(2.51)	---	---	34.6	(1.84)	A
Brazil	X	42.8	(1)	131.2	(3.06)	149.2	(3.48)	94.4	(2.20)	B
Ceylon	X	42.8	(1)	136.4	(3.18)	155.2	(3.63)	97.2	(2.27)	B
Kenya	X	42.8	(1)	131.6	(3.07)	148.4	(3.47)	96.0	(2.24)	B
Mozambique	X	42.4	(1)	139.6	(3.29)	154.4	(3.64)	103.2	(2.43)	B
Nigeria	X	38.8	(1)	128.0	(3.29)	144	(3.71)	93.2	(2.40)	B
		AN-15:0 methyl ether		AN-15:0 dimethyl ether		AN-15:0 dimethyl ether		AN-15:0 dimethyl anacardate		
Brazil	X,Y	25.6	(1)	74.4	(2.91)	65.4	(2.55)	118.0	(4.61)	C
Ceylon	X,Y	24.2	(1)	73.4	(3.03)	63.2	(2.61)	114.4	(4.73)	C
Kenya	X,Y	24.4	(1)	74.4	(3.05)	64.0	(2.62)	116.4	(4.77)	C
Mozambique	X,Y	25.2	(1)	76.0	(3.02)	65.8	(2.61)	121.2	(4.81)	C
Nigeria	X,Y	24.8	(1)	76.4	(3.08)	65.8	(2.65)	119.6	(4.82)	C
Tanzania	X,Y	24.8	(1)	74.6	(3.01)	64.4	(2.60)	115.6	(4.66)	C

[a]X=hydrogenated and anacardic acid diazomethane methylated. Y=methylated with dimethyl sulfate/potassium carbonate.

cashew nuts were obtained from R.G. Lowe, Federal Department of Forest Research, Ibadan, and Ceylonese cashew nuts from Mr. Anandappa, Mahdu Cashew Plantations, Colombo. Indian cashew nuts could not be obtained in time for this work.

Extraction of Natural CNSL

Cashew nuts were treated as described, and the CNSL extracted essentially by the method given (8). Between 45 and 75 g of each regional source of cashew nuts was used for solvent extraction. The regional source and % natural CNSL extracted at the 1st, 2nd, 3rd stages, and the total recovered were as follows, Brazil (21.3, 6.1, 1.1, 27.7%), Ceylon (16.9, 5.6, 1.8, 23.6%), Kenya (18.3, 7.3, 1.4, 26.2%), Mozambique (16.5, 8.7, 2.4, 27.6%), Nigeria (20.1, 4.3, 1.3, 25.3%), and Tanzania (15.2, 8.5, 2.3, 26.0%). The final totals are sometimes slightly lower than the sum of the three extractions due to traces of solvent present. During the extractions, 0.1% 4-methyl-2,6-di-t-butylphenol was used as an antioxidant. All CNSL samples were stored in tightly stoppered flasks at -20 C under nitrogen.

Thin Layer Chromatography

All TLC was carried out on Silica Gel G. Analytical plates were 10 x 8 cm (0.25 mm thick), and preparative plates were 20 x 20 cm (1.0 mm thick). Sample application, development, visualization elution, and recovery were carried out as described (4).

Gas Liquid Chromatography

All GLC was carried out as described (3,4) with a Pye Unicam 104 gas chromatograph with flame ionization detector. Glass columns were used (5 ft or 6 ft by 3/16 in.) with nitrogen (27 ml/min) as carrier gas. The column length, stationary phase, and temperature were as follows: A, 5 ft, 3% SE30, 220 G; B, 6 ft, 3% SE30, 180 C; C, 5 ft, 2% PEGA, 200 C. Peak areas were determined by triangulation which showed close agreement with the integration method.

It was essential to use 2% PEGA on a (100-120) BSS support (Diatomite C) for the separation of the methylated unsaturated constituents; (60-80) and (80-100) mesh sizes were unsatisfactory, although the latter was adequate for the AN-15:0 methyl ethers. All GLC results were the average of six determinations, and standard deviations were calculated for all results.

Mass Spectroscopy

Mass spectroscopy was carried out by D. Carter with an AEI MS902 instrument through the courtesy of the ULIRS scheme at The School of Pharmacy, University of London. TLC separations of natural CNSL (ca. 0.20 g) from the different sources were made on preparative plates, and the recovered materials, anacardic acid, cardol, 2-methyl cardol and cardanol, used for mass spectroscopy. Anacardic acid was converted (3) to methyl anacardate. The procedure and processing of results were carried out as described (5).

Hydrogenation and Methylation

Hydrogenation of natural CNSL extracted from the different sources was carried out as described (8,9) until no further uptake of hydrogen occurred and the ^{1}H-NMR spectrum showed no olefinic absorption.

The regional samples of natural CNSL in ethereal solution were treated first with ethereal diazomethane (3). Complete methylation of the CNSL (1 part) was carried out by refluxing for 3 hr with dimethyl sulfate (4 parts) and anhydrous potassium carbonate (25 parts) in benzene solution (10 ml). The methylated product was examined by GLC.

RESULTS AND DISCUSSION

Extraction of Natural CNSL

In the solvent extraction of natural CNSL, two sets of extractions had been carried out, both of which showed good agreement.

From weights of CNSL recovered by the three extractions, it is clear that the recovery is substantially complete at the second stage. In one analytical procedure (10) concerned with anacardic acid, the shell and kernel were apparently co-disintegrated. This greatly complicates the subsequent analytical steps, and preliminary separation of the two is desirable. Continuous ethereal extraction of the disintegrated shells would be useful, although this could not be investigated in the present work. The lowest and highest yields, namely from Ceylonese and Brazilian cashew nuts, do not differ by more than ca. 15%. The figures for the % anacardic acid afford an indication of the potential technical CNSL recoverably by the roasting process. At present, relatively little natural CNSL is recovered on an industrial scale by solvent extraction (1) and the natural product remains a potential source of the major component, anacardic acid, first isolated 130 years ago (11). The % recovery and composition of the glyceride oil from the cashew kernel will be reported elsewhere.

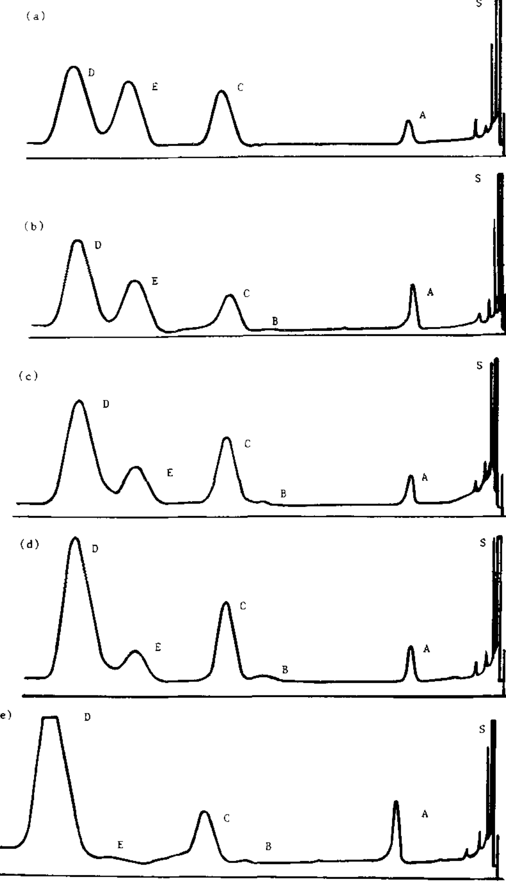


FIG. 1. Gas liquid chromatogram (on 2% PEGA) of progressive stages in the methylation of natural CNSL. (A), (AN-15:0) cardanol methyl ether, (B), (AN-15:0), 2-methyl cardol dimethyl ether, (C), (AN-15:0), cardol dimethyl ether, (D), (AN-15:0), dimethyl anacardate, (E), (AN-15:0), methyl anacardate, (S), solvent.

Relative Retention of AN-15:0 Hydrogenated Component Phenols and Their Methyl Ethers

The retention times (RT) and relative retentions (RR) of the AN-15:0 hydrogenated component phenols and their mehtyl ethers are shown in Table I.

The greater variation in relative retention of 2-methyl cardol and methyl anacardate on an SE30 column (B) compared with the fully methylated materials on a PEGA column (C) was due to skewed peaks. With PEGA, peaks were symmetrical and although the stationary phase was being used at the maximum recommended temperature, consistent performance was obtained. With SE30 columns, the life of the column was relatively short and silanization

TABLE II

Composition of C_{15} Component Phenols in Natural CNSL from Different Regions (Gas Liquid Chromatography on the Stationary Phase PEGA)[a]

Parameter	Anacardic acid[b]	Cardol	2-Methyl cardol	Cardanol
Relative response factors[c]	5.7465	5.7911	5.4757	6.5643
(4) CNSL (Brazil)	77.43 ± 0.88	18.17 ± 0.12	2.30 ± 0.19	2.09 ± .05
(4) CNSL (Ceylon)	75.48 ± 0.20	20.13 ± 0.14	2.13 ± .08	2.25 ± 0.18
(4) CNSL (Kenya)	75.72 ± 0.49	19.34 ± 0.18	2.58 ± 0.99	2.36 ± 0.09
(4) CNSL (Mozambique)	76.47 ± 0.74	15.69 ± 0.14	1.83 ± 0.04	6.01 ± 0.03
(4) CNSL (Nigeria)	76.46 ± 0.16	19.73 ± 0.18	2.61 ± 0.02	1.21 ± 0.06
(4) CNSL (Tanzania)	74.06 ± 0.68	15.03 ± 0.51	1.74 ± 0.24	9.17 ± 0.09

[a]AN-15:0 dimethyl anacardate (376) expressed as AN-15:0 anacardic acid (348); AN-15:0 cardol dimethyl ether (348) expressed as AN-15:0 cardol (320); AN-15:0 2-methyl cardol dimethyl ether (362) expressed as AN-15:0 2-methyl cardol (334); AN-15:0 cardanol methyl ether (318) expressed as AN-15:0 cardanol (304).
[b]Anacardic acid (344); cardol (314); 2-methyl cardol (328); cardanol (298).
[c]Relative response factors are expressed (3) as % component/g. Hence, in the correction step 2, the % is divided by the RRF.

did not prolong this.

For the hydrogenated AN-15:0 diazomethane-treated phenols, a considerable excess of dimethyl sulfate was required to convert phenolic methyl anacardate to dimethyl anacardate. During experiments where the course of the methylation reaction was monitored and later, where different molar proportions of dimethyl sulfate to CNSL phenols were used, a fifth peak in the gas chromatogram (column C, RT 95.4, RR 4.04) due to AN-15:0 methyl anacardate was present. This was adequately resolved for triangulation purposes from the succeeding peak of dimethyl anacardate. Its occurrence did not complicate the quantification since the relative response factor (RRF) of AN-15:0 methyl anacardate has been determined (6).

The volume of dimethyl sulfate (ml) and the ratio (from the peak areas) of dimethyl anacardate to methyl anacardate in the methylation of Mozambique CNSL (0.4 g) were as follows: 0.5 ml, 1.76; 0.9 ml, 4.79; 1.3 ml, 29.76 (Fig. 1). With the proportions of reagents given in the Experimental section, the methylations proceeded to completion, and no methyl anacardate was present.

Quantitative Composition of the Component Phenols in Regional Sources of CNSL

The first quantitative GLC analyses on hydrogenated natural CNSL from different regions were made with 3% SE30 as the stationary phase (9). Skewed peaks and insufficient resolution of cardol and 2-methyl cardol tended in the present series to result in underestimation of the former and inaccuracy in the latter, although electronic integration would probably have solved the problem. The use of a longer (6 ft) column was not wholly successful, and the difficulty in obtaining a column as effective as the original was due to the different nature and particle size of the support in the present work. However, since an alternative procedure based on methylation of the hydrogenated product and GLC examination on PEGA (3) was available, the use of SE30 was not pursued. A minor advantage of PEGA as a stationary phase is that it reveals the presence of AN-15:0, AN-15:1, AN-15:2, and AN-15:3 constituents, should the hydrogenation stage have been incomplete.

The final results for the component phenols in regional sources of CNSL have been summarized in Table II. The four steps (3) in the processing of results were: (a) calculation of the uncorrected normalized (%) composition from the peak area, (b) correction (% composition/RRF) and normalization of the % composition with the RRF values (at the head of the table), (c) calculation and normalization of the results as AN-15:0 phenols, with the molecular weights given at the foot of the table, (d) expression of the results shown in the table as unsaturated phenols (anacardic acid and cardanol being on average dienoid and cardol and 2-methyl cardol, trienoid).

In general, the results for the % anacardic acid show similarity, and the % cardol and cardanol show greater variation.

The differences in % anacardic acid between the present and earlier results (3) for natural CNSL of Mozambique origin are considered to be due to its decarboxylation. The same is also true of the result for Tanzanian CNSL. In the case of CNSL from Mozambique, expression of the difference of % cardanol (6.05-1.59) as % anacardic acid and normalization of the modified results gives 80.92% anacardic acid, 15.69% cardol, 1.83% 2-methyl cardol, and 1.59% car-

TABLE III

Composition of Unsaturated Constituents of the C_{15} Component Phenols in Natural CNSL from Different Regions

	Methyl anacardate	AN-15:0 (348)[a]	AN-15:1 (346)	AN-15:2 (344)	AN-15:3 (342)
Relative response factor (RRF)[b]		.66736 (1.0000)	1.2842 (1.9243	2.1004 (3.1473)	3.0843 (4.6216)
(3)	CNSL (Brazil)	2.69	25.03 ± 1.76	18.68 ± 0.89	53.60 ± 0.76
(3)	CNSL (Ceylon)	2.86	33.53 ± 0.80	17.76 ± 0.44	45.84 ± 0.40
(3)	(Kenya)	2.41	33.26 ± 2.39	19.69 ± 0.40	44.64 ± 1.46
(3)	CNSL (Mozambique)	2.23	32.04 ± 0.45	17.37 ± 0.42	48.35 ± 0.27
(3)	CNSL (Nigeria)	3.03	28.51 ± 1.41	32.13 ± 0.89	36.34 ± 0.50
(3)	CNSL (Tanzania)	2.49	27.78 ± 5.68	19.29 ± 3.24	50.43 ± 3.66
	Cardol	AN-15:0 (320)	AN-15:1 (318)	AN-15:2 (316)	AN-15:3 (314)
Relative response factor		.5074 (1.000)	2.7964 (5.5112)	2.2612 (4.4504)	2.5636 (5.0524)
(3)	CNSL (Brazil)	0.26	8.65 ± 0.29	21.59 ± 0.60	69.49 ± 0.82
(3)	CNSL (Ceylon)	0.31	11.83 ± 0.59	23.09 ± 1.03	64.76 ± 1.21
(3)	CNSL (Kenya)	0.27	11.92 ± 0.65	24.03 ± 0.34	63.78 ± 0.34
(3)	CNSL (Mozambique)	0.32	11.06 ± 0.46	21.54 ± 0.27	67.08 ± 0.70
(3)	CNSL (Nigeria)	0.38	8.27 ± 1.39	39.95 ± 1.14	51.40 ± 0.41
(3)	CNSL (Tanzania)	0.29	11.27 ± 0.48	20.90 ± 0.61	67.53 ± 0.39
	2-Methyl cardol	AN-15:0 (334)	AN-15:1 (332)	AN-15:2 (330)	AN-15:3 (328)
Relative response factors[c]		.5074 (1.000)	2.7964 (5.5112)	2.2612 (4.4504)	2.5636 (5.0524)
(3)	CNSL (Brazil)	1.19	14.55 ± 0.99	21.96 ± 3.46	62.30 ± 3.39
(3)	CNSL (Ceylon)	0.71	9.52 ± 0.31	13.09 ± 0.15	76.68 ± 0.71
(3)	CNSL (Kenya)	0.98	20.94 ± 0.49	21.62 ± 0.57	56.46 ± 0.91
(3)	CNSL (Mozambique)	1.19	18.30 ± 0.74	19.74 ± 0.91	60.67 ± 1.96
(3)	CNSL (Nigeria)	2.24	16.70 ± 1.62	38.21 ± 1.44	42.85 ± 2.12
(3)	CNSL (Tanzania)	0.95	20.25 ± 1.82	18.39 ± 0.55	60.42 ± 1.69
	Cardanol	AN-15:0 (304)	AN-15:1 (302)	AN-15:2 (300)	AN-15:3 (298)
Relative response factors		1.95730 (1.0000)	2.2621 (1.551)	2.5279 (1.2915)	6.5559 (3.3493)
(3)	CNSL (Brazil)	1.99	23.63 ± 0.60	16.21 ± 0.26	57.94 ± 0.37
(3)	CNSL (Ceylon)	2.35	32.54 ± 1.82	17.07 ± 1.34	48.04 ± 0.42
(3)	CNSL (Kenay)	2.68	32.64 ± 2.84	17.89 ± 0.37	46.79 ± 0.54
(3)	CNSL (Mozambique)	1.91	30.95 ± 1.41	13.32 ± 2.58	53.82 ± 0.51
(3)	CNSL (Nigeria)	3.08	25.94 ± 1.19	25.19 ± 0.98	45.79 ± 1.12
(3)	CNSL (Tanzania)	1.80	31.74 ± 0.72	14.63 ± 0.42	51.83 ± 0.79

[a]The figures in parentheses at the head of the table represent the molecular weight of the saturated, monoene, diene, and triene constituents of anacardic acid, respectively; similar mol wts are given elsewhere in the table for cardanol, cardol, and 2-methyl cardol.

[b]The relative response factors are expressed as g x 10^4/% constituent (5), and obtained from a standard mixture. The corrected % for the natural product was, therefore, obtained from % constituent x RRF (ref. 5). These values are of course different from those found in GLC examination.

[c]The same values as for cardol were used.

danol comparing favorably with the earlier figures (3), 81.98% anacardic acid, 13.78% cardol, 2.64% 2-methyl cardol, and 1.59% cardanol.

Quantitative Composition of the Unsaturated Constituents of the Component Phenols

Quantitative analysis of the AN-15:0, AN-15:1, AN-15:2, and AN-15:3 constituents has been carried out by GLC (4) on PEGA after preliminary TLC separation followed by methylation. Originally (12) the acetates were experimented with, but their slight instability made the methyl ethers more useful volatile derivatives, particularly in the case of anacardic acid. The long retention times of cardol dimethyl ether and dimethyl anacardate protracted the analysis when many samples were involved. A TLC/mass spectroscopic procedure (5) in which the separated component phenols were examined without the requirement for the preparation of a volatile derivative, was found to be more suitable for the examination of many samples.

Table III shows the final results for the four constituents of methyl anacardate, cardol, 2-methyl cardol, and cardanol. The steps in the procedure were: (a) calculation of the nor-

TABLE IV

Comparison of Results from Gas Liquid Chromatographic (GLC) and Mass Spectroscopic (MS) Analyses for the Constituents of Cardanol from Different Natural CNSL Sources[a]

Cardanol	AN-15:0	AN-15:1	AN-15:2	AN-15:3
CNSL (Brazil)				
(1) GLC	1.98 ± 0.54	22.93 ± 0.31	16.10 ± 0.30	58.98 ± 0.81
(2) MS	1.99	23.63	16.21	57.94
CNSL (Mozambique)				
(1) GLC	1.88 ± 0.19	30.74 ± 0.93	14.63 ± 0.30	52.75 ± 0.66
(2) MS	1.91	30.95	13.32	53.82
CNSL (Tanzania)				
(1) GLC	1.42 ± 0.12	31.83 ± 0.72	14.54 ± 0.32	52.21 ± 0.63
(2) MS	1.80	31.74	14.63	51.83

[a]Values taken from Table III (cardanol).

TABLE V

Total Average Unsaturation of C_{15} Component Phenols in Natural CNSL From Different Regions

Component and unsaturation	Regional source					
	Brazil	Ceylon	Kenya	Mozambique	Nigeria	Tanzania
Cardanol	2.09	2.25	2.36	6.01	1.21	9.17
(1) Average double bond (ADB)	2.297	2.108	2.088	2.190	2.137	2.165
Cardol	18.17	20.13	19.34	15.69	19.73	15.03
(2) Average double bond	2.603	2.523	2.513	2.554	2.424	2.557
2-Methyl cardol	2.30	2.13	2.58	1.83	2.61	1.74
(3) Average double bond	2.454	2.657	2.335	2.399	2.217	2.383
Methyl anacardate	77.43	75.48	75.72	76.47	76.46	74.06
(4) Average double bond	2.232	2.066	2.066	2.118	2.018	2.176
Total unsaturation [Σ(ADB x % composition)]/100	2.3056	2.1709	2.1598	2.1956	2.1047	2.2358

malized % composition from the peak heights of the relevant molecular ion (P) in the mass spectrum, (b) normalization of the % composition corrected for the (P+2)% contribution, and (c) normalization of the % composition corrected for the relative response differences (% composition x RRF).

The results show greater variations than in the case of the component phenols. This is particularly noticeable for Nigerian CNSL.

Comparison of the Gas Liquid Chromatographic and Mass Spectroscopic Methods for the Analysis of the Unsaturated Constituents

The results from GLC of the methyl ether compared with MS analysis of the phenol, for cardanol from the Mozambique, Brazilian, and Tanzanian sources is shown in Table IV which gives: (a) the % composition corrected and normalized, and (b) the % composition by MS. The agreement is excellent, provided relative response factors are applied in each set. Only cardanol can be used in the GLC method without the methylation stage, whereas the low volatility of the phenols caused no problem in the vacuum conditions of the mass spectrometer. The speed of analysis is considerably increased in the MS method.

Relative Total Unsaturation of the Component Phenols

In Table V, a comparison has been made of the average number of double bonds (ADB) for each component phenol from different regions. The fraction of diene and triene from Table III for each component phenol have been multiplied by two and three, respectively, and added to the value of the monoene to give the average values shown. The total unsaturation for each source has been obtained from the summed ADB value multiplied by % composition (from Table II). The order of total unsaturation with respect to country of origin is Brazil, Tanzania, Ceylon, Kenya, Mozambique, and Nigeria.

Influence of Regional Source upon the Derived Technical CNSL

The results for natural CNSL from different regions are of interest in connection with the potential technical CNSL derivable by industrial decarboxylation.

The CNSL from Mozambique and Tanzania is similar and differs from the Ceylon, Kenya, and Nigerian sources which have a higher % cardol although the other component phenols are present to the same extent in all, with the exception of the % cardanol originating from natural decarboxylation. The total unsaturation is similar for all the samples, the % triene being highest followed by the % diene and % monoene, with the exception of the Nigerian material in which the % triene and diene are comparable and the former lower than for the other samples.

The Mozambique, Tanzanian, Indian, and Brazilian regions are the primary sources of technical CNSL by decarboxylation of the natural product and are, therefore, of greater commercial and industrial interest. The Brazilian source has a higher % of cardol and this could be significant with regard to aldehyde polymerization reactions involving the phenolic ring. In East African and Brazilian natural CNSL, the unsaturation is somewhat similar, and derived technical CNSL should, other factors being equal, be similar in general composition. However, later work (part XII) has indicated the presence of varying proportions of polymeric material.

Factors Influencing the Unsaturation in Natural CNSL from Different Regions

The compositional results appear to indicate that the regional sources all belong to the same species *Anacardium occidentale*, simply cultivated in different parts of the world. The results for Nigerian CNSL may point to its being another variation. No botanical identification was possible in the present work with any of the sources.

In the case of essential oils, the same species grown in different parts of the world produces much the same composition (13) and the same is true of oils and fats where (4), "each species produces its own characteristic qualitative mixture of seed fatty acids which remain the same irrespective of the environment in which it is grown."

Only one of eight species of *Anacardium occidentale* is cultivated as a food producer (14), and it seems likely this is the one examined in the present work. The related *Anacardium semecarpus* (15) and *Anacardium giganteum* (16) are very different in composition. Similarly, the members of the related Rhus genus, *Rhus toxicodendron* (17,18) and *Phus vernicifera* (19), are widely different in % of unsaturated constituents. It is not wholly clear, however, to what extent % compositional variation may be expected within a species.

For the oils and fats, growth in cooler conditions gives seeds richer in the more unsaturated acids and poorer in oleic acid, and vice versa in warmer climates (20). Accordingly, a correlation appeared possible between the latitude of growth of the *Anacardium occidentale* source and the toal unsaturation in the CNSL. Cultivation in a cooler climate might lead to a higher % triene or higher total unsaturation. The difference between Brazilian CNSL (ΣADB 2.31, latitude 18°S) and Kenyan CNSL (ΣADB 2.16, latitude 0°) seems to show some connection. However, latitude alone without criteria of average temperature, humidity, rainfall, and other factors may be misleading for correlative purposes.

The kernel fatty acid composition, namely AN-16:0 6.4%, AN-18:0 13.3%, AN-18:1 74.1%, and AN-18:2 7.7% (21,22) (ADB value 0.895), compares strikingly with the predominantly unsaturated CNSL composition (ADB value ca. 2.10). Both in chain length and unsaturation the two are different. Palmitic acid is a biosynthetic precursor of the C_{15} phenols, and its low % in the kernel may be coincidental or related to its utilization in the phenolic shell liquid. As a result of the extensive work of Bloch and of Lynen, the pathways of biosynthesis of fatty acids have become clarified (23), and their involvement in the phenolic lipid biosyntheses in *Anacardium occidentale* are being examined currently.

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Isolation of Sterol Mutants in *Chlamydomonas reinhardi*: Chromatographic Analyses

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ABSTRACT

Several sterol mutants of *Chlamydomonas reinhardi* were isolated by selecting for single colonies resistant to the polyene antibiotic, nystatin. The sterol profiles of three isolates – KD7, KD16, and KD21 – as well as a wild type strain (137C) were determined by gas liquid chromatography and mass spectrometry. The wild type strain contained ergosterol (C_{28}-5,7,22-triene) as the predominant sterol, with smaller amounts of a C_{28}-5,7-diene, a C_{28}-7-ene, and C-24 ethyl analogs of these three sterols. The three mutants had altered sterol composition, but none required exogenous sterol for growth. KD7 contained a C_{28}-5,7,22,25-tetraene, a C_{28}-5,7,25-triene, a C_{28}-7,25-diene, and three C-24 ethyl analogs. The lesion in KD7 is apparently an inability to reduce the C-25 double bond required for the biosynthesis of ergosterol and an ethyl analog. The predominant sterols accumulated by KD16 and KD21 were a C_{28}-5,7-diene, a C_{28}-7-ene, and C-24 ethyl versions of these two sterols, suggesting that these strains are 22(23)-desaturase mutants.

INTRODUCTION

Interest in plant sterols stems from their roles as structural components of cell membranes and as possible regulators of hormone action (1). Sterol biosynthesis in plants has been investigated principally using radioactive acetate and mevalonate precursors to deduce the operative sequence of the pathway (1). An alternative method of elucidating the biosynthetic steps, particularly those following the formation of cycloartenol, was employed by Dickson and Patterson (2) who used the hypocholesteremic drug AY-9944 (trans-1,4-bis-[2-chlorobenzylaminomethyl] cyclohexane dihydrochloride). These investigators administered AY-9944 (4 ppm) to *Chlorella ellipsoidea* and found that this dose completely inhibited $\Delta^8 \rightarrow \Delta^7$ isomerization and reduction of the C-14 double bond. In this way, intermediates in the biosynthetic pathway could be isolated and determined.

An alternative approach to the elucidation of the sterol biosynthetic pathway has been to obtain mutants blocked at various steps. Such mutants have been reported in a variety of fungi – *Saccharomyces cerevisiae* (3,4), *Candida albicans* (5,6), *Candida tropicalis* (7), and *Neurospors crassa* (8), by selecting for resistance to polyene antibiotics such as nystatin. Polyenes act by complexing with sterols in the cell membrane causing loss of selective permeability and cell death (9). Resistance to polyene antibiotics results from a genetic lesion in the sterol pathway such that only sterol intermediates accumulate. These mutant strains neither require nor take up exogenous sterol from the medium (3). The intermediate sterols that accumulate have less affinity for nystatin than for the end product sterol. For example, yeast strains possessing Δ^8 sterols are more resistant to polyenes than strains possessing Δ^7 sterols, which in turn are more resistant than strains possessing $\Delta^{5,7}$ sterols (6). Indeed, this method of selection for nystatin resistance was also successful in the isolation of a sterol mutant in LM mouse fibroblast cells (10).

We report here the isolation of sterol mutants in the alga *Chlamydomonas reinhardi* by selecting for resistance to the antibiotic, nystatin. We have isolated two mutants both of which do not synthesize the wild type end product sterol, ergosterol. To our knowledge, this is the first report of ergosterol as the major sterol in an algal genus other than *Chlorella* (11).

For convenience in referring to the complex mixtures of C-24 methylated and ethylated sterols present in the mutants, we have used the notation C_{28} to indicate methylated and C_{29} to indicate ethylated sterols. Use of these notations does not indicate whether the C-24 methyl and ethyl groups are in the R or S configurations.

MATERIALS AND METHODS

Organism

A wild type strain of *Chlamydomonas reinhardi*, 137C, obtained from R. Togasaki, Indiana University, was the parent strain of all sterol mutants.

Media and Growth Conditions

Cells were cultured at 30 C on Tris-acetate-phosphate medium (TAP), pH 7.3,

TABLE I

Range of Nystatin Resistance Levels of Wild Type and Mutant Strains of *Chlamydomonas reinhardi*

Strain[a]	Resistance[b]				
	0	5[c]	10	15	20
137C	++	+	-	-	-
KD7	++	+	+	±	-
KD21	++	+	+	+	±
KD16	++	+	+	+	±

[a]Recorded 72 hr after replica plating to TAP plates containing nystatin.

[b]++Heavy confluent growth; +Confluent growth for at least 50% of the determinations; ±Spotted growth or appreciable thinning out of cell streak; -No growth; cell streak bleached white.

[c]Units of nystatin per ml.

prepared according to the procedure of Gorman and Levene (12). Cultures were grown either on solid medium (2% agar) with continuous illumination provided by fluorescent tubes supplying an intensity of 50 μeinsteins m^{-2} sec^{-1} or in liquid medium on a rotary shaker at ca. 200 rpm with continuous illumination provided by an overhead flood light supplying an intensity of 110 μeinsteins m^{-2} sec^{-1}.

Mutant Selection and Nystatin Resistance Determinations

Approximately 10^6 cells were plated on TAP medium containing 5-50 units (U) of nystatin per ml. Nystatin was added separately to autoclaved medium at 50 C, and plates were used the same day. Single colonies appeared spontaneously after 120 hr. Similarly, 10^6 cells were plated on TAP plus nystatin followed by UV irradiation for 30-120 sec using a chromato-vue UV box (Ultra-Violet Products Inc., San Gabriel, CA). Again, single colonies appeared after 120 hr. All nystatin-resistant colonies were restreaked on fresh TAP plates, incubated for 72 hr, and replica plated on TAP plus 0-20 U/ml nystatin plates.

Sterol Extractions and Sterol Derivatization

Algal cultures grown in 250 ml flasks for 72-96 hr were pelleted and nonsaponifiable sterols extracted into n-heptane (10 ml) by the method of Breivik and Owades (13). The n-heptane was subdivided into three fractions for derivatization. Each fraction was evaporated to dryness under a stream of nitrogen and converted into acetyl, trimethylsilyl (TMS), and permethyl derivatives as follows.

Acetyl derivatives were prepared by adding 1 ml of acetic anhydride-pyridine (5:1) to the dried fraction and the mixture allowed to react for 1 hr at 60 C. The excess acetylating reagent was removed under a stream of nitrogen and extracts reconstituted in ethyl acetate. TMS derivatives were prepared by adding 50-100 μl of *bis*-trimethylsilylacetamide in pyridine (Tri Sil BSA, Pierce Chemical Co., Rockford, IL) to the dried extract and the mixture allowed to react for 15-20 min at 60 C. Permethyl derivatives were prepared by adding 150 μl of distilled dimethylsulfoxide to the dried extract, and sterols were permethylated with the methylsulfinylmethide carbanion and methyl iodide after the method of Leclercq and Desiderio for peptides (14). Acetyl, TMS, and permethyl derivatives of 5α-cholestan-3β-ol were prepared in a similar manner and co-injected with sterol mixtures to establish the appropriate relative retention times.

Gas Liquid Chromatography (GLC) and Mass Spectrometry (MS)

The analytical separations were carried out on either a Shimadzu 6AM or a Tracor Model 550 chromatograph. The glass columns (1.87 or 2.0 m) were packed with either 1% OV-17 or 1% SE-30 on 100/120 mesh Gas-Chrom Q; the phases were prepared in this laboratory by solution coating. Operating parameters included: nitrogen flow rate, 40 ml/min; inlet temperature, 250-285 C; detector temperature, 285-300 C; and column oven temperature, 230-250 C. All analyses were isothermal.

The mass spectra were collected oscillographically on an LKB9000-S combination GLC-MS instrument with a 1.87 m 1% OV-17 column as the GLC inlet. The source temperature was 270 C, column oven temperature was 230 C, accelerating and ionizing potentials were 3.5 kV and 70 eV, respectively, and the trap current was 60 μA.

RESULTS

Isolation and Description of Mutants

A wild type strain of *Chlamydomonas reinhardi,* 137C, was plated at an approximate cell concentration of 10^6 cells/ml on TAP medium containing 10, 20, or 50 U/ml systatin. Nystatin resistance was indicated by single colony growth, and 54 separate colonies were isolated from these plates. Additionally, before plating onto nystatin plates, cells were UV irradiated with doses of UV which subsequently proved to be nonlethal. Twenty-eight individual colonies obtained from UV-irradiated cells were isolated as nystatin resistant. Of the combined 82 colonies isolated as resistant to nystatin, only 3 proved to be consistently resistant upon retesting. Resistant strain KD7 was isolated as a

TABLE II

Relative Retention Times (RRT) on 1% OV-17 of Gas Liquid Chromatograhic Peaks in Sterol Fractions From Wild Type and Mutant Strains

Sterol fraction	Peak no.[a]	RRT[b] – acetyl	RRT-TMS	RRT-permethyl
137C	1	1.36	1.39	1.38
	2	1.63	1.60	1.62
	3	1.75	1.76	1.78
	4	---	2.05	2.08
KD7	6	1.45	1.49	1.49
	7	1.58	1.64	1.64
	8	1.85	1.91	1.91
	9	2.03	2.07	2.09
KD21	2	1.56	1.61	1.61
	4	1.97	2.04	2.05
Ergosterol		1.37	1.39	1.39

[a]Peak no. refers to those in Figure 1.

[b]Relative to corresponding 5α-cholestan-3β-ol derivative.

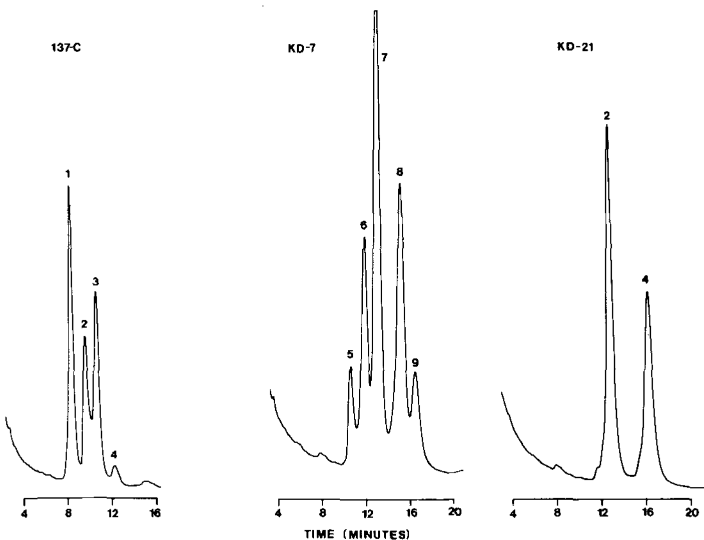

FIG. 1. Gas liquid chromatographic separations of TMS derivatives of sterols extracted from Strains (a) 137C, (b) KD7, and (c) KD21.

single colony from a 10 U/ml nystatin plate, and KD16 and KD21 were isolated as single colonies from 20 U/ml nystatin plates after prior treatment with a nonlethal UV dose.

Table I records the ranges of resistance levels of the KD mutants and wild type strain, 137C, after 72 hr growth. Sensitivity to nystatin becomes obvious after 24 hr growth as nonresistant cells bleach due to an apparent loss of chlorophyll. After 72 hr growth, strain 137C is resistant to 5 U/ml nystatin and sensitive to 10 U/ml. KD21 and KD16 are equally nystatin resistant and both are more resistant than KD7.

Sterol Composition of Wild Type and Mutant Strains

Relative retention times (RRT) on OV-17 of each of the components of the wild type and mutant sterol mixtures compared to 5α-cholestan-3β-ol are presented in Table II. Figure 1 shows representative chromatograms of TMS derivatives of strains 137C, KD7, and KD21. Peak 1 in wild type strain 137C has the same RRT as ergosterol (as acetyl, TMS, and permethyl derivatives). Table III contains partial mass spectral data obtained for the acetyl and TMS derivatives, including molecular ions of the major sterols and other significant ions. Several of the GLC peaks are due to mixtures

TABLE III

Partial Mass Spectra of the Components in Wild Type and Mutant Sterol Fractions, Acetyl, and TMS Derivatives

Strain	Peak no.	Acetyl derivatives		TMS derivatives	
		MW	Other ions: m/e (R.I.)	MW	Other ions: m/e (R.I.)
137C	1	438 (4.5)	253(45),363(28),378(80)	468(25),472(15)	253(28)255(8.4),337(49),363(68), 367(4.4),378(16),382(1.4),453(2.2), 457(3.5)
	2	440(0),442(22)	253(47),255(27),365(15),380(33), 427(7)	470(9.2),472(55)	253(13),255(50),339(29),365(28), 380(10),455(1),457(13)
	3	452(7)	253(42),377(26),392(72)	482(27),486(10)	253(30),255(9.2),351(50),377 (73),392(20),467(2.5)471(2.5)
	4	--	----	486(33)	255(45),353(9),379(14),471(7)
KD7	5	--	---	454(12),456(48)	253(9),255(10),349(14),351(18), 439(4),441(24)
	6	436(8),440(9)	253(42),255(15),313(14),361(25), 376(62),425(5)	466(34),470(11)	253(27),255(10),335(49),361(72), 376(20),455(6)
	7	438(2),440(25)	253(22),255(45),313(87),363(9), 365(11.4),378(40),380(10),425(22)	468(20),470(37)	253(18),255(36),337(37),343(44), 363(54),365(16),378(16),380(10), 455(23)
	8	450(9),454(3)	253(49),313(16),375(28),390(69)	480(48),484(5)	253(38),255(12),343(11),349(60), 375(98),390(24),465(4)
	9	454(19)	253(18),255(34),313(68),379(10), 393(7),439(15)	480(5),482(11), 484(28)	253(24),255(38),343(51),377(25), 379(10),392(6),394(7),469(26)
KD21	2	440(3.6),442(15)	253(19),255(15),365(22),380(57), 427(3.6)	470(34),472(15)	253(15)255(12),339(81),365(100), 380(32),455(4),457(2.5)
	4	454(8),456(11)	253(36),255(13),379(44),394(100), 441(3)	484(35),486(11)	253(17),255(5),353(68),379(100), 394(33),369(4)

of two or more sterols, thereby accounting for the multiple molecular ions. Both C_{28}- and analogous C_{29}- based derivatives are present in all sterol mixtures.

GLC and GLC-MS analyses again indicate that KD21 and KD16 are similar and thus may be regarded as two isolates of the same mutation.

DISCUSSION

The aim of this study was twofold: (a) to obtain sterol mutants in a photosynthetic organism and (b) to elucidate the sterol biosynthetic pathway in a plant species by analyzing the sterol composition in mutants blocked at discrete steps in the pathway. These considerations will be discussed in turn.

The results of this study are similar in nature to studies done with yeast and other fungi in that sterol mutants are not auxotrphs, i.e., they do not require exogenous end product sterol for growth. In addition, preliminary results using a Clark-type oxygen electrode system indicate that KD7 and KD21 both show wild type levels of dark oxygen uptake and light-dependent oxygen evolution (R. Togasaki, unpublished results). Studies in a number of laboratories suggest that sterols play an important role in the development and maintenance of respiratory competence (15,16). For example, Astin and Haslam (17) have demonstrated that sterols help to maintain the impermeability of yeast mitochondrial membranes to protons and thus the ability to couple phosphorylation to oxidation. Haslam and Bard (unpublished results) and Thompson and Parks (18) find that several yeast sterol mutants have a reduced capacity to utilize ethanol, a nonfermentable carbon source. Thus, the effects of altered sterol composition of the photosynthetic mechanism remain as yet unknown, and the problem requires fuller analyses of chloroplast functioning in these and other sterol mutants.

An unequivocal analysis of the sterols found in the wild type and mutant strains was hampered by the absence of sterol standards. However, the GLC and GLC-MS analyses did allow the following observations and interpretations.

The mass spectra of the acetyl and trimethylsilyl derivatives of the algal sterols determined in this study (Table III) contain two common features: (a) molecular ions of varying intensities with those of the TMS derivatives being equal to or greater than those of the acetates and (b) losses of a methyl radical (15 atomic mass units, amu), acetic acid (60 amu), tri-methylsilanol (TMSOH, 90 amu), and the side chain at C-17 (123, 125, or 127 amu in the C_{28} and 137, 139, or 141 amu in the C_{29} series). Loss of acetic acid or TMSOH from the molecular ion is favored in these sterols, which appear to be Δ^5 or $\Delta^{5,7}$, because of the conjugated systems ($\Delta^{3,5}$ or $\Delta^{3,5,7}$) that occur in the resulting ions. The mass spectra of sterols which do not have a C-5,6 double bond contain molecular ions of higher relative intensity than sterols containing this double bond (see also Nes et al. (19) for confirmation of these general correlations). The ions at *m/e* 253 and *m/e* 255, having the structures below, are indicative of the number of double bonds in the steroid nucleus or in the side chain of the original sterol derivative.

m/e 253 *m/e* 255

For example, a sterol acetate with a molecular weight (MW) of 438, such as ergosterol acetate, which gives an ion at *m/e* with a molecular weight (MW) of 438, such as ergosterol acetate, which gives an ion at *m/e* 253, must have two double bonds in the nucleus and one in the side chain. If this same sterol gave an ion at *m/e* 255, then two double bonds must be in the side chain, e.g., $\Delta^{22,24(28)}$. All of the above considerations were important in determining the probable structures of the sterols produced by the wild type and mutant strains.

The primary sterol produced by the wild type strain, 137C, was ergosterol (peak 1, Fig. 1a) as determined by GLC and GLC-MS comparisons to derivatives of standard ergosterol. The C-24 ergosterol isomer (epiergosterol) may also be present in this peak, however. None of the mutants contained these sterols. Two minor sterols from wild type, both present in peak 2, Figures 1a and 1c, and also present in strain KD21 were characterized as a C_{28}-7-ene and a C_{28}-5,7-diene mainly on the basis of the mass spectral data, but also by comparison of RRT to those of standard acetate compounds reported by Bard et al. (20). The monoene, Δ^7, MW acetate = 442, must have one double bond in the steroid nucleus (*m/e* 255); it cannot be Δ^8 because the latter has an RRT of 0.16 units lower than Δ^7 (20). Additionally, this compound does not lose either acetic acid or

TABLE IV

Sterol Composition of Wild Type and Mutant Strains Identified by Gas Liquid Chromatography and Mass Spectrometry

Strain	Sterols	
137C	C_{28}-7-ene	C_{29}-7-ene
	C_{28}-5,7-diene	C_{29}-5,7-diene
	C_{28}-5,7,22-triene (ergosterol)	C_{29}-5,7,22-triene
KD7	C_{28}-7,25-diene	C_{29}-7,25-diene
	C_{28}-5,7,25-triene	C_{29}5,7,25-triene
	C_{28}-5,7,22,25-tetraene	C_{29}-5,7,22;25-tetraene
KD21	C_{28}-7-ene	C_{29}-7-ene
	C_{28}-5,7-diene	C_{29}-5,7-diene

TMSOH from the molecular ion and thus the possibility of the monoene being Δ^5 was eliminated. On the other hand, the diene, MW acetate = 440, does show these losses as well as the ion at *m/e* 253 indicating two double bonds in the nucleus. Therefore, it was assigned the $\Delta^{5,7}$ structure. Wild type also produces C-24 ethyl analogs of these three sterols (peaks 3 and 4, Fig. 1a and 1c) and likewise KD21 produces C-24 ethyl analogs of the monoene Δ^7 and diene $\Delta^{5,7}$ (peak 4, Fig. 1c). From the above evidence, it can be concluded that KD21 is a C-22 desaturase mutant since the sterols in KD21 appear to be similar to those in the wild type strain except KD21 sterols lack the C-22 double bond.

The sterols detected in strain KD7 were not as easily characterized as those in 137C and KD21 although all are probable precursors of ergosterol or a C-24 ethyl analog. Peak 6 (Fig. 1b) represents two compounds, the major one being a tetraene (MW acetate = 436) and the minor one, a diene (MW acetate = 440). The tetraene had an identical RRT (1.45) to that published by Bard et al. (20) for ergosta-5,7,22,24(28)-tetraen-3β-ol. However, a C_{28}-5,7,22-25-tetraene is a more probable sterol structure for the following reasons: (a) it has been demonstrated that 25-methylene and 25-ethylidene are the sterol precursors for C_{28} and C_{29} sterols, respectively (21), in three species of Chlorophyta; (b) a tetraene such as C_{28}-5,7,22,24(28) would be expected to show a UV maximum at 230-235 nm due to a conjugated diene system (13). The nonsaponifiable fraction did have UV maxima at 271,282, and 293 nm indicative of a B-ring conjugated double bond system but no 230-235 nm peak. The mass spectral evidence (Table III) also supports a structure with two double bonds each in the side chain and the steroid nucleus with a $\Delta^{5,7}$ pattern necessary for the significant losses of acetic acid and TMSOH under electron impact. No significant structure could be assigned to the minor diene which has one double bond each in the side chain and in the steroid nucleus – $\Delta^{8,25}$ is a possibility.

Two sterols were also present in peak 7 (Fig. 1b). One was a triene (MW acetate = 438) with two double bonds in the steroid nucleus and one in the side chain, but chromatographically different from ergosterol, and the other was a diene (MW acetate = 440) with one double bond in each moiety. From the available evidence, the structures C_{28}-5,7,25-triene and C_{28}-7,25-diene were assigned, respectively, to these two compounds. As in the wild type and KD21 strains, C-24 ethyl analogs were also present (peaks 8 and 9).

From the sterol structures which could be determined, we conclude that the mutant strain KD7 is a C-25 reductase mutant since all major sterols in this strain have this C-25 double bond. Reduction of this double bond is necessary in the formation of ergosterol.

A minor peak labeled 5 in Figure lb is probably due to two unsaturated derivatives of cholesterol as evidenced by the molecular weights (454, 456; MW of cholesterol-OTMS = 458) and fragmentation of the TMS derivatives (Table III). Since we have observed small amounts of cholesterol in old cultures (120 hr growth) of wild type, the presence of these sterols in cultures of this mutant was not surprising.

The sterols in the wild type and mutant strains consistent with this analysis are summarized in Table IV.

Studies of ergosterol biosynthesis in yeast using sterol mutants and radioactively labeled precursors of ergosterol have led to the conclusion that these is no specific sequential biosynthetic route to ergosterol; ergosterol can be formed by any one of several tracks through a complex network of reactions (20,22-24) indicating that the enzymes involved have a

broad range of substrate specificities. Similarly, there seems to be two possible biosynthetic routes to cholesterol synthesis in animal cells (25). Several biosynthetic routes have been proposed for the synthesis of stigmasterol and β-sitosterol, two end-product higher plant sterols (1). While our results with KD7 and KD21 do not permit us to suggest multiple biosynthetic routes, we suggest that an analysis of a full complement of sterol mutants plus radioactive labeling experiments may reveal whether multiple biosynthetic tracks do exist.

In addition to the two main considerations discussed above, i.e., the role of sterols in the photosynthetic mechanism and the elucidation of the sterol biosynthetic mechanism, the isolation of plant mutants with altered sterol composition may help to elucidate the role played by sterols as possible hormone effectors. Application of these selection techniques may be applied to higher plant protoplasts. Using plant tissue culture techniques, nystatin-resistant protoplasts may be used to regenerate whole plants with altered sterol composition.

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Effects of Ethanol Ingestion and Dietary Fat Levels on Mitochondrial Lipids in Male and Female Rats[1]

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ABSTRACT

The effects of sex, dietary fat levels, and ethanol ingestion on rat liver mitochondrial lipids have been studied. Two groups of male animals were fed either a low-fat diet for about 76 days or a high-fat diet for about 52 days, and two groups of female animals were fed the same low-fat diet for about 50 days or the high-fat diet for about 37 days. Ethanol was substituted isocalorically for carbohydrate and amounted to 36% of total calories. The total as well as individual concentrations of fatty acids, phospholipids, and neutral lipids were determined in all eight groups of animals. Variable changes were observed in the total fatty acid composition of mitochondria from each of the four groups of animals. After ethanol ingestion, there was a decrease in arachidonate/linoleate ratio in males, while no change was observed in females. Increasing the fat content of the diet decreased this ratio in both controls and experimentals, but it did not alter the effects of ethanol on either sex. Presumably, this was due to the fact that corn oil was the only source of lipid. After ethanol ingestion, the total fatty acid concentration increased in all groups of animals except the males fed the low-fat diet. A decrease was observed in this group. The same pattern of change was reflected in changes in total phospholipid concentrations. In each case, the majority of the concentration change in total phospholipid could be accounted for by changes in phosphatidylcholine (PC). Measurement of choline oxidase (C.O.) showed that ethanol ingestion increased C.O. activity only in the low-fat group of males. No change was observed in the other three groups. Chronic ethanol ingestion is known to increase the methylation of phosphatidylethanolamine (PE); therefore, in order to decrease PC, the increase in C.O. in the low-fat males must have been of sufficient magnitude to offset the increase in PE methylation. Increasing the fat content of the diet offset the effect of ethanol on C.O. in males. Neither ethanol nor fat exerted much effect on C.O. in females. These results emphasize the importance of dietary levels of fat as well as sex in the study of liver mitochondria structure and function in relation to ethanol metabolism.

INTRODUCTION

Depending upon the sex of the animal, chronic ethanol ingestion affects the oxidation of certain mitochondrial substrates differently (1,2). These differences have been related to the male sex hormone, testosterone (2). In studies not related to ethanol ingestion, estradiol and testosterone have been shown to affect the biosynthesis of phosphatidylcholine (PC) (3,4). Other researchers (5-7) have reported sex differences in total liver lipid composition as well as in liver lipid metabolism (4,8-11), and a fractionation of liver PC has shown sex differences in certain molecular species of PC (12). Chronic ethanol ingestion results in an increase in total liver PC (13). However, an increase also occurs in oxidation of choline by liver mitochondria (14). Because of the interrelationships between ethanol ingestion, lipids, membranes, mitochondrial function, and sex, it becomes important to understand more about how chronic ethanol ingestion affects mitochondrial lipid composition as well as mitochondrial function. Therefore, this investigation is an attempt to demonstrate the effects of chronic ethanol ingestion on the lipid composition of mitochondria from both male and female animals. Furthermore, it correlates the effect of ethanol ingestion on choline oxidase with mitochondrial PC concentrations.

MATERIALS AND METHODS

Procedure for Preparing the Diet

Constituents used in preparing the liquid diet are listed in Table I and II. In one beaker, the protein, lactalbumin hydrolysate, was dissolved in about 300 ml deionized water. In a second beaker, dextrose was added (with stirring) to 150 ml boiling deionized water. After this second solution was cooled, the remaining ingredients were added as follows: (a) citric acid; (b) vitamin fortification mixture; (c) salt solutions (Table II); (d) Tween-80; (e) corn oil. The final volume of this second mixture should be about 400 ml. The dextrose-salt-lipid mixture was now placed in a Waring Blender and blended at 27,000 rpm for 2-5 min. It was then combined with the protein and stirred rapidly with a magnetic stirrer until mixing was com-

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TABLE I

Composition of Low- and High-Fat Liquid Diets

	Low-fat			High-fat		
	g/l	Cal/l	% Cal	g/l	Cal/l	% Cal
Dextrose	276.0	1104.0	69.9	182.1	728.4	46.1
Protein[a]	92.0	368.0	23.3	69.1	276.4	17.5
Lipid[b]	8.0 (8.7 ml)	72.0	4.6	60.0 (65.5 ml)	540.0	34.2
Vitamin mixture[c]	4.0	13.6	.9	4.0	13.6	.9
Citric acid	2.0	---	---	2.0	---	---
Salt solutions[d]	17.6	---	---	17.6	---	---
Tween-80	5.4 (5.0 ml)	52.5	1.4	5.4 (5.0 ml)	22.5	1.4
	405.0	1580.1		340.2	1580.9	

[a]Lactalbumin hydrolysate (extra soluble) peptone no. 60 dri-form (No. 1401800, Gibco, Santa Clara, CA).

[b]Mazola brand pure corn oil (Best Foods, Englewood Cliffs, NJ).

[c]Vitamin fortification mix (No. 104654, ICN Nutritional Biochemical, Corp., Irvine, CA).

[d]See composition of stock salt solutions in Table II.

TABLE II

Salt Solutions[a]

Salt	Stock solutions (g/l)	Volume stock solution used (ml/l)	Final salt concentration (mg/l)
Potassium phosphate monobasic (KH_2PO_4)	173.25	40	6,930.0
Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$)	433.9	20	8,678.0
Sodium chloride (NaCl)	195.35	8	1,562.8
Magnesium sulfate ($MgSO_4 \cdot H_2O$)	49.9	4	199.6
Manganese sulfate monohydrate ($MnSO_4 \cdot H_2O$)	6.05	4	24.2
Potassium iodide (KI)	0.25	4	1.0
Ammonium molybdate ($(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.125	4	0.5
Cupric sulfate ($CuSO_4 \cdot 5H_2O$)	12.2	4	48.8
Ferric ammonium citrate (green)	31.15	4	124.6
Zinc chloride ($ZnCl_2$)	2.55	4	10.2

[a]Stock salt solutions are added to the dextrose solution (see Methods) in the order in which they appear in the table.

pleted. After this initial mixing, the sitrring was stopped for 5-10 min to allow trapped air to rise to the top. After adjusting the final volume with deionized water to 1 liter, a very slow but steady mixing speed was used to complete the homogenization of the diet. The resulting light brown liquid diet was stored at 4 C until used. During a 24 hr period, there was no settling of the diet.

Animals and Diet

Male Long-Evans rats were obtained from Simonsen Labs, Gilroy, CA. The animals weighed 140-160 g upon arrival and were maintained on ad libitum Purina Laboratory Chow and water for about 2-3 days. The animals were then paired by weight before pair-feeding the different liquid diets. Richter tubes were used for measuring the daily consumption of the liquid diets, and a 7:30 a.m. light, 7:30 p.m. dark lighting schedule was used. Because these animals were used for other experiments not discussed in this report, different feeding times were used. The length of time each group of animals was maintained is noted in Table III.

Mitochondria Isolation and Lipid Extraction

Rats were sacrificed by decapitation, and their livers were immediately removed and placed in ice-cold 0.25 *M* sucrose containing 3.4 m*M* Tris-HCl (pH 7.4). Mitochondria were isolated according to the procedure of Chappell and Hansford (15). Each liver was homogenized in 0.25 *M* sucrose containing 1 m*M* EGTA, 3.4 m*M* Tris-HCl (pH 7.4), and 1% defatted bovine serum albumin. The procedure of Goodman was used to defat the albumin (16). The protein concentration was determined by the procedure

TABLE III

Effects of Ethanol on Liver Weights in Male and Female Rats Fed High- and Low-Fat Diets

	Initial weight (g)	Control			Ethanol		
		Final weight (g)	Weight Gain (g/day)	Liver weight (g/kg)	Final weight (g)	Weight gain (g/day)	Liver weight (g/kg)
Low-fat male (6)[a]	143	370	2.99	33.1 ± 0.5	375	3.05	33.1 ± 0.1
High-fat male (10)	151	342	3.67	32.7 ± 2.2	355	3.92	55.2 ± 2.7[b,c]
Low-fat female (5)	157	232	1.50	31.9 ± 0.7[c]	229	1.44	31.4 ± 0.9
High-fat female (10)	148	219	1.91	38.3 ± 1.0	225	2.08	53.2 ± 1.9[b,c]

[a]The numbers in parentheses represent the number of animals in each group. Each number represents the mean for each group. Because liver weights were the main emphasis of this table, SEM values are presented for liver weights only. Days on diet: Low-fat male = 74-79; high-fat male = 46-60; low-fat female = 45-53; High-fat female = 35-40.

[b]Compared to the control, this value was statistically significant at the $P < 0.001$ level. A paired t-test was utilized.

[c]Compared to the low-fat treated animals, these values were statistically significant at the $0.01 > P > 0.001$ level. The Student's t-test was used. All other comparisons were not statistically significant.

of Lowry et al. (17), and the lipids were extracted by the procedure of Bligh and Dyer (18).

Lipid Fractionation and Quantification

The phospholipids were separated by thin layer chromatography (TLC) using a solvent consisting of chloroform-methanol-acetic acid-water (100:60:16:8). The various phospholipids were located with 2,6-dichlorofluorescein, eluted from the TLC scrapings according to Arvidson (19), and quantitated by the total phosphate procedure of Bartlett (20). The neutral lipids were separated by TLC using a solvent system consisting of petroleum ether-ether-acetic acid (90:10:1). An internal standard fatty acid, hepadecanoic acid, was added to the TLC scrapings prior to elution by the procedure of Arvidson (19). Cholesterol esters, triglycerides, and free fatty acids were quantitated by gas chromatography of their component fatty acids as described previously (21). Total mitochondrial fatty acids were assayed similarly. Total cholesterol was determined by the Searcy and Bergquist procedure (22).

Choline Oxidase

Measurements of the choline oxidase system were performed polarographically. The total reaction mixture contained 0.23 *M* mannitol, 0.07 *M* sucrose, 0.02 *M* Tris-HCl (pH 7.4), 0.005 *M* potassium phosphate, 0.05 *M* choline, and 1 m*M* $CaCl_2$. The reaction was initiated by adding 0.05 ml of a mitochondrial suspension which contained 1.5-2.0 mg protein. This was a modification of the procedure outline by Kagawa et al. (23).

Materials

All reagents were reagent grade quality.

RESULTS

A significant visual difference was observed between the livers of male and female animals maintained on the different diets. In the male animals, ethanol affected liver appearance and weight only in the high-fat diet group. The weight of the livers in this group was increased about 69% (Table III), and the livers were distinguished by a light brown color rather than the normal reddish brown color. When the amount of fat was increased in the diet, there was no observed effect on liver weights in the control male rats (low-fat diet liver weight, 33.1 g/kg compared to high-fat liver weight, 32.7 g/kg). When the ethanol-treated animals were compared between the two diets, there was a 67% increase in liver weights in the animals fed the high-fat diet compared to the low-fat diet. On the other hand, the female rats fed the high-fat diet had livers that were increased in weight 20% in controls and 69% in experimentals (Table III) when compared to those maintained on the low-fat diet. The livers from the ethanol-treated high-fat females also had the light brown color similar to those of the high-fat males fed the ethanol diet. Ethanol affected liver weights only in the high-fat group of female animals. An increase of 39% was observed.

Tables IV-IX are the results of the lipid analyses of the liver mitochondria obtained from the animals in each of the four groups.

TABLE IV

A Comparison of the Effects of Chronic Ethanol Ingestion on Total Mitochondrial Fatty Acids from Male and Female Rats Fed a Low-Fat Diet

	Male		Female	
	Control	Ethanol	Control	Ethanol
		(nmole %)		
16:0	18.1 ± 0.4	18.9 ± 0.4	19.9 ± 0.4	14.9 ± 0.1[a,c]
16:1	4.4 ± 0.2	3.0 ± .01	3.8 ± 0.2	3.4 ± 0.2
18:0	20.8 ± 0.4	19.3 ± 0.2	26.2 ± 0.4[c]	29.2 ± 0.5[b,c]
18:1	14.7 ± 0.1	14.0 ± 0.3	14.6 ± 0.4	16.1 ± 0.7
18:2ω6	10.5 ± 0.2	16.0 ± 0.1[a]	9.8 ± 0.3	10.5 ± 0.4[c]
20:3ω9	2.0 ± 0.05	2.2 ± 0.1	0.3 ± 0.02	0.2 ± 0.02
20:3ω6	2.4 ± 0.08	1.5 ± 0.05	1.0 ± 0.05	1.2 ± 0.1
20:4ω6	23.9 ± 0.6	21.9 ± 0.3	20.8 ± 0.4[c]	19.5 ± 0.2
22:4ω6	0.5 ± 0.02	0.4 ± 0.05	0.4 ± 0.07	0.5 ± 0.05
22:5ω6	1.5 ± 0.04	1.5 ± 0.06	1.9 ± 0.07	2.5 ± 0.1
22:5ω3	0.2 ± 0.03	0.2 ± 0.01	-	-
22:6ω3	1.3 ± 0.03	1.4 ± 0.1	1.1 ± 0.06	1.9 ± 0.07
20:4/18:2	2.30 ± 0.09	1.37 ± 0.03[a]	2.14 ± 0.11	1.87 ± 0.08[c]
Total fatty acid (nmole/mg protein)	606.8 ± 5.1	544.3 ± 1.0[a]	463.7 ± 9.8	622.2 ± 13.3[a]

[a]Ethanol different from control at $P<0.001$. (Each value represents mean ± SEM of four or five animals.)

[b]Ethanol different from control at $P<0.05$.

[c]Male different from female at $P<0.01$ or better.

TABLE V

A Comparison of the Effects of Chronic Ethanol Ingestion on Total Mitochondrial Fatty Acids from Male and Female Rats Fed a High-Fat Diet

	Male		Female	
	Control	Ethanol	Control	Ethanol
		(nmole %)		
16:0	18.1 ± 0.04	12.8 ± 0.2[a]	14.8 ± 0.2[c]	10.0 ± 0.2[a,c]
16:1	0.5 ± 0.04	0.6 ± 0.03	0.5 ± 0.02	0.3 ± 0.02
18:0	22.2 ± 0.2	28.0 ± 0.2[a]	25.1 ± 0.3[c]	31.1 ± 0.3[a,c]
18:1	5.8 ± 0.1	8.6 ± 0.3[a]	7.6 ± 0.2[c]	7.5 ± 0.2
18:2	20.2 ± 0.1	25.0 ± 0.3[a]	21.1 ± 0.3	21.0 ± 0.2[c]
20:3ω9	2.1 ± 0.05	2.2 ± 0.1	0.7 ± 0.02	1.1 ± 0.04
20:3ω6	1.1 ± 0.03	2.0 ± 0.03	0.5 ± 0.03	0.7 ± 0.04
20:4ω6	21.5 ± 0.3	18.1 ± 0.4[a]	22.6 ± 0.5	21.7 ± 0.3[c]
22:4ω6	1.4 ± 0.05	0.3 ± 0.02	0.8 ± 0.04	0.9 ± 0.03
22:5ω 6	2.4 ± 0.1	0.5 ± 0.02	2.2 ± 0.2	1.9 ± 0.08
22:5ω3	1.4 ± 0.02	0.2 ± 0.03		
22:6ω3	3.6 ± 0.07	1.6 ± 0.06[a]	3.9 ± 0.04	3.7 ± 0.1[c]
20:4/18:2	1.07 ± 0.02	0.72 ± 0.02[a]	1.07 ± 0.03	1.03 ± 0.02[c]
Total fatty acid (nmole/mg protein)	596.1 ± 4.6	619.8 ± 3.0[b]	592.3 ± 5.7	642.6 ± 7.3[a]

[a]Ethanol different from control at $P < 0.001$. (Each value represents the mean ± SEM from four or five animals.)

[b]Ethanol different from control at $P < 0.02$.

[c]Male different from female at $P < 0.001$.

Tables IV and V show the effect of chronic ethanol ingestion on the total mitochondrial fatty acid concentrations of both male and female rats fed either the high-fat or low-fat liquid diet. On the low-fat diet (Table IV), chronic ethanol ingestion resulted in a significant decrease of 10.3% in the fatty acid concentration of the mitochondria from the males. In contrast, an increase of 34.2% was observed in the females. When the high-fat diet was fed

TABLE VI

A Comparison of Effects of Ethanol on Mitochondrial Lipids of Male and Female Rats Fed Low-Fat Diet[a]

		Male		Female	
		Control	Ethanol	Control	Ethanol
Triacylglycerol (nmole %)	16:0	41.6	30.1	14.7	15.4
	16:1	—	10.2	—	—
	18:0	41.1	36.3	50.0	52.8
	18:1	16.3	18.5	31.9	22.9
	18:2	1.4	5.3	3.4	9.3
Total (nmoles/mg protein)		21.4 ± 0.2	22.6 ± 0.5	23.2 ±0.6	21.4 ± 0.6
Free fatty acids (nmole %)	16:0	41.1	31.7	12.2	11.3
	16:1	15.8	—	—	2.6
	18:0	20.5	23.3	29.9	17.5
	18:1	11.2	22.5	23.0	14.8
	18:2	11.7	25.0	5.7	11.5
	20:3ω6	—	—	—	1.4
	20:4ω6	—	—	25.0	33.1
	22:5ω6	—	—	4.3	4.6
	22:6ω3	—	—	—	3.3
Total (nmoles/mg protein)		41.9 ± 0.4	24.6 ± 0.4[b]	104.5 ± 3.1	211.5 ± 3.5[b]
Cholesterol esters (nmole %)	16:0	42.8	53.2	40.4	65.1
	16:1	—	—	—	—
	18:0	33.3	22.7	35.5	19.2
	18:1	15.6	17.0	18.6	11.6
	18:2	6.7	7.1	6.0	4.1
Total (nmoles/mg protein)		18.0 ± 0.2	14.1 ± 0.3[b]	18.3 ± 0.2	14.6 ± 0.5[b]
Total cholesterol (nmoles/mg protein)		—	—	36.4 ± 1.4	36.8 ± 1.2

[a]Four animals were used to determine each number, but the SEM was omitted from the nmole % values to simplify the table. The total values are the mean ± SEM. The various lipids were separated as described in the Methods section, and the methyl esters were prepared, separated, and quantitated as described previously (21).

[b]Control different from ethanol at $P < 0.01$ or greater.

(Table V), significant increases of 4.0% and 8.4% were observed after ethanol ingestion in males and females, respectively. Thus chronic ethanol ingestion increased the total mitochondrial fatty acid concentrations of all animals except the males fed the low-fat diet. The relative concentrations were increased more in the females than in the males.

Table IV also compares the effects of ethanol on the total fatty acid composition of the male liver mitochondria to those of the female when the low-fat diet was used. In the males, only linoleic acid (18:2ω6) was altered significantly by ethanol ingestion. It was increased 52.4%. In the females, palmitate (16:0) was decreased 25%, and stearate (18:0) was increased 11.5% by ethanol. Another difference between males and females was that ethanol decreased the arachidonate/linoleate (20:4ω6/18:2) ratio significantly only in the males (40.5%). When the data from the male control were compared to those of the female control, only two fatty acids were different: 18:0 increased 26% and 20:4ω6 decreased 13%. When the ethanol-treated were compared, three acids were different in composition: 16:0 decreased 21%, 18:0 increased 51%, and 18:2ω6 decreased 34%.

Table V compares the effects of ethanol on the total fatty acid composition of the male liver mitochondria to that of the female when the high-fat diet was used. In the male, three fatty acids were decreased (16:0, 29.3%, 20:4ω6, 15.8%, 22:6ω3, 55.6%) and three fatty acids were increased (18:0, 26.1%; 18:1, 48.3%; 18:2, 23.7%). These data were quite different from those obtained from males fed the low-fat diet, where only 18:2ω6 was found to

TABLE VII

A Comparison of Effects of Ethanol on Mitochondrial Lipids of Male and Female Rats Fed a High-Fat Diet[a]

		Male		Female	
		Control	Ethanol	Control	Ethanol
Triacylglycerol (nmole %)	16:0	41.1	46.8	18.6	23.1
	16:1	—	—	—	—
	18:0	41.6	21.1	36.1	27.2
	18:1	14.9	10.1	31.1	31.8
	18:2	2.3	22.0	16.4	18.5
Total (nmoles/mg protein)		21.4 ± 0.4	21.8 ± 0.9	18.3 ± 0.2	17.3 ± 0.5
Free fatty acids (nmole %)	16:0	50.4	43.0	17.1	10.4
	16:1	10.8	—	.7	.9
	18:0	15.2	21.5	19.6	14.3
	18:1	5.9	14.8	14.5	13.0
	18:2	17.7	21.1	18.7	28.8
	20:3ω6	—	—	—	.9
	20:4ω6	—	—	25.3	27.6
	22:5ω6	—	—	2.9	2.0
	22:6ω3	—	—	1.8	2.1
Total (nmoles/mg protein)		49.2 ± 1.2	23.7 ± 1.3[b]	174.2 ± 2.7	270.7 ± 2.8[b]
Cholesterol esters (nmole %)	16:0	27.3	31.7	41.0	56.8
	16:1	—	5.0	—	11.4
	18:0	29.1	11.0	35.5	17.0
	18:1	33.5	21.7	18.6	10.2
	18:2	10.5	30.5	5.6	4.5
Total (nmoles/mg protein)		100.0 ± 0.7	105.3 ± 1.9	18.3 ± 0.2	26.4 ± 0.9[b]
Total cholesterol (nmoles/mg protein)		—	—	30.5 ± 0.7	35.3 ± 1.7

[a]Four animals were used to determine each number, but the SEM was omitted from the nmole % values to simplify the table. The total values are the mean ± SEM. The various lipids were separated as described in the Methods section and the methyl esters prepared, separated, and quantitated as described previously (21).

[b]Control different from ethanol at $P < 0.01$ or better.

be altered. In the females, 16:0 was increased 32.4% and 18:0 was decreased 23.9%. This was almost identical to changes observed when the low-fat diet was used (Table IV). Therefore, increasing the fat content of the diet markedly altered the effects of ethanol in the male. The increase had little effect on the female. Further, the 20:4ω6/18:2 ratio was decreased significantly in males only. When male and female controls were compared, 16:0 was decreased 18.2%, while both 18:0 and 18:1 were increased 13.1% and 31.0%, respectively. When the ethanol-treated aniamls were compared, five acids were different: 16:0 and 18:2 were decreased 21.9% and 16.0%, respectively, while 18:0, 20:4ω6, and 22:6ω3 were increased 11.0%, 19.9%, and 131.3%, respectively. Thus, as might be predicted, the high-fat diet seems to augment the effects of ethanol on mitochondrial fatty acid composition.

Table VI compares the manner in which ethanol affects fatty acid composition as well as concentration of triacylglycerol (TG), free fatty acids (FFA), and cholesterol esters (CE) in male and female liver mitochondria. A low-fat diet was fed to the animals used in the analyses presented in this table. Ethanol seemed to affect the fatty acid composition of each lipid differently; thus, it is very difficult to make any generalizations concerning the effects of ethanol on the fatty acids of these three lipid fractions. Only in the female FFA fractions were there fatty acids of the C_{20} and C_{22} groups. Ethanol resulted in increases in the major polyenes, but there was still a marked decrease in the 20:4ω6/18:2 ratio. The FFA fraction was decreased 41.3% in males and increased 102.4% in females. Further, this frac-

TABLE VIII

Effects of Chronic Ethanol Ingestion on the Phospholipids of Liver Mitochondria from Animals Fed a Low-Fat Diet

Lipid	Males		Female	
	Control	Ethanol	Control	Ethanol
	(nmoles P/mg protein)			
CL	35.8 ± 1.4	27.5 ± 0.3[a]	30.7 ± 0.6	29.6 ± 1.0
PE	76.8 ± 1.7	80.4 ± 1.3	45.5 ± 1.4	52.9 ± 0.6[a]
PS-PI	20.2 ± 1.1	19.9 ± 1.1	18.7 ± 0.4	20.1 ± 1.1
PC	133.5 ± 1.3	113.4 ± 1.1[a]	76.1 ± 1.5	91.8 ± 1.2[a]
Sph	3.8 ± 0.2	4.3 ± 0.3	8.3 ± 0.1	8.7 ± 0.1
LPC	2.9 ± 0.1	2.3 ± 0.2	18.6 ± 0.5	25.3 ± 1.1[a]
% Recovery	94.2	91.3	82.7	83.8
Total	289.4 ± 2.1	271.4 ± 2.9[a]	245.4 ± 2.1	276.8 ± 3.1[a]

[a]Control different from ethanol at $P < .02$ or greater. (Each value represents the mean ± SEM from four animals.) Abbreviations: CL, cardiolipin; PE, phosphatidylethanolamine; PS-PI, phosphatidylserine-phosphatidylinositol; PC, phosphatidylcholine; Sph, sphingomyelin; LPC, lysophosphatidylcholine.

TABLE IX

Effects of Chronic Ethanol Ingestion on the Phospholipids of Liver Mitochondria from Animals Fed a High-Fat Diet

Lipid	Males		Females	
	Control	Ethanol	Control	Ethanol
	(nmoles P/mg protein)			
CL	21.1 ± 0.5	16.8 ± 0.2[a]	37.8 ± 2.6	41.4 ± 0.6
PE	67.4 ± 0.3	73.0 ± 0.7[a]	63.2 ± 2.1	71.6 ± 1.2[a]
PS-PI	3.0 ± 0.1	6.0 ± 0.2[a]	20.8 ± 0.3	19.6 ± 0.7
PC	93.2 ± 0.6	117.6 ± 1.8[a]	104.2 ± 2.9	128.0 ± 1.7[a]
Sph	2.9 ± 0.2	3.0 ± 0.2	6.0 ± 0.6	6.0 ± 0.3
LPC	0.6 ± 0.2	0.8 ± 0.1	34.0 ± 1.9	23.1 ± 0.3[a]
% Recovery	89.2	87.7	81.9	81.1
Total	210.7 ± 1.6	249.5 ± 0.9[a]	329.0 ± 4.5	366.4 ± 2.3[a]

[a]Control different from ethanol at $P < 0.02$ or greater. (Each value represents the mean ± SEM from four animals.) Abbreviations: CL, cardiolipin; PE, phosphatidylethanolamine; PS-PI, phosphatidylserine-phosphtidylinositol; PC, phosphatidylcholine; Sph, sphingomyelin; LPC, lysophospatidylcholine.

tion was quite large in both female groups compared to males, and, in fact, accounted for well over 50% of the total neutral lipids.

The cholesterol ester fraction was decreased similarly in both males and females by ethanol. No change was observed in total cholesterol in females.

Table VII compares the neutral lipids of mitochondria from male and female rats fed a high-fat diet. Again, ethanol affected the fatty acid pattern in each lipid fraction differently, and no generalization can be made. The FFA fraction of the female group was the only fraction containing C_{20} and C_{22} polyenes, and the 20:4ω6/18:2 ratio still was decreased about 30% by ethanol. Similar to the low-fat diet, ethanol decreased the total FFA concentration in males and increased it in females. In the controls, there was a 3.6-fold increase in FFA in females compared to males, and in the ethanol-treated, an 11.3-fold increase was observed.

Whereas ethanol ingestion affected the CE concentration similarly in males and females fed the low-fat diet, marked differences were observed between these two groups after feeding a high-fat diet. Ethanol did not affect CE concentrations in the males, but these values were 4 to 5-fold higher than in females. Contrary to the low-fat diet, a significant increase was observed in females fed the high-fat diet. Little change was observed in the total cholesterol concentration in females, but because of the tremendous increases in CE concentrations

TABLE X

Effects of Ethanol and Dietary Fat on Choline Oxidase in Male and Female Rats

	Control	Ethanol	Effect of ethanol % change
	(nmoles/min/mg protein)		
Low-fat male	16.8 ± 0.43	24.80 ± 0.77[a]	+47.6
High-fat male	24.39 ± 0.17	24.87 ± 0.28	+ 2.0
Effect of fat (% change)	+56.4	0	
Low-fat female	13.50 ± 0.37	13.52 ± 0.30	+ 0.1
High-fat female	9.61 ± 0.20	9.39 ± 0.27	- 2.3
Effect of fat (% change)	-28.8	-30.5	

[a]This value is statistically different from the control at the $P < 0.001$ level of significance. No difference existed between the other control and ethanol-treated. (Each value represents the mean ± SEM for five animals.)

in males, no generalization can be made concerning mitochondrial cholesterol concentration in males fed the high-fat diet.

Ethanol did not affect the TG fraction in either sex, and both sexes had about the same TG concentrations.

When the phospholipid fraction, the major lipid fraction of mitochondria, was studied, cardiolipin (CL) and phosphatidylcholine (PC) were the only lipids significantly decreased in males fed the low-fat diet (Table VIII). In females, however, lysophosphatidylcholine (LPC), PC, and phosphatidylethanolamine (PE) were increased by chronic ethanol ingestion. As a result of these changes, the total phospholipid concentration was decreased in males and increased in females. When comparing males to females, the major differences were that PC and PE were decreased while LPC and sphingomyelin (Sph) were increased in the females no matter whether the controls or the ethanol-treated were compared. The rather large increase in LPC concentration in females (2.6-fold in controls and 11.0-fold in experimental) could contribute to the significant increase in FFA shown in Table VI. It may be noteworthy to point out that the mitochondria from the female animals had been frozen and thawed several times prior to the lipid extractions.

Major differences were observed in the phospholipids of males fed the high-fat diet (Table IX). Only CL was decreased. PE, phosphatidylserine-phosphatidylinositol (PS-PI), and PC were increased by ethanol ingestion, and as a result, the total phospholipids were increased. In females, only PE and PC were increased. LPC was decreased, but the total phospholipid fraction was increased. When comparing males to females, all phospholipids except PE were increased in females, and as a result, there was a marked increase in total phospholipids in females no matter whether the controls or the ethanol-treated animals were compared.

When the fatty acid concentrations of each individual lipid fraction were totaled and compared to the total concentrations given in Tables IV and V, recoveries were low in the mitochondria from the male animals fed the high-fat diet (88% in control and 90% in ethanol-treated) and high in the mitochondria from the female animals fed the same diet (119% in control and 133% in ethanol-treated). However, about 100% recovery was obtained in the other four groups of animals; thus, all data reported were internally consistent.

In all four groups of animals, only the low-fat males had less phospholipid after ethanol-treatment than did the controls. In all other groups of animals, ethanol ingestion increased the phospholipid concentration. Because PC was the major mitochondrial phospholipid, changes in total phospholipid concentration were governed largely by changes in PC concentration. This suggested that a correlation may exist between choline oxidase activity and mitochondrial PC concentration. Table X shows the effect of dietary ethanol on the activity of choline oxidase in male and female animals maintained on either the low-fat or high-fat liquid diet. There was a 47.6% increase in the rate of choline oxidation in the low-fat males as a result of chronic ethanol ingestion. Choline oxidase was not effected in any of the other groups of animals as a result of chronic ethanol ingestion. Feeding a high-fat diet resulted in a 47.5% increase in choline oxidation rate in the male control animals; whereas, in the ethanol-treated males, there was no effect on choline oxidation. In both the control and ethanol-treated females, there was about a 30% decrease in the rate of choline oxidation as a result of the high-fat diet.

TABLE XI

A Comparison of the Ethanol Treatment to the Control and the Low-Fat Diet to the High-Fat Diet in Terms of Choline Oxidase Activities and Phosphatidylcholine Concentrations

	Choline oxidase activity	Phosphatidylcholine concentration
Ratio of ethanol/control		
Male low-fat	1.48	0.85
Male high-fat	1.02	1.26
Female low-fat	1.00	1.21
Female high-fat	0.98	1.23
Ratio of low-fat/high-fat		
Male control	0.69	1.43
Male ethanol	1.00	0.96
Female control	1.42	0.73
Female ethanol	1.44	0.72

Because the low-fat males were the only group to show both a decrease in mitochondrial PC and an increase in choline oxidase activity, a comparison was made between the rates of choline oxidation and the mitochondrial PC content. A ratio of the choline oxidation rates between the ethanol-treated animals and the control animals (Table XI) showed that only the male animals fed the low-fat diet had values which differed from unity. Further, the ratio of the PC content in the ethanol-treated animals compared to the control animals showed the low-fat males to be unique when determining the effects of chronic ethanol ingestion on rat liver mitochondria. This ratio was below unity for the low-fat males, and above unity for all other groups.

Table XI also shows another correlation between choline oxidase and PC concentration. When the ratios of choline oxidase activity in the low-fat/high-fat were compared to the same ratios for PC concentration, an inverse relationship was observed. As the ratios for choline oxidase increased, the ratios for PC concentration decreased. Thus, these correlations suggest that choline oxidase plays an important role in controlling mitochondrial PC concentration.

DISCUSSION

The change in total mitochondrial fatty acid concentrations as a result of chronic ethanol feeding was very interesting. Earlier work (14,24) has shown alterations in mitochondrial fatty acids. These earlier data were obtained from low-fat males and were essentially the same as that presented in Table IV. A similar alteration in the profile of mitochondrial fatty acids was observed in males fed a high-fat diet. The major changes were an overall decrease in the polyenes accompanied by an increase in linoleic acid. Because 18:2ω6 was the major fatty acid component of the diet, an increase in 18:2ω6 was not surprising. The 20:4ω6/18:2ω6 ratio was markedly decreased in both groups of males. However, in the females, the major polyenes were either not changed (high-fat) or increased (low-fat), the 20:4ω6/18:2ω6 ratio was only slightly affected.

In males, the decreased 20:4ω6/18:2ω6 ratio has been equated with an effect on the elongation desaturation pathway as a result of ethanol metabolism (25). In this respect, it is of interest that chain elongation is dependent upon cellular levels of ATP for the activation of fatty acids prior to elongation (26). Chronic ethanol ingestion has been shown to depress ATP levels in male rat liver (27-29). Furthermore, no effect has been observed in the female rat liver. The ATP levels remained similar to control levels (27). The different effects of ethanol metabolism on hepatic ATP levels suggest that no changes were observed in the female mitochondrial 20:4ω6/18:2ω6 ratio because sufficient ATP was present for fatty acid activation, thus allowing chain elongation and desaturation to occur normally.

The lack of changes in ATP levels also could account for the effects of chronic ethanol feeding on the rates of choline oxidation. In the low-fat male, cellular ATP levels (27) and mitochondrial ATP levels (30) have shown to be decreased. In addition, we also have observed no change in ATP levels in mitochondria isolated from female rats chronically fed the low-fat ethanol containing diet (Thompson and Reitz, unpublished observations). Choline oxidase is under direct control by ATP (23,32). In fact, an inverse linear relationship between choline oxidase and mitochondrial ATP concen-

tration has been noted in both control and ethanol-treated animals (30). Hence, this may explain why choline oxidation is preferentially increased in the male (low-fat). High-fat diets in conjunction with ethanol also have been found to result in decreased levels of hepatic ATP (28). However, no differences were observed in choline oxidation (Table X). This possibly could be due to the effects of the high-fat diet on the control animals. Even more interesting was the fact that the high-fat diet actually decreased choline oxidase activity in the female animals. Because of a lack of effect on ATP levels (27 and Thompson and Reitz, unpublished observations), ethanol metabolism has no effect on the activity of choline oxidase in either group of female animals; however, there seemed to be an inhibitory effect of dietary fat on this enzyme system in females.

Because the changes in mitochondrial phospholipid concentrations were largely due to changes in the concentrations of only one phospholipid fraction, the phosphatidylcholines, the metabolism of the phosphatidylcholines must be affected by chronic ethanol ingestion. Fallon et al. (13) have observed that chronic ethanol ingestion resulted in an increase in the methylation of phosphatidylethanolamines (PE) in male rats fed a low-fat diet. No change was observed in the CDP-choline pathway. The increased oxidation of choline by mitochondria isolated from male rats fed the low-fat diet (Table X) could contribute to an increase in the methylation of PE in two ways. First, it would directly produce a decrease in the availability of choline for PC synthesis. Second, an increase in the total oxidation of choline to yield glycine would contribute three one-carbon units into the one-carbon pool: one unit directly via methionine formation, and two units indirectly via formation of 5,10-methylene tetrahydrofolate. Thus, by decreasing choline availability while at the same time increasing the cofactor required for methylation, the increased oxidation of choline should result in an increase in PE methylation. The fact that ethanol ingestion did not alter choline oxidase activity in either female group or in the high-fat male group and that an increase in PC concentration was observed in these three groups would suggest that the methylation pathway was increased in all three groups.

Our data from either the control groups of animals or from the ethanol-treated groups suggest that choline oxidase plays an important role in controlling mitochondrial PC concentrations. Table XI emphasized this by showing that as the ratio of choline oxidase activity between the low-fat animals and the high-fat animals increased, the PC ratio decreased proportionately. However, when the ethanol-treated groups were compared to their control groups, the story is not so clear cut. This probably arises from the effects of ethanol on other pathways of lipid metabolism, specifically the methylation of PE to form PC (13). This also was emphasized by the data in Table X which showed that ethanol ingestion had no effect on choline oxidase in either group of females or in the high-fat males. As a result, PC actually increased in concentration. It was most interesting to compare the low-fat males to these three groups. In the low-fat males, ethanol must have stimulated choline oxidase more than methylation because the concentration of PC decreased. Thus, the data presented in this report strongly suggest that in studying the effects of ethanol on liver lipid metabolism both sexes must be used and that the use of a low-fat diet may be the most effective way to assess the effects of ethanol per se.

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Δ15,18-Tetracosadienoic Acid Content of Sphingolipids from Platel and Erythrocytes of Animals Fed Diets High in Saturated or Polyunsaturated Fats

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ABSTRACT

The effect of diets high (15%) in saturated (beef tallow) or polyunsaturated (corn or cottonseed oil) fatty acids on the fatty acid composition of sphingomyelin from canine erythrocytes and platelets and sphingomyelin and neutral glycosphingolipids of swine erythrocytes was determined. Sphingolipids of platelets and erythrocytes from animals fed high levels of corn or cottonseed oil exhibited a dramatic alteration in their fatty acid composition, most notable of which was a 50% reduction in nervonic acid (24:1ω9) as compared to levels observed in control or tallow fed animals. This decrease was compensated for by a quantitatively similar increase in a C_{24} dienoic acid. The long chain dienoic acid was isolated by silver nitrate thin layer chromatography and determined by analysis of its oxidation products to be Δ15,18-tetracosadienoic acid (24:2ω6). When the animals were fed the diets high in polyunsaturates, the 24:2ω6 represented 13, 20, and 9% of the sphingomyelin fatty acids from canine erythrocytes, platelets, and swine erythrocytes, respectively, and 5% of the neutral glycosphingolipid fatty acids of swine erythrocytes. In contrast, the 24:2ω6 represented less than 4% of the total cellular sphingolipid fatty acids in animals fed the control or high beef tallow diets. The 24:1ω9 in the sphingolipids of the animals fed the polyunsaturated diet was roughly equal to that of 24:2ω6, whereas in the sphingolipids of animals fed the control or saturated fat (beef tallow) diet, the 24:1ω9 was twice these values. Since sphingomyelin is a membrane component, the increase in unsaturation (24:2ω6) in its fatty acid moiety induced by dietary polyunsaturates may affect membrane fluidity and may alter membrane properties.

INTRODUCTION

Sphingomyelin of erythrocytes (1), platelets (2), and other tissues (3) characteristically contains significant amounts of C_{16} to C_{24} saturated fatty acids, as well as nervonic acid (24:1ω9). In addition, trace amounts of tetracosadienoic acid (24:2) have been observed in the sphingolipids of various tissues (4-6). During lipid analyses of erythrocytes and platelets from animals on experimental diets, we observed the presence of large amounts of a long chain dienoic acid in the sphingomyelin and neutral glycosphingolipids of those fed corn or cottonseed oil diets, but not in those animals fed tallow or control diets. The purpose of this report is to describe in detail the effect of feeding saturated (beef tallow) or polyunsaturated (corn or cottonseed oil) fatty acids on the fatty acid composition of sphingomyelin from canine erythrocytes and platelets and sphingomyelin and neutral glycosphingolipids from swine erythrocytes.

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MATERIALS AND METHODS

Experimental Animals and Diets

Nine-month-old male foxhounds were thyroidectomized and randomly assigned to be fed one of six experimental diets. These diets consisted of the basal (control) diet (diet 1) [Zeigler Brothers, Inc., Gardners, PA. Composition of the basal diet is available from the authors.]; or the basal diet supplemented with either 0.75% taurocholate (diet 2); 15% beef tallow (diet 3); 1.5% safflower oil, 0.75% taurocholate, 10.5-13.0% beef tallow, and 0.5-3.0% chlesterol (diet 4); 15% cottonseed oil (diet 5); or 12.0-14.5% cottonseed oil, 0.5-3.0% cholesterol, and 0.75% taurocholate (diet 6). Blood cholesterol was maintained at ca. 1500 mg/dl by adjusting the cholesterol intake of animals fed diets 4 and 6. During the 23 week feeding trial, dogs were housed in indoor runs with water available on an ad libitum basis. Dogs were fasted for 18 hr before blood samples were drawn.

Miniature swine (Sinclair Farms, University of Missouri, Columbia, MO) were fed a diet (7) similar to the dog diet for 26 weeks. One pig was randomly selected for bleeding from each of three groups that received the basal diet with 15% tallow, 15% corn oil, or 15% cottonseed

oil. The feed manufacturer (Zeigler Brothers, Inc., Gardners, PA) mixed the fats into the basal swine diet.

Preparation of Platelets and Erythrocytes

Blood was drawn from the foxhounds through a 2 in., 16 Ga polyethylene catheter into 0.3 volume of BSG-citrate (pH 7.4) [BSG-citrate (g/1): NaCl, 6.832; Na citrate dihydrate, 38.00; glucose 2.00; Na_2HPO_4, 1.22; KH_2PO_4, 0.218]. Platelets and erythrocytes were then isolated by modification of the Stractan II (an arabino-galactan polysaccharide, St. Regis Paper Company, Tacoma, WA) procedure of Corash et al. (8,9). The blood-BSG-citrate was layered on an equal volume of 20% Stractan and centrifuged at 980 x g for 15 min. Erythrocytes from the Stractan layer were washed 3 times with 10 volumes of phosphate buffered saline (PBS). "Platelet rich plasma" which remained above the 20% Stractan was layered on a discontinuous gradient composed of 1/2 volume of 12% Stractan and 1/2 volume of 20% Stractan. After centrifugation for 5 min at 4,000 x g, platelets were isolated from the interface between the 12 and 20% Stractan. Swine erythrocytes were isolated by centrifugation at 1500 x g for 10 min (4 C) from blood drawn into 0.2 volume of BSG-citrate. Packed erythrocytes were washed 3 times with 10 volumes of PBS. All samples were stored at -70 C.

Isolation of Sphingomyelin and Neutral Glycosphingolipids

Lipid extraction and purification was carried out on 10 ml of packed erythrocytes as described by Booth (10) and Nelson (11). Due to the smaller sample size, platelets from 200 ml of whole blood were extracted and purified with 20% of the solvent volume described for erythrocyte extraction, and the Sephadex column chromatography was scaled down proportionally. Sphingomyelin and neutral glycosphingolipids were isolated by two-dimensional thin layer chromatography (TLC) and visualized with 2′,7′-dichlorofluorescein (10,11). The spots were scraped, and methyl esters prepared in the presence of Silica Gel H by heating with 3N methanolic HCl in a sealed vial at 70 C for 6 hr. Under these conditions, esterification was essentially complete in 6 hr.

Isolation and Identification of the Dienoic Acid

Methyl esters were prepared from sphingomyelin isolated from erythrocytes of dogs on the cottonseed oil diet. About 100 μg of a dienoate methyl ester fraction was isolated by argentation TLC of the total methyl ester fraction (12). The esters were oxidized by a permanganate-periodate procedure (13). Following the addition of sodium bisulfite, one drop of ethanolic NaOH (1N) was added, and the sample was freeze dried. The residue was then acidified with a minimum volume of 10% aqueous H_2SO_4 saturated with diethyl ether and an aliquot injected into the gas liquid chromatograph (GLC) for analysis of short chain free fatty acids (FFA). The remainder of the sample was extracted with petroleum ether and esterified with methanolic HCl to determine the long chain mono- and dicarboxylic acid content.

Analysis of Fatty Acid Methyl Esters

GLC analyses were conducted on a Hewlett Packard 5830A gas chromatograph, equipped with dual hydrogen flame detectors, using either glass capillary or packed columns. The gas chromatograph with the capillary column was equipped with an all glass splitter (J&W Scientific, Orangevale, CA). Nitrogen make-up gas was heated by passage through copper tubing in the GLC oven prior to entering the detector at 47 ml/min. Helium carrier-gas pressure was 36 psi at the inlet with a split of ca. 100 to 1.

A 60 m x 0.25 mm ID open tubular glass capillary column coated with SP 2340 (J&W Scientific, Orangevale, CA) was used for the quantitative analysis of all methyl esters. Samples were injected at 145 C and separated by temperature programming with heating rates of 0.6 C/min for the first 27 min, 0.7 C/min for the next 20 min, and 0.8 C/min until the final temperature of 185 C was reached. The final temperature was maintained for 20 min. Under these conditions, 24:1ω9 eluted in ca. 65 min. The concentration of methyl esters injected was ca. 20 μg/μl. Correction factors for individual methyl esters were determined from repeated injections of quantitative standards purchased from Nu-Chek-Prep, Elysian, MN.

Packed glass columns (2 mm ID x 180 cm) were used in the identification of the unknown components. Methyl esters were analyzed on columns packed with 10% SP 2330 on 100-120 mesh Chromosorb W AW (polar column) or on 3% SP 2100 on Supelcoport 100-120 mesh (nonpolar column). The former was operated at 180 C and the latter at 205 C. Both packings were purchased from Supelco, Inc., Bellefonte, PA. Free fatty acids were separated on a column of 7.5% EGA-2% H_3PO_4 on 90-100 mesh Anakrom ABS (the packing was a gift from Dr. D.P. Schwartz, USDA, Eastern Regional Research Center, Philadelphia, PA). Methyl esters were hydrogenated in methanol with Adam's

Catalyst (Pfaltz & Bauer, Inc., Stamford, CT) in a microhydrogenator (Supelco, Inc., Bellefonte, PA) (14).

RESULTS AND DISCUSSION

The fatty acid composition of the sphingomyelin from the erythrocytes of animals on the control diet (Table I) was similar to that reported for erythrocytes of other species (1). Sphingomyelin from erythrocytes contained predominantly (72%) saturated fatty acids ranging in chain length from C_{16} to C_{24}. The major unsaturated acid of the sphingomyelin was 24:1ω9 which represented 24.0% of the total fatty acid. The 24:1ω9 content averaged 23.2% for the control (diets 1 and 2) and tallow (diets 3 and 4) diets, but only 13.2% for the cottonseed oil and cottonseed oil cholesterol fed animals (Tables I and II). This reduction in the sphingomyelin 24:1ω9 was compensated for by an increase in an unknown component (subsequently identified as 24:2ω6) from a mean of 2.6% in the control and tallow fed animals to 13.2% in those fed cottonseed oil.

The dienoic acid was identified by analysis of methyl esters prepared from the sphingomyelin of erythrocytes obtained from dogs fed cottonseed oil (diets 5 and 6). The chain length of the dienoic acid was determined to be 24 carbons by GLC analysis of the combined methyl esters on an SP 2100 column (nonpolar) and by GLC analysis of a portion of the sample which had been hydrogenated. The unknown cochromatographed with 24:1ω9 on the nonpolar column suggesting that it was a C_{24} unsaturated acid. GLC analysis of the hydrogenated sample yielded a peak for lignoceric acid (24:0) equal to the sum of the original lignocerate, nervonate, and the dienoate component. This observation confirmed the C_{24} chain length. The remainder of the sample when fractionated by $AgNO_3$ TLC yielded three fractions: saturated, monounsaturated, and the dienoate component which migrated like the methyl ester of a long chain dienoic acid. To locate the position of the double bonds, the fatty acid methyl ester fraction of the uncharacterized component which had been iso-

TABLE I

Fatty Acid Composition of Sphingomyelin from Platelets and Erythrocytes of Dogs on Control Diets

Fatty acid	Erythrocytes	Platelets
14:0	0.5 ± 0.1[a]	0.2 ± 0.1
16:0	51.7 ± 1.7	22.4 ± 2.4
18:0	14.7 ± 1.0	5.6 ± 1.0
18:1ω9	0.2 ± 0.1	0.2 ± 0.1
20:0	1.4 ± 0.1	2.0 ± 0.2
22:0	1.5 ± 0.3	8.6 ± 1.0
22:1ω9	0.2 ± 0.2	1.0 ± 0.2
22:1ω7[b]	—	0.4 ± 0.1
23:0	0.3 ± 0.3	0.8 ± 0.1
24:0	2.4 ± 0.4	6.6 ± 1.1
24:1ω9	24.0 ± 0.7	47.1 ± 3.0
24:1ω7[b]	tr	1.2 ± 0.1
24:2ω6	2.7 ± 0.4	3.2 ± 0.8
Other[c]	0.6 ± 0.2	0.8 ± 0.1

[a]Weight percent reported as the mean ± SD for analysis of samples from four animals. Two each on the control diets (diets 1 and 2).

[b]Tentative identification.

[c]The sum of unidentified components.

TABLE II

Fatty Acid Composition of Sphingomyelin from Erythrocytes and Platelets of Dogs on Experimental Diets

Fatty[a] acid	Diet[b] Erythrocytes: Tallow	Tallow-SFO cholesterol	CSO	CSO cholesterol	Platelets: Tallow	Tallow-SFO cholesterol	CSO	CSO cholesterol
16:0	53.4[c]	49.2	53.5	53.4	23.4	23.0	24.8	20.6
18:0	15.2	13.8	10.0	8.4	7.2	7.2	6.4	6.3
22:0	1.8	2.6	2.8	2.0	11.2	10.8	9.2	12.4
24:0	1.8	3.5	3.0	5.4	6.7	5.9	7.2	7.0
24:1ω9	21.8	23.8	12.7	13.8	42.4	39.4	24.0	24.9
24:2ω6	1.0	4.2	12.8	13.7	1.0	5.5	20.6	21.2
Other	5.0	2.9	5.2	3.3	8.1	8.2	7.8	7.6
(n)[d]	(2)	(2)	(2)	(2)	(2)	(3)	(2)	(1)

[a]Data for the major fatty acids are reported individually. Fatty acids present in lower amounts are summed under other.

[b]See text for composition of diets; SFO, safflower oil; CSO, cottonseed oil.

[c]Weight percent – reported as the mean (n = 2 or 3) or result of a single analysis (n = 1).

[d](n) – number of animals.

TABLE III

Fatty Acid Composition[a] of Sphingomyelin and Neutral Glycosphingolipids from Erythrocytes of Swine Fed Diets Containing High Amounts of Tallow, Corn Oil, or Cottonseed Oil

Fatty acid	Diet[b]					
	Tallow	Corn oil	Cottonseed oil	Tallow	Corn oil	Cottonseed oil
	Sphingomyelin			Neutral glycosphingolipids		
16:0	31.1	26.3	26.5	3.7	1.8	1.9
16:1ω7	1.4	0.2	0.4	1.0	0.2	0.2
18:0	7.6	4.7	5.8	2.2	1.2	1.5
18:1ω9	2.9	0.3	0.7	1.5	0.2	0.2
20:0	2.3	3.2	3.4	1.2	1.2	1.8
22:0	6.2	9.2	11.6	17.5	18.4	25.1
22:1ω9	tr	tr	tr	1.8	0.7	0.6
24:0	23.0	34.4	28.4	48.5	53.7	49.7
24:1	20.6	10.4	10.0	15.6	8.6	6.4
24:2ω6	1.1	7.6	10.1	0.8	5.4	5.1
26:0	0.3	0.9	1.0	2.7	4.0	2.9
26:1	0.8	0.7	tr	1.4	1.0	0.6
26:2	---	1.2	0.6	---	2.3	2.2
Other[c]	2.6	1.5	1.5	2.2	1.2	1.7

[a]Results of analysis of samples from a single animal on each diet. Reported as weight percent of methyl esters.

[b]For composition of the diets, see ref. 7.

[c]The sum of minor components.

lated by $AgNO_3$ TLC was oxidized. Methyl linoleate and methyl nervonate were also oxidized to serve as controls and gave the expected products. GLC analyses of the oxidation products from the unknown detected the presence of hexanoic acid and a C_{15} dicarboxylic acid. This indicated that the unknown acid was Δ15,18-tetracosadienoic acid (24:2ω6). No other isomers of 24:2 were noted. This fatty acid is also present in trace amounts in the sphingomyelin of normal brain (4) and in higher amounts in ascites tumors (15).

The fatty acid composition of sphingomyelin derived from canine platelets (Tables I and II) differed from that found in the erythrocytes. The major differences observed were lower percentages of 16:0 and 18:0, and higher percentages of 22:0 and 24:1ω9 in the platelets when compared to the erythrocytes. Feeding cottonseed oil altered the percentage of 24:1ω9 and 24:2ω6 in the platelet sphingomyelin as it did in the erythrocytes. The sphingomyelin from platelets of animals on the tallow and control diets contained 40 to 47% nervonic acid, whereas those on the cottonseed oil and cottonseed oil cholesterol diets contained an average of 25% of this acid. The reduction in 24:1ω9 was again compensated for by an increase in the 24:2ω6 from 1 to 3% in the tallow fed and control animals to a mean of 20.9% in the cottonseed oil fed dogs. It is of interest that the addition of safflower oil (1.7%) to the tallow cholesterol diet caused a slight elevation of 24:2ω6, relative to control and tallow fed animals, in the sphingomyelin of platelets and erythrocytes (Tables I and II). These results suggested that the 24:2ω6 was formed as a result of the high content of 18:2ω6 in the diets of the cottonseed oil fed animals. However, it was possible that the increase in 24:2ω6 observed in the cottonseed oil fed groups was mitigated by the presence of traces of cyclopropene acids which inhibit desaturation of 18:0 to 18:1ω9 (16,17), the precursor of 24:1ω9. Cyclopropene acids are known components of cottonseed oil. Furthermore, it has been demonstrated that feeding cyclopropenes causes a reduction of 24:1ω9 in the sphingomyelin of rat brain and liver with an increase in the percentages of 16:0 and 18:0 (3). The occurrence of 24:2 was not reported, however (3).

In order to extend our observations to another species and to determine if the increase in 24:2ω6 was mediated by the presence of cyclopropenes, miniature swine were fed diets containing beef tallow, cottonseed oil, or corn oil. Corn oil was chosen for comparison because of its high linoleate content and lack of cyclopropenes.

The diet-dependent, reciprocal relationship between 24:1ω9 and 24:2ω6 noted in dogs was also observed in swine. In the sphingomyelin fraction isolated from swine erythrocytes, the 24:2ω6 increased from 1.1% in the tallow fed animals to a mean of 8.8% in the corn and cottonseed oil fed animals (Table III). A similar but smaller increase was found for the

24:2ω6 in the neutral glycosphingolipid fraction isolated from swine erythrocytes (Table III). Since feeding corn oil, which contains no cyclopropenes, had an effect similar to cottonseed oil on the fatty acid composition of the sphingolipids, the increased 24:2ω6 observed in these studies was related to the high content of 18:2ω6 in the diet. (20).

The fatty acid composition of sphingomyelin from swine erythrocytes (Table III) differed from that of canine erythrocytes (Tables I and II). The former contained a mean of 28% 16:0 and 28.6% 24:0, whereas the latter contained 52% and 3% of these acids, respectively. In addition, in both the sphingomyelin and neutral glycosphingolipids isolated from swine erythrocytes, two or three components eluted from the GLC column after 24:2ω6. These peaks were tentatively identified as 26:0, 26:1, and 26:2 on the baisis of their retention time when analyzed on the SP2330 packed column which was operated isothermally. The peak assignment of 26:0 was based on the expected retention time for 26:0 calculated by extrapolation of the regression of the log of the retention time on the chain length of saturated fatty acid methyl ester standards. The separation factors (retention time of component divided by the retention time of the parent compound) of the two unknown peaks relative to the 26:0 peak were the same as those for 24:1ω9 and 24:2ω6 relative to 24:0. Thus, these two peaks were tentatively identified as 26:1 and 26:2. These assignments were consistent with the migration of these compounds during argentation TLC. The peaks identified as 26:0, 26:1, and 26:2 were associated with the saturated, monounsaturated, and diunsaturated fractions, respectively. The content of 26:2ω6 was increased in the glycosphingolipids from the animals fed cottonseed or corn oil as compared to those fed tallow (Table III).

The presence of 24:2ω6 in sphingomyelin of platelets and erythrocytes has not been previously reported. However, 24:2 has been observed in the glycosphingolipids of rabbit erythrocytes (6). The highest amount of 24:2 in sphingomyelin reported for any tissue occurs in certain rat and mouse hepatomas (representing 5-7% of the sphingomyelin fatty acids) (18). However, normal and host liver lacks detectable 24:2 (19) despite the capability of rat liver microsomes to convert 18:2 to 24:2 (20). [Linoleic acid is generally desaturated and elongated by the following pathway: 18:2ω6 → 18:3ω6 → 20:3ω6 → 20:4ω6. Liver microsomes do, however, contain an enzyme system which is capable of the elongation of 18:2ω6 to 24:2ω6 (18:2ω6 → 20:2ω6 → 22:2ω6 → 24:2ω6).] The appearance of 24:2 in hepatomas may reflect an alteration in 18:2ω6 metabolism resulting from an overaccumulation of 18:2 secondary to impaired conversion of 18:2 to 20:4 (21). An impaired conversion has been noted in cultured Morris hepatoma cells which lack the ability to convert 18:2 to 20:3ω6 (22). In fact, the amount of 24:2 in the sphingomyelin of a hepatoma cell line grown in culture was increased to 23% by supplementing the medium with linoleic acid (23). The present study comparing the fatty acid composition of erythrocytes and platelets from animals on diets high or low in linoleate further supports the concept that high levels of 18:2ω6 may lead to the occurrence of 24:2ω6. Since sphingomyelin is a component of the cell membrane, the increase in 24:2ω6 in the sphingomyelin fatty acids in the erythrocytes and platelets of animals fed the diets high in polyunsaturates could have a profound effect on the properties of the membrane. Whether this alteration in composition induced by dietary polyunsaturates affects the properties of the cellular membranes and, hence, metabolism, should prove an interesting area of future study.

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Synthesis and Mass Spectrometry of 1-Acyl and 3-Acyl-*sn*-Glycerol Carbonates

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ABSTRACT

sn-Glycerol-1,2-carbonate was prepared from *D*-serine, *sn*-glycerol-2,3-carbonate from *L*-serine, via 1-*0*- or 3-*0*-benzyl-*sn*-glycerol, respectively. *sn*-Glycerol-2,3-carbonate was also prepared from *D*-mannitol or *D*-serine following the *sn*-glycerol-3-β,β,β-trichloroethylcarbonate route. *sn*-Glycerol-1,2-carbonate and *sn*-glycerol-2,3-carbonate were acylated with saturated and unsaturated fatty acid chlorides to form 3-acyl-*sn*-glycerol-1,2-carbonates and 1-acyl-*sn*-glycerol-2,3-carbonates, respectively. The mass spectra of the enantiomeric monoacyl-*sn*-glycerol carbonates showed molecular ions and acyl cations (RCO^+) of high intensity. The heterocyclic dioxolan-2-one ring was remarkably stable during electron impact.

INTRODUCTION

We attempted to synthesize mixed acid 1,2-diacyl-*sn*-glycerols using the β,β,β-trichloroethylcarbonate group for protection of the free hydroxy moiety. This method was previously used in the synthesis of 1,2-diacyl-*sn*-glycerols containing identical fatty acid residues (1-3).

Starting with *sn*-glycerol-3-β,β,β-trichloroethylcarbonate (*VI*), we tried to acylate stepwise the two free hydroxy groups of the glycerol backbone with different fatty acid chlorides (4). Though a monoacylated product was formed in the first acylation step, no 1-acyl-*sn*-glycerol-3-β,β,β-trichloroethylcarbonate (*XIII*) could be isolated. In all experiments, however, 1-acyl-*sn*-glycerol-2,3-carbonate (*VIII*) was formed in good yields. Until now only one compound of the 1-acyl-*sn*-glycerol-2,3-carbonates (*VIII*), 1-stearoyl-*sn*-glycerol-2,3-carbonate, was described by any physical data (2).

We synthesized both enantiomeric forms, *sn*-glycerol-1,2-carbonate (*VIIa*) and *sn*-glycerol-2,3-carbonate (*VII*) (1,3-dioxolan-4-(R) and (S)-(hydroxymethyl)-2-one). From these carbonates, two homologous series of enantiomeric monoacyl-*sn*-glycerol derivatives could be prepared by acylation with fatty acid chlorides. The synthesized monoacyl-*sn*-glycerol carbonates were investigated by mass spectrometry (MS).

EXPERIMENTAL PROCEDURES

Mass spectra were recorded on a Varian CH 7 mass spectrometer. The ionization voltage was 70 eV, accelerating voltage 3 kV, electron emission 300 μA, and the ion source temperature 170 C. All compounds were introduced into the mass spectrometer by direct inlet, heated to 90-130 C depending on the volatility of the individual compounds. ^{1}H-NMR spectra were taken on a Varian T-60 nuclear magnetic resonance spectrometer. Spectra were measured in deuterochloroform with tetramethylsilane as internal standard. Infrared spectra were recorded on a Perkin-Elmer PE 297 grating infrared spectrophotometer. Optical rotation values were measured on a Perkin-Elmer 241 polarimeter. $[\alpha]_D$-values were observed at 20 C on chloroform solutions, and the unacylated glycerol carbonates on aqueous solutions at a concentration of c = 1.

All reagents and solvents, unless mentioned otherwise, were obtained from E. Merck (Darmstadt, Germany). Fatty acid chlorides and pure fatty acids were purchased from Sigma Chemical Co. (St. Louis, MO) and Nu-Chek-Prep (Elysion, MN). Sodium-bis-(2-methoxyethoxy)-aluminum hydride, Red-Al®, is available from Aldrich Chemical Co. (Milwaukee, WI).

All synthetic steps described or cited were carried out with both enantiomeric forms.

1,2-*O*-Isopropylidene-*sn*-Glycerol (IV)

A solution of 10 g (0.06 mole) of methyl 2,3-isopropylidene-*D*-glycerate (*XI*) and a twofold excess of Red-Al in 100 ml toluene-diethyl ether (1:1, v/v) was refluxed for 1 hr. Excess reagent was destroyed by adding a mixture of ethyl acetate and diethyl ether, followed by a small amount of ethanol-water (1:1, v/v). After filtration, the precipitate was washed with diethyl ether, and the combined layers were dried over $MgSO_4$. The solvents were evaporated, and distillation of the residue yielded 7.15 g (81%) 1,2-*O*-isopropylidene-*sn*-glycerol (*IV*), bp 47 C/0.1 mm; $[\alpha]_D^{20}$ +12.8° (neat); Lit. 5, $[\alpha]_D^{22}$ +13.1°, bp 75.5-76 C/10 mm. Calculated for $C_6H_{12}O_3$: C, 54.54; H, 9.09. Found: C, 54.56; H, 9.10.

TABLE I

C, H-Values of 1- and 3-Acyl-*sn*-Glycerol Carbonates

Fatty acid residue	$C_nH_mO_q$	Calculated		Found	
		C	H	C	H
1-Palmitoyl	$C_{20}H_{36}O_5$	67.38	10.18	67.28	10.17
3-Palmitoyl	$C_{20}H_{36}O_5$	67.38	10.18	67.35	10.14
1-Stearoyl	$C_{22}H_{40}O_5$	68.71	10.49	68.66	10.45
3-Stearoyl	$C_{22}H_{40}O_5$	68.71	10.49	68.80	10.45
1-Arachidoyl	$C_{24}H_{44}O_5$	69.86	10.75	69.85	10.79
3-Arachidoyl	$C_{24}H_{44}O_5$	69.86	10.75	69.81	10.73
1-Oleoyl	$C_{22}H_{38}O_5$	69.07	10.01	69.10	10.00
3-Oleoyl	$C_{22}H_{38}O_5$	69.07	10.01	69.12	10.05
1-Linoleoyl	$C_{22}H_{36}O_5$	69.44	9.54	69.39	9.61
3-Linoleoyl	$C_{22}H_{36}O_5$	69.44	9.54	69.40	9.63

3-*O*-Benzyl-*sn*-Glycerol-1,2-Carbonate (XVIIa)

3-*O*-Benzyl-*sn*-glycerol (*XVIa*) (17 g, 0.09 mole), 40 ml of diethylcarbonate, and 170 mg of $KHCO_3$ were mixed in a round bottom flask connected to a distillation apparatus. The mixture was heated and kept at 100 C for 4 hr. During this time, 10 ml of ethanol was removed by distillation. Upon cooling to room temperature, 400 ml of water was added slowly, and the solution was extracted three times with 100 ml portions of diethyl ether. The combined diethyl ether extracts were washed with 1N sulfuric acid, then with 5% $NaHCO_3$-solution, water, and were dried over $MgSO_4$. The diethyl ether was evaporated, and the residue was distilled giving 3-*O*-benzyl-*sn*-glycerol-1,2-carbonate (17 g, 88%), bp 150 C/0.1 mm; n_D^{23} 1.5196; $[\alpha]_D$ -12.14° c = 0.98; Calculated for $C_{11}H_{12}O_4$: C, 63.46; H, 5.77. Found: C, 63.43; H, 5.70.

sn-Glycerol-1,2-Carbonate (VIIa)

3-*O*-Benzyl-*sn*-glycerol-1,2-carbonate (*XVIIa*) (10 g, 0.04 mole) in 150 ml of glacial acetic acid was hydrogenated over 500 mg palladium/charcoal as a catalyst. The rate of cleavage of the benzyl group was monitored by thin layer chromatography (TLC). When the reaction was completed, the catalyst was filtered off, the solvent was removed, and *sn*-glycerol-1,2-carbonate (*VIIa*) was obtained (5.3 g, 93.4%), $[\alpha]_D$ -58.3° (neat). Lit.6,$[\alpha]_D$ -59° (neat). Calculated for $C_4H_6O_4$: C, 40.68; H, 5.08. Found: C, 40.66; H, 5.12.

Acylation with Fatty Acid Chlorides

Glycerol carbonate, 1 mmole, was dissolved in 10 ml of freshly distilled dry methylene chloride and 2 ml of fresh dry pyridine. The solution was cooled to 0 C, and a solution of 2 mmoles of fatty acid chloride in 5 ml of dry methylene chloride was added dropwise using a nitrogen atmosphere and stirring. Stirring was continued for 30 min at 0 C and for 2 hr at 25 C. Methylene chloride, 50 ml, was added, and the mixture was successively washed with dilute hydrochloric acid, water, 5% $NaHCO_3$-solution, and water. After drying over Na_2SO_4, the solvent was evaporated, and the crude product was purified by preparative TLC. Elemental analyses of the monoacyl-*sn*-glycerol carbonates are shown in Table I.

Acylation with Fatty Acids in the Presence of Thionyl Chloride

Glycerol carbonate, 2 mmoles, and 2-3 mmoles of fatty acid were stirred in 30 ml anhydrous benzene using a nitrogen atmosphere. Thionyl chloride, 0.2 ml, was added, and the suspension was stirred at 70 C for 48 hr under exclusion of moisture, air, and light. A clear solution was obtained when the reaction was completed. The solvent and excess thionyl chloride were removed under reduced pressure at room temperature, and the crude product was purified by TLC.

Purification of Acyl Glycerol Carbonates

Purification of the synthesized 1-acyl-*sn*-glycerol-2,3-carbonates (*VIII*) and 3-acyl-*sn*-glycerol-1,2-carbonates (*VIIIa*) for mass spectrometric investigation was achieved by preparative TLC. Thin layer plates coated with 1 mm of Silica Gel H (type 60) were developed with 1,2-dichloroethane. The R_f of all species was in the region of 0.4, well separated from by-products. Monoacyl glycerol carbonate, 30 mg, could be separated easily onto a 20 x 20 cm plate. Bands were made visible by iodine vapor or water, if the compounds were unsaturated, and eluted with chloroform-methanol (2:1, v/v). After evaporation of solvents, the pure compounds were dried in vacuo over P_2O_5 at 25 C for 24 hr.

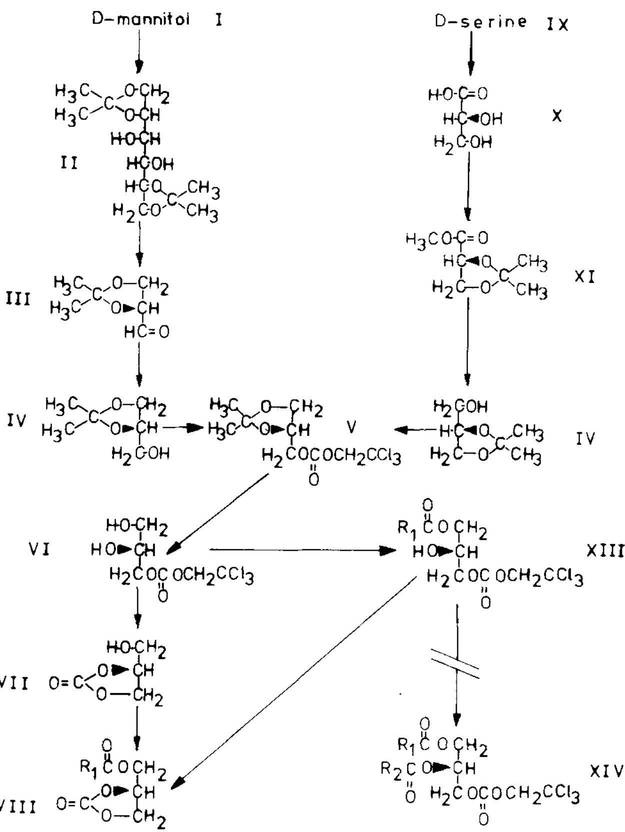

FIG. 1. *sn*-Glycerol-3-β,β,β-trichloroethylcarbonate route for the synthesis of 1-acyl-*sn*-glycerol-2,3-carbonate starting from *D*-mannitol or *D*-serine.

RESULTS AND DISCUSSION

1,2-*O*-Isopropylidene-*sn*-glycerol (*IV*) was synthesized starting from *D*-mannitol (*I*) or *D*-serine (*IX*) according to established methods (7,8). The first approach, using *sn*-glycerol-β,β,β-trichloroethylcarbonate, is shown in Figure 1. 1,2-*O*-Isopropylidene-*sn*-glycerol upon reaction with β,β,β-trichloroethylchloroformate gave 1,2-*O*-isopropylidene-*sn*-glycerol-3-β,β,β-trichloroethylcarbonate (*V*) (2), from which *sn*-glycerol-3-β,β,β-trichloroethylcarbonate (*VI*) was obtained by removal of the isopropylidene group with dilute hydrochloric acid or with an acidic ion exchange resin (2,9). *sn*-Glycerol-3-β,β,β-trichloroethylcarbonate (*VI*) was cyclized in pyridine at 80 C to form *sn*-glycerol-2,3-carbonate (*VII*) (10).

β,β,β-Trichloroethanol was formed as a by-product in the cyclization step in addition to *sn*-glycerol-2,3-carbonate. β,β,β-Trichloroethanol could be removed by distillation, but this led to extensive decomposition of *sn*-glycerol-2,3-carbonate (*VII*) (6). Chromatographic separation methods could not be applied, because both preparative TLC and column chromatography, either on alumina or silica gel, effected cleavage of the carbonate group or total racemization of the product. β,β,β-Trichloroethanol, however, consumed up to 50% of the acylating reagents during acylating procedures and as an acylated compound interfered with the purification.

These difficulties were overcome by an alternative synthetic approach via the benzyl-*sn*-glycerol route, starting from *D*- and *L*-serine (*IX* and *IXa*). This synthesis is shown in Figure 2. According to Lok et al. (5), the enantiomeric isopropylidene-*sn*-glycerols (*IV* and *IVa*) were prepared in three steps. An alternative reduction procedure of methyl 2,3-*O*-isopropylidene-*D*-glycerate (*XI*) was used. Reduction with Red-Al gave better yields and was simpler. Isopropylidene-*sn*-glycerols (*IV* and *IVa*) protected in the 1- or 3-positions by a benzyl group (*XV* and *XVa*) (11) were cleaved by treatment with hydrochloric acid, and the 1-*O*- or 3-*O*-benzyl-*sn*-glycerols (*XVI* and *XVIa*)

FIG. 2. 1-*O*-Benzyl-*sn*-glycerol route for the synthesis of 1-acyl-*sn*-glycerol-2,3-carbonate starting from *L*-serine. 3-Acyl-*sn*-glycerol-1,2-carbonate was the final product if started from *D*-serine.

TABLE II

Physical Properties of 1-Acyl-*sn*-Glycerol-2,3-Carbonates (VIII) and 3-Acyl-*sn*-Glycerol-1,2-Carbonates (VIIIa)

Fatty acid acyl residue	*sn*-Glycerol-2,3-Carbonate		*sn*-Glycerol-1,2-Carbonate	
	$[\alpha]^{20}_{D}$	mp [C]	$[\alpha]^{20}_{D}$	mp [C]
Palmitoyl	$+7.3^{0}$	67	-7.4^{0}	67
Stearoyl	$+6.05^{0}$ [a]	82	-6.1^{0}	82
Arachidoyl	$+4.44^{0}$	84	-4.4^{0}	85
Oleoyl	$+5.1^{0}$	21	-5.0^{0}	21
Linoleoyl	$+6.2^{0}$	<-10	-6.4^{0}	<-10
Unacylated *sn*-Glycerol carbonates	$+36.9^{0}$ [b]		-37.2^{0}	

[a]Lit. 2, $[\alpha]^{22}_{D}$ $+6.9^{0}$ (c = 1.08, chloroform).
[b]Lit. 17,18, $[\alpha]^{20}_{D}$ $+ 36.8^{0}$ (c = 1, chloroform).

were isolated and distilled. During our experiments, another simple synthesis of 3-*O*-benzyl-*sn*-glycerol (*XVIa*) starting from *D*-mannitol (*I*) was reported (12).

While carbonate can be introduced with phosgene (6), $KHCO_3$ in dimethylsulfoxide (13,14), or with diethylcarbonate (15,16), we selected the diethylcarbonate method, which gave the best yields. The benzyl group was removed by catalytic hydrogenolysis over palladium/charcoal in glacial acetic acid. In the *D*- and *L*-series, both enantiomeric glycerol carbonates (*VII* and *VIIa*) were obtained after removal of catalyst and solvent without further purification giving correct optical rotations (17-19). A recent investigation by Chebyshev et al. (17,18) showed that, under the conditions applied, no isomerization occurred during hydrogenolysis.

Hexadecanoic, octadecanoic, eicosanoic, *cis*-9-octadecenoic, and *cis*-9-*cis*-12-octadecadienoic acids were introduced by two different acylation methods. The first made use of fatty acid chlorides in dry chloroform/pyridine and the second of fatty acids and thionyl chloride in anhydrous benzene, avoiding preparation of fatty acid chlorides (20). Physical properties of 1-acyl-*sn*-glycerol-2,3-carbonates (*VIII*) and 3-acyl-*sn*-glycerols-1,2-carbonates (*VIIIa*) are given in Table II.

The $[\alpha]_D$-values of the monoacyl-*sn*-glycerol carbonates are in good agreement with the values reported by Baer and Fischer (7) for the 1-acyl-2,3-*O*-isopropylidene-*sn*-glycerols, which belong to the same class of 1,3-dioxolanes, and the value reported by Pfeiffer et al. (2) for the stearoyl species.

Attempts to synthesize diacylglycerols with different acyl residues via *sn*-glycerol-3-β,β,β-trichloroethylcarbonate (*VI*) were not successful. The tendency of the β,β,β-trichloroethylcarbonate group to decompose rapidly in pyridine,

TABLE III

Mass Spectrometric Data of Acylated Glycerol Carbonates

Fatty acid acyl residue	M^+ m/e	M^+ %	$[M - C_3H_7]^+$ m/e	$[M - C_3H_7]^+$ %	RCO^+ m/e	RCO^+ %	m/e 160 %	m/e 173 %
1-Palmitoyl	356	66	313	28	239	33	68	100
3-Palmitoyl	356	59	313	31	239	35	74	95
1-Stearoyl	384	93	341	30	267	32	61	100
3-Stearoyl	384	89	341	28	267	30	57	100
1-Arachidoyl	412	94	369	35	295	34	77	100
3-Arachidoyl	412	90	369	31	295	33	78	100
1-Oleoyl	382	32	339	4	265	74	53	100
3-Oleoyl	382	27	339	4	265	57	51	100
1-Linoleoyl	380	100	337	5	263	59	44	75
3-Linoleoyl	380	73	337	5	263	43	32	55

yielding the cyclic carbonate (*VII*) and β,β,β-trichloroethanol, was probably responsible for this (10).

The acylated glycerol carbonates were characterized by ^{1}H-NMR. δ-Values of the glycerol backbone protons were: H_2C-O δ 4.25; HC-O δ 4.7, and the fatty acid acyl residue protons appeared at O=C-CH_2 δ 2.25; $(CH_2)_n$ δ 1.2; CH_3 δ 0.9. If the acyl residue was unsaturated, the protons attached to the double bond(s) occurred at C=CH δ 5-6. In 1-stearoyl-*sn*-glycerol-2,3-carbonate, the integral ratio of the glycerol protons to the acyl protons was 1:7.19 corresponding to the proton ratio in the molecule of 1:7.

The infrared spectra of the acyl glycerol carbonates showed two carbonyl vibrations, one ranging from 1780-1805 cm^{-1}, the other from 1640-1735 cm^{-1}, depending on the fatty acid residues of the molecules. The vibrations at higher wave number due to the carbonate group were more intense than the vibrations at lower wave number, which were produced by the carbonyl groups of the fatty acid moieties.

The main fragments in the mass spectra which were of interest for the general characterization of the enantiomeric 1- and 3-acyl-*sn*-glycerol carbonates (*VIII* and *VIIIa*) are shown in Table III. Two classes of fragments were observed in the mass spectra of the acylated cyclic glycerol carbonates. The first was produced by the stepwise degradation of the long acyl chains, the other consisted of the heterocyclic *sn*-glycerol carbonate and C_1- and C_2-fragments. The degradation was monitored in the case of saturated fatty acid residues to the fragment $[M - C_{14}H_{29}]^+$. Among the peaks of these fragments, which used to appear at distances of *m/e* 14, the peaks of the acyl cations were found. The RCO^+ fragments showed an intensity of 30% in saturated species increasing up to 60-70% in unsaturated species. The enlarged stability of the unsaturated acyl cations corresponded to the low intensity of the $[M - C_nH_{2n+1}]^+$ peaks. The peak at *m/e*173, formed by cleavage of the acyl chain between C_3 and C_4, was the most intense fragment, giving rise to the base peak, except in the spectrum of 1-linoleoyl-*sn*-glycerol-2,3-carbonate where M^+ had 100% intensity. Another very intense peak resulted from the McLafferty rearrangement at *m/e*160 showing intensities

m/e 160 *m/e* 173

from 50-70%. The alkyl fragments corresponding to *m/e* 160 and *m/e* 173 were missing in all spectra.

Especially in the range of *m/e* <110, the spectra of the unsaturated species were complicated by a number of peaks, which were formed by proton shifts and rearrangements within the alkyl radical cations. The high intensity of the molecular ion was noteworthy, ranging from 30-100%. This seems to be due to the stability of the dioxolan-2-one ring, which tends to retain the charge.

The mass spectra of the acyl-*sn*-glycerol carbonates differed from the spectra of other monoacylglycerol derivatives, such as diacetyl and trimethylsilyl, as reported by several authors (21-24). The spectra of these derivatives did not exhibit a molecular ion or one of low intensity. Furthermore, the trimethylsilyl ethers of monoacylglycerols usually gave mass spectra consisting mainly of intense fragments arising from trimethylsilyl groups and the glycerol moiety. Few degradation products were formed relating to the acyl group.

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Liver Lipid Alterations in Rats Fed Arginine Deficient Diets

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ABSTRACT

Arginine deficiency is associated with a marked increase in liver lipids in the rat. Triglyceride accumulation accounts for most of the fatty infiltration. Cholesterol concentration per gram of liver increased approximately 280% above control rats receiving dietary arginine. The percentage of phospholipids was significantly decreased in the arginine-deficient rat liver compared to controls. The fatty acid composition revealed a significant reduction in the percentage of palmitic, palmitoleic, oleic, and linoleic acids. However, both stearic and arachidonic acids were increased approximately 250 and 160%, respectively, in arginine-deficient livers compared to controls. Arginine deficiency in the rat causes a marked alteration in lipid metabolism similar to that observed with orotic acid feeding. The similarities of arginine deficiency and orotic acid feeding are discussed.

INTRODUCTION

Arginine deficiency is associated with a marked increase in the blood and urinary concentration of orotic and citric acids (1-5). Increased orotic acid excretion has been shown to occur during arginine deficiency in the rat, mouse, hamster, rabbit, guinea pig, and dog (3,4). Orotic aciduria induced by arginine deficiency is thought to result from a shunting of mitochondrial carbamoyl phosphate into cytoplasmic pyrimidine biosynthesis (1-4).

Orotic acid feeding is known to result in severe alterations in lipid metabolism (5-8). Massive hepatic lipid infiltration appears within 3 days in rats fed diets containing 1% orotic acid. The accumulated fat, which consists primarily of triglycerides, cholesterol, and phospholipids, appears to be derived from de novo synthesis (9,10). Orotic acid is thought to lead to fatty infiltration of the liver by inhibiting the incorporation of plasma apoproteins into lipoproteins (6,7). Therefore, the release of triglycerides and β-lipoproteins from the liver into the blood appears to be inhibited. Orotic acid induced fatty livers are not prevented by the usual lipotropic agents such as choline, methionine, inositol, folic acid, or vitamin B_{12} (11). However, supplementation of a diet containing 1% orotic acid with adenine has been shown to completely restore the liver lipid concentrations to normal (8,12).

The present studies were undertaken to examine the effect of dietary arginine and the associated increased orotic acid on liver lipids. These data are compared to previously reported studies on the effect of orotic acid feeding on liver lipids.

EXPERIMENTAL PROCEDURES

Male Sprague-Dawley rats were obtained from Harlan Industries, Indianapolis, IN. All rats were adapted for 4 days to a complete purified L-amino acid diet (2). After the adaptation period, the rats were randomly assigned to the complete diet (C) or the same diet devoid of arginine. After 17 days of feeding, the animals were sacrificed by decapitation and their livers removed for subsequent analysis.

All livers were analyzed for proteins, DNA, total lipids, phospholipids, triglycerides, and cholesterol. In addition, the total liver fatty acid compositions were determined. The protein content of liver homogenates was determined using the buiret reaction (13) with bovine serum albumin as a standard. DNA concentrations were determined by the method of Hubbard et al. (14). Total lipids were determined by the method of Folch et al. (15). Liver cholesterol was analyzed by the method of Zlatkin et al. (16). Total triglycerides were determined by Marzo et al. (17). Phospholipids were assayed according to the method of Eng and Noble (18).

Extraction of Liver

A portion of the isolated liver was washed repeatedly in saline water to remove blood and connective tissue. The wet weight of each liver was taken before homogenization in a hand homogenizer. Lipids were extracted with chloroform-methanol (2:1) and washed with saline water. The chloform layer was separated, dried over sodium sulfate, and the solvent removed using a rotary evaporator. The residual lipid was then redissolved in ca. 20 ml of chloroform-methanol (2:1) and filtered to remove nonlipid debris. The clear solution was then again evapoarated with a rotary vacuum evaporator. The isolated lipid was weighed and stored under N_2 in the freezer (-10 C).

Preparation of Methyl Esters

An aliquot of total liver lipid (1-10 mg) was converted to the corresponding methyl ester by

TABLE I

Effect of Dietary Arginine on Liver Size and Composition[a]

	Control	-Arginine
Liver weight (g/kg body weight)	4.71 ± 0.11^A	5.57 ± 0.79^B
Liver		
Protein (mg/g)	249 ± 7^A	232 ± 11^A
DNA (μg/g)	1799 ± 67^A	1775 ± 79^A
Lipid (g/100 g)	3.1 ± 0.2^A	8.5 ± 1.5^B
Cholesterol (mg/g)	1.73 ± 0.16^A	4.29 ± 0.87^B
Phospholipid (mg/g)	11.5 ± 2.4^A	13.6 ± 1.7^A
Triglycerides (mg/g)	12.0 ± 1.9^A	64.2 ± 5.5^B

[a]Means ± SEM for four animals per experimental diet. Horizontal means with unlike superscripts (A or B) differ $P < 0.05$.

TABLE II

Effect of Arginine Deficiency on the Percentage Liver Fatty Acids[a]

Fatty acid	Control	-Arginine
14:0	1.5 ± 0.2^A	3.7 ± 0.2^B
16:0	26.4 ± 0.1^A	24.8 ± 0.3^B
16:1	6.3 ± 1.9^A	3.7 ± 0.9^B
18:0	5.9 ± 0.4^A	15.7 ± 0.2^B
18:1	26.3 ± 2.3^A	21.0 ± 3.7^B
18:2	23.2 ± 1.4^A	16.2 ± 1.8^B
18:3	0.5 ± 0.1^A	0.2 ± 0.1^B
20:4	7.7 ± 1.5^A	13.0 ± 1.5^B

[a]Mean ± SEM for four animals per experimental diet. Horizontal means with unlike superscripts (A or B) differ $p < 0.05$. Minor percentages resulting from fatty acids less than 14 carbons or odd chains are not included.

heating with 25 ml of anhydrous methanol containing 1% concentrated H_2SO_4. After heating under reflux conditions for 4 hr, the reaction mixture was cooled to room temperature and transferred to a separatory funnel. An equal volume of distilled water and 25 ml of hexane was added. After shaking and extraction, the hexane layer was removed and the aqueous portion extracted with 25 ml hexane. The combined hexane extracts were then washed twice with equal volumes of water and dried over anhydrous sodium sulfate. Hexane was then removed to a constant volume (1-2 ml) in vacuo with a rotary evaporator and this was stored under N_2 at -10 C until analyzed.

Gas Chromatography

Methyl esters of fatty acids were analyzed by gas chromatography on a flame ionization detector gas chromatograph (HP 5830A) equipped with an electronic integrator. Separations were achieved with a 6 ft x 2 mm ID glass column packed with 10% SP-2340 coated on 100/120 mesh Supelcoport (Supelco, Inc., Bellefonte, PA) at a column temperature of 190 C; the detector and injector temperatures were 350 C and 240 C, respectively. The flow rate of carrier gas employed was 20 ml per minute. Quantitation and identification of the esters were achieved by comparison of retention times and amounts with standard mixtures of exters (Nu-Chek-Prep, Elysian, MN and Supelco, Inc.) on an electronic integrator.

RESULTS

Arginine deficiency results in an increase in the proportion of liver per kg of body weight (Table I). The increased liver size was not attributable to increased protein or DNA. However, the lipid composition of the arginine-deficient liver is dramatically altered. After 17 days of feeding an arginine-deficient diet, the liver lipid content increased ca. 280% above controls (Table I). Accompanying the increased total lipid was a similar increase in the triglyceride content. Although total phospholipids were similar, the percent of the total lipid present as phospholipids was significantly reduced by feeding an arginine-deficient diet (Table I). Liver cholesterol increased proportionally to the increased lipid content of the arginine-deficient livers.

Examination of the liver fatty acid composition revealed a significant reduction in the percentage of 16:0, 16:1, 18:1, and 18:2 fatty acids in livers obtained from arginine-deficient rats compared to controls (Table II). Arginine deficiency did significantly increase the percentage composition of liver 18:0 and 20:4 fatty acids compared to controls (Table II).

DISCUSSION

Arginine deficiency results in a dramatic increase in liver lipids. Previous reports have shown that arginine deficiency is consistently associated with an orotic aciduria (1-4). The fatty infiltration caused by arginine deficiency

is similar to that resulting from orotic acid feeding (9,10). Both cause a marked increase in liver triglycerides and cholesterol (this report, 9,10). Many of the alterations in liver fatty acid composition observed during arginine deficiency are also observed during orotic acid feeding (10). Both tend to cause an increase in the content of saturated fatty acids while decreasing in the content of unsaturated fatty acids. Increased arachidonic acid content observed during arginine deficiency has not been observed during orotate feeding.

The fatty accumulation accompanying arginine deficiency does not seem to be associated with liver toxicity as would be seen with carbon tetrachloride poisoning. Rats have been maintained on diets deficient in arginine for more than 35 days without any signs of necrosis despite lipid accumulations which may reach 20% of the wet weight of the liver (unpublished data).

Although arginine deficiency may cause a fatty liver by numerous mechanisms, no clear mechanism can be established from the present data. Clearly, increased orotic acid production by the arginine-deficient rat may be instrumental in causing fatty infiltration.

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Long and Medium Chain Triglycerides Increase Plasma Concentrations of Ketone Bodies in Suckling Rats

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ABSTRACT

The potential of medium chain triglyceride (MCT) and long chain triglyceride (LCT) as sources of plasma ketones was investigated in suckling rats. Initially high concentrations of plasma ketones in 6-, 10-, and 17-day-old rats increased 2- to 3-fold after acute feeding of MCT. This feeding had the same effect in fed or fasted adult rats. Corn oil (as a source of LCT) induced a large increase in the plasma ketone concentration of suckling rats and a relatively small but significant increase in fasted adult rats. The LCT treatment did not affect plasma ketone levels in fed adult rats. The results show clearly that feeding either LCT or MCT will enhance hyperketonemia in suckling rats. In the livers of all animals, regardless of age, the capacity for incorporation of [1-^{14}C]octanoate into CO_2 and acetoacetate far exceeded that for [1-^{14}C]palmitate. The hyperketonemic action of LCT in suckling rats was accompanied by an increased activity of carnitine palmityltransferase and increased level of carnitine.

INTRODUCTION

Mild ketosis occurs during the postnatal period in the rat (1-4) and in man (5). Although the physiological functions of ketone bodies (KB) are not completely understood, plasma KB can be oxidized for energy by extrahepatic tissues of developing rats (6-8). Further, we and others have recently demonstrated that KB serve as important precursors for lipid synthesis in the developing brain (8-10). Thus, moderately elevated levels of plasma ketones, as induced by dietary manipulation, might be beneficial to newborn development.

In an earlier study with fed adult rats, we showed that the plasma level of KB was increased dramatically by medium chain triglyceride (MCT) feeding but not by long chain triglyceride (LCT), e.g., corn oil feeding (11). The difference in ketogenicity between MCT and LCT stems from the regulation of fatty acid oxidation (11). The oxidation of long chain fatty acid depends on the activity of carnitine acyltransferase (12,13), whereas that of medium chain fatty acid is independent of the enzyme (14-16). Under fasting conditions leading to increased carnitine acyltransferase activity and carnitine levels (17,18), long chain fatty acid stimulated ketogenesis in perfused livers (17,19). Similarly, plasma levels of KB increased in fasted rats that had received long chian fatty acid by infusion (20).

These observations, together with previous studies showing that the activity of carnitine acyltransferase and the level of carnitine in the livers of developing rats were respectively 2- to 3- and 2- to 6-fold higher than values for adult rats (12,21,22), prompted us to investigate the potential of LCT and MCT as inducers of mild hyperketonemia in the developing rat. The results show that acute feeding of MCT as well as LCT provides a satisfactory means of elevating the plasma level of KB in developing rats.

MATERIALS AND METHODS

Animals

Sprague-Dawley rats were used throughout the experiments. Developing rats were the offspring of rats bred in this laboratory, as described previously (23), and were suckled by their dams at all times. Adult male rats (200-250 g), supplied by ARS/Sprague-Dawley, Inc., Madison, WI, were either fed Purina rat chow ad libitum or fasted for 24 hr. Before each experiment, either MCT, corn oil (as a source of LCT), or 0.9% NaCl solution, each at 1 ml/100 g body wt, was administered directly into the stomachs of suckling rats by use of an animal feeding-needle (20 G x 1.5 in.) obtained from Popper and Sons, Inc., New Hyde Park, NY. Equivalent concentrations of fats were force fed to adult rats through an infant stomach-feeding tube.

Analysis of Plasma Metabolites

At 2 hr after fat feeding, blood samples were drawn from the jugular veins of suckling rats and from the tail veins of adult rats and collected in heparinized tubes. Blood collected from adult rats before fat feeding served as the control. Plasma prepared by centrifugation was used for determination of ketone bodies (24). Glucose was measured by the glucose oxidase method, using Glucostat. Free fatty acids were

TABLE I

Effects of Medium and Long Chain Triglyceride Feeding on Plasma Concentrations of Metabolites in Developing and Adult Rats[a]

Treatment group	Ketones (μmol/ml)	Free fatty acids (μEq/ml)	Glucose (mg/100 ml)
6-day-old			
Saline	1.18 ± 0.04	0.79 ± 0.07	117 ± 7
MCT	3.78 ± 0.43[A]	2.08 ± 0.44[A]	134 ± 5
Corn oil	2.55 ± 0.12[A]	2.53 ± 0.34[A]	116 ± 6
10-day-old			
Saline	0.89 ± 0.16	0.92 ± 0.08	130 ± 5
MCT	2.59 ± 0.63[A]	1.52 ± 0.16[A]	156 ± 21
Corn oil	1.58 ± 0.17[A]	2.73 ± 0.34[A]	128 ± 7
17-day-old			
Saline	0.86 ± 0.18	0.81 ± 0.11	136 ± 5
MCT	3.76 ± 0.89[A]	1.35 ± 0.12[A]	142 ± 13
Corn oil	2.17 ± 0.14[A]	2.15 ± 0.28[A]	125 ± 6
Adult fed			
None	0.22 ± 0.03 (8)	0.38 ± 0.02 (8)	142 ± 3 (8)
MCT	8.40 ± 0.48[A]	0.77 ± 0.09[A]	105 ± 5[A]
Corn oil	0.40 ± 0.08	1.41 ± 0.14[A]	132 ± 4
Adult fasted			
None	1.29 ± 0.09 (8)	1.82 ± 0.14 (8)	116 ± 5 (8)
MCT	7.33 ± 1.17[A]	0.71 ± 0.13[A]	69 ± 4[A]
Corn oil	1.67 ± 0.08[A]	2.57 ± 0.09[A]	112 ± 2

[a]Rats were fed madium chain triglyceride (MCT), corn oil, or saline (1 ml/100 g body wt) 2 hr before blood samples were obtained. The body weights of 6-, 10-, 17-day-old, adult fed, and adult fasted rats were 13.6 ± 0.2, 18.2 ± 0.4, 29.6 ± 0.8, 234 ± 8, and 203 ± 3 g, respectively. All values represent means ± SEM for four rats unless otherwise indicated in parentheses. [A]Denotes significant difference ($P < 0.05$) from saline-treated or untreated rats.

determined by the titration procedure of Ko and Royer (25).

Ketone Synthesis

Liver slices (80-200 mg) prepared with a Stadie-Riggs hand microtome immediately after rats had been decapitated at 2 hr after fat feeding, were used for studies of ketogenic and oxidative capacities. The slices were incubated with [1-^{14}C]octanoate or [1-^{14}C]palmitate. The rates of [^{14}C]acetoacetate synthesis and $^{14}CO_2$ production from [^{14}C]fatty acids were determined by previously described methods (23).

Liver Enzyme and Carnitine

Liver mitochondria, prepared according to the procedure of Foster and Bailey (21), were frozen and thawed to determine the total activity of carnitine palmityltransferase (palmityl CoA:L-carnitine-o-palmityltransferase, EC 2.3.1.23). The enzyme activity was assayed by the method of Hoppel and Tomec (26) ysing the extraction procedure of Bremer (27). For measurement of tissue total carnitine, livers were homogenized in cold H_2O by the method of Borum and Broquist (28). Carnitine concentration was then assayed by the radioisotopic method of Cederblad and Lindstedt (29).

Chemicals

[1-^{14}C]Palmitate, [1-^{14}C]octanoate, and [1-^{14}C]acetyl CoA were purchased from New England Nuclear, Boston, MA, and DL-[methyl-^{14}C]carnitine from Amersham/Searle, Arlington Heights, IL. Medium chain triglyceride, containing $C_{8:0}$, 58%; $C_{10:0}$, 20%; and $C_{12:0}$, 1%, was obtained from Mead Johnson, Evansville, In. Corn oil, containing $C_{16:0}$, 12.5%; $C_{18:0}$, 2.5%; $C_{18:1}$, 29.0%; $C_{18:2}$, 55.0%; and $C_{18:3}$, 0.5%, was the product of ICN Nutritional Biochemicals, Cleveland, OH. β-Hydroxybutyrate dehydrogenase and carnitine acetyltransferase used for metabolite assays were obtained from Mannheim and Boehringer Biochemicals, Indianapolis, IN. Acetyl CoA and palmityl CoA were purchased from PL Biochemicals, Inc., Milwaukee, WI. Glucostat was provided by Worthington Biochemical Corp., Freehold, NJ. Other chemicals were obtained from Sigma Chemicals Co., St. Louis, MO.

Statistical Analysis

The data were analyzed for statistical significance by Student's t-test (30).

TABLE II

Incorporation of [1-^{14}C]Palmitate and [1-^{14}C]Octanoate into Acetoacetate and CO_2 in Liver Slices in Developing and Adult Rats[a]

	[1-^{14}C]Palmitate		[1-^{14}C]Octanoate	
	CO_2	Acetoacetate	CO_2	Acetoacetate
	(nano carbon atom incorporated/2 hr per 100 mg of liver)			
Developing rats				
4-day-old	250 ± 18^{A,B}	614 ± 57^{A,B}	1414 ± 109^{A,B}	1506 ± 70^{A,B}
10-day-old	148 ± 11^{A}	402 ± 66^{A,B}	443 ± 56^{B}	1433 ± 131^{B}
16-day-old	143 ± 4^{A,B}	164 ± 16^{A}	345 ± 22^{B}	644 ± 84^{B}
Adult rats				
Fed	84 ± 6	49 ± 6	354 ± 24	963 ± 146
Fasted	175 ± 9^{A}	209 ± 27^{A}	673 ± 61^{A}	2010 ± 58^{A}

[a]Liver slices were incubated in 2 ml of Krebs-Ringer phosphate buffer containing [1-^{14}C]palmitate (0.27 μCi radioactivity and 400 nmoles of palmitate) or [1-^{14}C]octanoate (0.27 μCi radioactivity and 800 nmoles of octanoate). The incubation medium was supplemented with 0.28% bovine serum albumin. The data are expressed as means ± SEM for five developing and four adult rats. ^{A,B}Denote significant differences from adult fed and adult fasted rats, respectively, at $P < 0.05$.

RESULTS

Changes in Plasma Concentrations of Metabolites

In agreement with previous studies (1-4), plasma concentrations of ketones (sum of β-hydroxybutyrate and acetoacetate) in 6- to 17-day-old rats were 3- to 4-fold higher than corresponding values for adult rats fed ad libitum (Table I). These initially high ketone levels in developing rats were further increased by 2.2-, 1.9- and 3.4-fold, at 2 hr after MCT feeding. Medium chain triglyceride had a much more pronounced effect when administered to fed or fasted adult rats: respective concentrations of plasma ketones 2 hr after treatment were 37.1- and 4.7-fold higher than pretreatment values.

In contrast to MCT, corn oil induced an increase of plasma ketone levels in suckling but not adult fed rats. Values for suckling rats had increased from 80 to 150% at 2 hr after corn oil was administered. When adult rats were deprived of food for 24 hr, corn oil feeding caused a slight but significant increase in plasma concentrations of ketones. In a related experiment, we found that if MCT or corn oil was administered 1 hr before blood sampling, only MCT increased plasma concentrations in either suckling rats or adult fasted rats (data not shown).

Administration of fats, corn oil in particular, caused plasma concentrations of free fatty acids (FFA) to increase in both suckling and adult fed rats. Although corn oil feeding increased initially high levels of FFA in adult fasted rats, MCT feeding produced the opposite effect. As demonstrated previously (11), lower levels of FFA in MCT compared to corn oil-fed rats are in part attributable to the method (25) used for determining long and medium chain fatty acids derived from the two triglycerides. Although this method is sensitive to long chain fatty acids, it detects only 66-77% of medium chain-fatty acids (25). It should be stressed, however, that changes in circulating fatty acids were not always correlated to changes in plasma concentrations of KB.

Plasma concentrations of glucose in suckling rats were not affected by feeding either MCT or corn oil, whereas in adult rats fed ad libitum or fasted the concentrations decreased after MCT feeding but remained unchanged after corn oil treatment.

Hepatic Capacities for Ketogenesis from Medium and Long Chain Fatty Acids

We have established that, in vitro, acetoacetate (AcAc) accounts for more than 85% of the total ketone bodies (β-hydroxybutyrate plus AcAc) produced and that [^{14}C]AcAc synthesized from ^{14}C-labeled fatty acid reflects the hepatic capacity for ketone synthesis (23). To determine the rates of ketogenesis and oxidation of medium and long chain fatty acids, we measured the incorporation of [1-^{14}C]octanoate and [1-^{14}C]palmitate into [^{14}C]AcAc and $^{14}CO_2$ in liver slices. Similar to the rates of ketogenesis reported previously (4), the hepatic capacities for [^{14}C]AcAc synthesis from [1-^{14}C]palmitate were 2.4- to 11.5-fold higher in suckling than in adult fed rats (Table II). Synthetic capacities were highest in 4-day-old pups and decreased with age. After 24 hr of fasting, the ketogenic capacity utilizing [1-^{14}C]palmitate was sharply stimulated in the adult rat. Coincident with ketogenesis, the oxidation of [1-^{14}C]palmitate through Krebs cycle was higher in suckling and adult fasted rats than in fed adults. An estimated 53 to 73%

TABLE III

Specific Activity of Carnitine Palmityltransferase and Concentration of Carnitine in the Livers of Developing and Adult Rats[a]

Group	Carnitine palmityltransferase (nmoles/min per mg protein[b])	Carnitine (nmoles/g liver)
Developing rats		
5-day-old	8.27 ± 0.14 (4)^{A,B}	904 ± 24 (4)^{A,B}
10-day-old	9.95 ± 0.61 (4)^{A,B}	652 ± 18 (4)^{A,B}
15-day-old	5.94 ± 0.25 (4)^{A,B}	635 ± 15 (5)^{A,B}
Adult rats		
Fed	3.97 ± 0.07 (4)	184 ± 17 (5)
Fasted	4.86 ± 0.17 (4)^{A}	361 ± 13 (8)^{A}

[a]Values are means ± SEM for number of rats indicated in parentheses.
[b]The enzyme activity is expressed on the basis of mitochondrial protein. ^{A,B}Denote significant differences from adult fed and adult fasted, respectively, at $P < 0.01$.

of total [1-^{14}C]palmitate oxidized was converted to [^{14}C]AcAc in suckling rats, compared with 36% in adult fed rats.

The capacities for oxidation and ketogenesis from [1-^{14}C]octanoate in the liver of adult fed rats were similar to the oxidative and ketogenic capacities in pups. In 4-day-old pups and adult fasted rats, however, the rates of both $^{14}CO_2$ and [^{14}C]AcAc production were higher than in adult fed rats. A greater proportion of [1-^{14}C]octanoate was partitioned into [^{14}C]AcAc than into $^{14}CO_2$. The ratios of [1-^{14}C]octanoate incorporated into [^{14}C]AcAc/$^{14}CO_2$ ranged from 2:1 to 3:1 in 10-day-old, 16-day-old, and adult rats, either fed or fasted. In all age groups, capacities for ketogenesis and CO_2 production from [1-^{14}C]octanoate were 1.5- to 19-fold higher than corresponding capacities utilizing [1-^{14}C]palmitate.

Carnitine Palmityltransferase Activity and Carnitine Level

Carnitine palmityltransferase was assayed in frozen-thawed mitochondria of the liver. The assay measured total activity of carnitine palmityltransferase I and II of the inner mitochondrial membrane. The activities of the enzyme in 5-, 10-, and 15-day-old suckling rats were 2.0-, 2.5-, and 1.5-fold, respectively, of values for adult fed rats (Table III). The activity of adult rats increased by 20% after 24 hr of fasting. The high activities of the transferase observed in suckling and adult fasted rats were accompanied by increased levels of carnitine in the liver. However, the difference in carnitine levels between suckling and adult fed rats was greater than the difference in carnitine palmityltransferase activities.

DISCUSSION

The ketogenicity of MCT in adult laboratory animals and man is well established (11,31-35). Described here are experiments in which the triglyceride effectively induced increases in plasma concentrations of ketones in suckling rats. Although MCT-fed suckling rats had elevated concentrations of plasma FFA, the increase in fatty acid flux cannot by itself account for enhanced hyperketonemia (3,11,23,36). This is clearly indicated by the obersvation that despite an increase in FFA, plasma concentration of ketones was not altered in adult fed rats treated with corn oil (Table I). As in adult rats (11), the hyperketonemic effect of MCT in suckling rats results in part from rapid oxidation of medium chain fatty acids and a consequent increase in the incorporation of acetyl CoA into ketones (Table II). The accelerated rate of oxidation of medium chain fatty acids can be attributed to activation of medium chain fatty acids to form acyl CoA in mitochondria (15). Lee and Fritz have shown that the rate of ketone production from octanoylcarnitine was higher than that from octanoate in adult and fetal livers (37). This observation suggests a possible role of carnitine medium chain acyltransferase in fatty acid oxidation. In fact, the activity of carnitine octanoyltransferase has been demonstrated in the rat liver (19,38). Further, Foster and Bailey observed that the activity of carnitine decanoyltransferase was higher in the liver of suckling rats than adult rats (21). However, because a similar rate of ketogenesis from ^{14}C-octanoate was obtained in 10- and 17-day-old and adult fed rats (Table II), it is unlikely that carnitine medium chain acyltransferase regulates the oxidation of medium chain fatty acids.

It is interesting to note that while plasma concentration of KB of adult fasted rats was dramatically elevated by MCT-feeding, plasma concentrations of FFA decreased. The reason for suppresion of FFA by MCT is obscure. Since MCT and ketones are known to simulate

insulin secretion (11,39), it is conceivable that MCT feeding inhibits fasting-induced lipolysis and lowers plasma FFA by increasing insulin.

Ketogenesis from long chain fatty acids is determined by the rate of fatty acid transport into mitochondria (40). This transport process is regulated by the activity of carnitine long chain acyltransferase (12,13) and carnitine level (22). Earlier studies by others have clearly shown that an increased capacity to synthesize ketones from long chain fatty acids was accompanied by an increased activity of carnitine long chain acyltransferase (18). Because of the high activity of the transferase (Table III), it is not surprising that LCT feeding produced hyperketonemia in suckling but not in adult fed rats (Table I). The ketogenicity of LCT was not only related to increased transferase activity but was closely associated with elevated levels of carnitine in the liver (Table III). However, our data were not adequate for assessing the relative role of the transferase activity and carnitine level in the induction of hyperketonemia. It is important to note that the quantitative difference in ketone production in adult-fed vs. suckling rats was greater than the difference in carnitine level, which in turn was greater than the difference in transferase activity. These relationships suggest that ketogenesis depends more on carnitine level than on the transferase activity. In this respect, McGarry and his collaborators (22,41) have recently proposed that an elevated level of carnitine is one of the major prerequisites for the development of newborn ketosis.

The developmental changes in plasma concentrations of ketones were closely related to changes in the hepatic capacities to synthesize ketones from long chain but not medium chain fatty acids (Tables I and II). When compared with 10- to 17-day-old pups, the adult fed rat had lower concentration of plasma ketones which was accompanied by a decreased rate of ketogenesis from ^{14}C-palmitate and by an unchanged rate of the synthesis from ^{14}C-octanoate. However, our results clearly demonstrate that both MCT and LCT are effective in increasing plasma ketones in suckling rats. Although the liver can more readily synthesize ketones from MCT than from LCT, the latter triglyceride is physiologically more important since fatty acids available for ketogenesis during the suckling period are derived from milk triglyceride, which, in most species except rabbit, consists mainly of long chain fatty acids (42). On the other hand, MCT provides a more versatile ketone precursor, one that would not be affected by nutritional conditions such as deficiency of carnitine or carnitine acyltransferase and lipid malabsorption.

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COMMUNICATIONS

Effects of Synthetic and Natural Lysophosphatidic Acids on the Arterial Blood Pressure of Different Animal Species

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ABSTRACT

Intravenous injection of lysophosphatidic acid was found to cause hypertension in rats and guinea pigs, but hypotension in cats and rabbits. The potencies of the pressor and depressor effects of synthetic lysophosphatidic acids in rats and cats depended on their chain length and the degree of unsaturation of their fatty acyl moieties.

INTRODUCTION

We previously observed a transient pressor response after intravenous injection of crude soybean lecithin into rats (1). The substance with pressor activity in the preparation was isolated and identified as lysophosphatidic acid (1-acyl-*sn*-glycero-3-phosphate) (2). It contained a high percentage of unsaturated fatty acids and about five times more pressor activity than synthetic 1-palmitoyl-*sn*-glycero-3-phosphate, suggesting that the fatty acid moiety in these molecules greatly influenced their pressor activity. Therefore, we examined nine lysophosphatidic acids for their effects on the systemic arterial blood pressure when injected intravenously into various experimental animals.

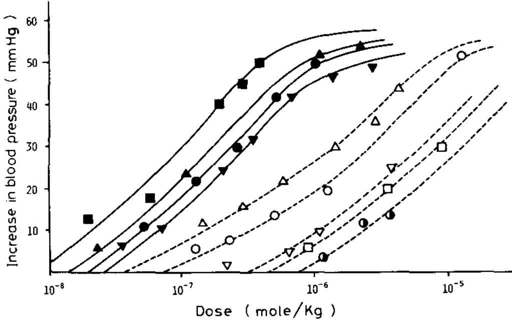

FIG. 1. Dose-response curves for the effects of synthetic LPA on the systemic arterial blood pressure of rats. Values are means of three observations. - - - ◑ - - - Decanoyl-LPA (10:0), - - - □ - - - Lauroyl-LPA (12:0), - - - ○ - - - Myristoyl-LPA (14:0), - - - △ - - - Palmitoyl-LPA (16:0), - - - ▽ - - - Stearoyl-LPA (18:0), —●— Oleoyl-LPA (18:1), —▼— Linoleoyl-LPA (18:2), —■— Linolenoyl-LPA (18:3), —▲— Arachidonyl-LPA (20:4).

MATERIALS AND METHODS

Lysophosphatidic acid (LPA) from crude soybean lecithin (Nakarai Kagaku, Kyoto, Japan) was prepared as described previously (1,2). Crude soybean lecithin was treated with cold acetone, and acetone-insoluble materials were chromatographed on a silicic acid column with chloroform-methanol mixtures increasing the polarity. The active fraction eluted with chloroform-methanol (4:6) was further purified by chromatography on Sephadex LH-20 with chloroform-methanol (1:1) and rechromatography with chloroform-methanol-water (60:35:8). Its fatty acid composition was as follows: palmitic acid, 24.7%; stearic acid, 7.5%; oleic acid, 8.7%; linoleic acid, 50.0%; and linolenic acid, 7.0%. Synthetic 1-palmitoyl-, 1-decanoyl-, and 1-oleoyl-LPA were purchased from Serdary Research Laboratories (London, Ontario, Canada) and purified by Sephadex LH-20 column chromatography with chloroform-methanol (1:1, v/v). The purified preparations migrated as single compounds by chromatography on silica gel plates (Merck, Silica gel plate 60, 250 μm-thick) using chloroform-methanol-water (60:35:5, v/v/v) or chloroform-methanol-28% ammonia (65:35:5, v/v/v) as developing solvents. Lauroyl-, myristoyl-, stearoyl-, linoleoyl-, linolenoyl-, and arachidonyl-LPA were prepared by the method of Long et al. (3,4) from the corresponding 1-acyl-*sn*-glycero-3-phosphocholines (Sigma Chemical Co., St. Louis, MO, and Serdary Research Laboratories) by hydrolysis with phospholipase D (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4: Sigma). 1-Acyl-*sn*-glycero-3-phosphocholine (25 mg) was sus-

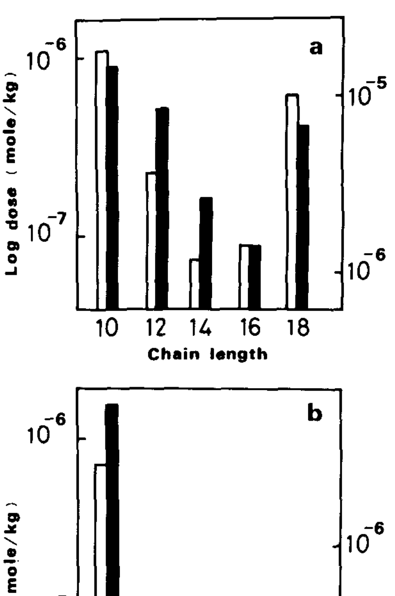

FIG. 2. The dependence of vasoactivity on the acyl chain length (a) and unsaturation of acyl chain (b) for lysophosphatidic acids. (a) Numbers represent the acyl chain length of saturated LPAs. (b) Numbers (0,1,2,3) represent degree of unsaturation of C_{18} acyl chain in LPAs. S: LPA from soybean lecithin, 4: arachidonyl-LPA. White bar shows the mean dose of LPA producing a half maximum fall in blood pressure of cats (left ordinates). Black bar shows the mean dose producing a half maximum rise in blood pressure of rats (right ordinates).

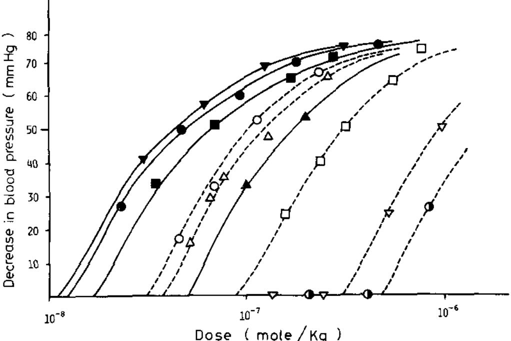

FIG. 3. Dose-response curves for the effects of synthetic LPA on the systemic arterial blood pressure of cats. Values are means of two observations. Symbols are the same as those in Figure 1.

pended in 1.2 ml of water. Acetate buffer, pH 5.8 (0.2 M; 3.1 ml) and 1 M $CaCl_2$ (0.75 ml) were added, followed by 1.25 ml of enzyme solution (25 mg). The reaction mixture was incubated at 38 C for 24 hr. The products were extracted with a chloroform-methanol mixture from the reaction solution adjusted to pH 3 with 1 M citrate. Lysophosphatidic acid was purified by column chromatography on Sephadex LH-20 with chloroform-methanol (1:1) to remove a by-product (cyclic phosphatidic acid). The purified fractions appeared homogeneous on thin layer plates coated with silica gel and developed with the two solvent systems described above. The purified LPA preparations were assayed biologically as reported in detail previously (1). In brief, systemic arterial blood pressure was measured manometrically via a cannula inserted into the carotid artery. The test materials as suspension in 200 μl of saline were injected from a microsyringe into the cannulated jugular veins of the anesthetized rats (200-250 g, urethane; 1.8 g/kg, i.p.) and guinea pigs (350-400 g, sodium pentobarbital; 40 mg/kg, i.p.), and femoral veins of the anesthetized male cats (2.8-3.2 kg, sodium pentobarbital; 40 mg/kg, i.p.) and rabbits (3.0-3.5 kg, urethane; 1.5 g/kg, i.p.).

RESULTS AND DISCUSSION

In 1960, McQuarrie and Anderson (5) detected a substance in commercial soybean lecithin with depressor activity on cats, but they did not examine it further either chemically or pharmacologically. Lysophosphatidic acid isolated from soybean lecithin was found to be hypertensive to rats and guinea pigs but hypotensive to cats and rabbits. This species specificity is the same as that of the F-series of prostaglandins (6-8) but different from that of histamine (9,10) which is hypertensive to rabbits and guinea pigs but hypotensive to cats and dogs. The present findings on the species specificity of LPA suggest that their depressor substance may have been LPA.

Cats were the most sensitive of the animals tested to isolated LPA (threshold doses, 2-3 μg/kg), rabbits being much less sensitive (threshold doses, 400-600 μg/kg. Rats were about three times more sensitive than guinea pigs, the threshold doses of LPA for the two being 10-15 μg/kg and 30-40 μg/kg, respectively. These values were in the same order as those of prostaglandin $F_{2\alpha}$ except those for rabbits. The qualitative and quantitative similarity in the vasoactive effects of LPA and prostaglandin $F_{2\alpha}$ may be indicative of similar mechanisms of

action of LPA.

Nine molecular species of LPA were assayed on cats and rats, which were used as representatives of animals showing depressor and pressor reactions, respectively. Figure 1 shows the log dose-pressor response relationships of these nine compounds on rats. The curves were similar in shape and horizontally parallel. Palmitoyl-LPA was the most active of the saturated LPA. The activity decreased progressively with increase or decrease from 16 in the fatty acid hydrocarbon chain length. Incorporation of *cis*-double bonds into the hydrocarbon chain of LPA resulted in increase in the activity in the order, 18:3>18:2=18:1>16:0≫18:0 (Fig. 2). Arachidonyl-LPA had a slightly higher pressor activity than did oleoyl-LPA.

As shown in Figure 3, similar log dose-depressor response relationships of LPA preparations were observed in cats, but myristoyl-LPA was slightly more active than palmitoyl-LPA. The most active molecular species was linoleoyl-LPA, and arachidonyl-LPA had low depressor activity relative to its pressor activity in rats (Fig. 2).

In the previous paper (1), we suggested from data obtained with different pharmacological blockers that LPA might exert a direct effect on cell membranes of cardiovascular smooth muscles, but not influence afferent and efferent sympathetic nervous systems. The present results suggest that certain limited hydrophobic regions in the molecules are required for acute, potent vasoactive effects. The presence of at least one *cis* double bond in the hydrocarbon chain resulted in high pressor and depressor activites, possibly by the optimal stereospecific interactions with some receptor sites. The requirement for hydrophobic regions may reflect the important hydrophobic interactions of LPA with the cell membranes of smooth muscles, causing activation of intracellular contraction systems.

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Isolation and Characterization of the Monounsaturated Long Chain Fatty Acids of *Mycobacterium tuberculosis*

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ABSTRACT

The positions of double bond in the monounsaturated C_{15}-C_{32} fatty acids of *Mycobacterium tuberculosis* H37Ra were established by gas chromatography/mass spectrometry of the ozonized esters and their pyrrolidide derivatives. The monounsaturated C_{15}-C_{21} fatty acids had the double bond primarily at the Δ^9 position while the monounsaturated longer chain fatty acids (C_{22}-C_{32}) had the double bond in several positions. Many of the latter acids, especially the odd-numbered series, were very complex isomeric mixtures. Quantitation showed the most abundant even-numbered long chain fatty acid isomers to be as follows: C_{22}, Δ^4; C_{24}, Δ^5; C_{26}, Δ^7 and Δ^9; C_{28}, Δ^9; C_{30}, Δ^{11} and Δ^{13}; C_{32}, Δ^{13} and Δ^{15}.

INTRODUCTION

Mycobacterium tuberculosis H37Ra was shown to contain C_{27}-C_{56} fatty acids (1). The synthesis of these long chain fatty acids was found to be isoniazid sensitive just as the synthesis of mycolic acids was drug sensitive (1,2). These observations suggested that the long chain fatty acids and mycolic acids might be on the same biosynthetic pathway. Moreover, the long chain fatty acids could be precursors of mycolic acids. As the first step in our efforts to elucidate the pathway to the synthesis of mycolic acids, we have now begun to isolate and characterize these long chain fatty acids. From these determinations, we hope to establish the structural relationship between these acids and mycolic acids. In this communication, we report on the structural determination of the monounsaturated C_{15}-C_{32} fatty acids in *M. tuberculosis* H37Ra.

Literature review showed that the monounsaturated fatty acids greater than C_{26} had not been studied in detail in mycobacteria. Hung and Walker (3) studied the monounsaturated C_{18}-C_{26} fatty acids of both *Mycobacterium smegmatis* and *Mycobacterium bovis* BCG. Asselineau et al. (4) isolated a series of monounsaturated fatty acids from *Mycobacterium phlei* with the size range of C_{20}-C_{27}. In these studies, no thorough qualitative or quantitative determinations of the fatty acid isomers were made. We have now isolated and characterized the monounsaturated C_{15}-C_{32} fatty acids of the H37Ra strain. The results showed an interesting and complex pattern of isomeric distribution for the monounsaturated C_{25}-C_{32} fatty acids.

PROCEDURES

Chemicals

Pyrrolidine was obtained from Aldrich Chemical Co., Inc., Milwaukee, WI. Standard normal (C_{14}, C_{16}, C_{18}, C_{20}, C_{22}, C_{24}, C_{26}, C_{28}, and C_{30}) and monoenoic (C_{16} and C_{18}) fatty acid methyl esters were obtained from Analabs, Inc., North Haven, CT. All other chemicals used were reagent grade.

Growth of Bacteria

The H37Ra strain of *M. tuberculosis* was grown at 37 C in glycerol-alanine-salts medium (medium A) (1) in a New Brunswick Microferm 28-1 fermentor, New Brunswick Scientific Co., Inc., Edison, NJ.

Isolation of Methyl Esters of Long Chain Fatty Acids from *M. tuberculosis*

The procedure used in the preparation of the various methyl esters (including Fraction D) is described in a previous publication (1). Fraction D (a nonmycolate ester fraction) was prepared from 300 g of harvested cells of *M. tuberculosis* H37Ra grown in medium A. Two cycles of Sephadex LH-20 column (2 x 144 cm) chromatography of Fraction D with chloroform-methanol (2:1, v/v) yielded a methyl ester fraction (350 mg) that was enriched with respect to the C_{26} chain length. Gas liquid chromatography (GLC) analysis showed the range of esters to be C_{20}-C_{36}. This fraction was applied to a 3 x 18 cm silicic acid-$AgNO_3$ column (Hi-Flosil-Ag, Applied Science Laboratories, Inc., State College, PA), and the saturated and unsaturated esters were separated (1) yielding 263 mg of saturated esters and 53 mg of unsaturated esters. GLC showed the chain

length range to be C_{20}-C_{34} for the unsaturated esters. These monounsaturated esters (5 mg) were fractionated by preparative GLC on a 10% SE-30 column at a load of 1 mg per injection. Each fraction was analyzed by GLC and shown to be pure. The samples were kept under N_2 before and after fractionation.

Isolation of Unsaturated C_{15}-C_{21} Methyl Esters from *M. tuberculosis*

A methyl ester fraction obtained from the Sephadex LH-20 column chromatography (two cycles) of Fraction D (1) and enriched with respect to C_{15}-C_{19} chain length (about 500 mg) was further fractionated on a silicic acid-$AgNO_3$ column as previously described and provided 70 mg of unsaturated esters. GLC of the unsaturated esters revealed the actual range to be C_{15}-C_{21}, and this preparation was used to determine the position of the double bond.

Analytical Procedures

Fatty acids were methylated with diazomethane. The pyrrolidide derivatives of the fatty acids were prepared by reacting the methyl esters with pyrrolidine in the presence of acetic acid at 100 C for 30 min as described by Andersson and Holman (5). Fourier transform nuclear magnetic resonance spectra were determined with a Bruker model HX-90E spectrometer at 90 MHz.

Both preparative and analytical GLC of the methyl esters were carried out on a Packard model 419 gas chromatograph with an all-glass 1.83 m x 4 mm column containing either 3% or 10% SE-30 on (80-100 mesh) Supelcoport (Supelco, Inc., Bellefonte, PA). The injection temperature was 260 C, and the thermal conductivity detector temperature was 310 C. The column temperature was programmed at 5 C/min from 180-305 C, and the flow rate was 60 ml helium/min.

Ozonolysis of the individually fractionated C_{22}-C_{32} monounsaturated esters and the pyrrolidide derivatives of C_{15}-C_{21} monounsaturated acid mixture was carried out by dissolving the samples in 50-100 μl of methylene chloride, to which 1 μl of pyridine was added. The samples were chilled in a dry ice-acetone bath and ozonized with a Micro-ozonizer (Supelco, Inc.) for 80 sec. A stream of N_2 was bubbled through the solution. The resulting aldehyde and ester products were analyzed by GC/MS. The mass spectra for these samples were obtained with Dupont 21-491B mass spectrometer operated at 20 or 70 eV interfaced with a computer (A.E.I. DS-50 system). The gas chromatograph (Varian Aerograph series 2700) was equipped with an all-glass 1.83 m x 2 mm column containing 3% OV-1 on (100-200 mesh) Varaport 30 programmed at 10 C/min from 40-310 C with a flow rate of 23 ml helium/min and was interfaced to the mass spectrometer via a glass transfer line and single-stage jet separator. The fatty aldehyde content in the ozonized sample was quantitated by GLC with a flame ionization detector.

RESULTS

Preliminary Structural Analysis of the Unsaturated Methyl Esters of C_{20}-C_{34} Fatty Acids

Nuclear magnetic resonance analysis of the unsaturated C_{20}-C_{34} ester mixture showed the presence of olefinic protons (5.28, 5.34, and 5.39 ppm). The unsaturated methyl esters were purified by preparative GLC, and mass spectrometry (MS) suggested that each fraction contained one double bond.

Positional Analysis of C_{15}-C_{21} Monounsaturated Esters by the Pyrrolidide MS Method

GC/MS analysis of the pyrrolidide derivatives of the monounsaturated short chain esters indicated the position of unsaturation in the fatty acids. The fatty amides produced were shown to contain the unsaturation predominantly at the Δ^9 position by the Andersson-Holman rule (5) and this was confirmed by GC/MS analysis of the ozonized pyrrolidides.

Positional Analysis of C_{22}-C_{32} Monounsaturated Esters by the Ozonolysis Method

The C_{22}-C_{32} monounsaturated esters were fractionated by GLC and analyzed by the ozonolysis method using GC/MS to determine the positions and distribution of the double bond. The products of the ozonolysis reaction were found to be complex mixtures of many aldehydes and esters. Figure 1 shows a computer-generated GLC tracing of the ozonized C_{27} ester. The upper tracing (a) gives the total ion current from the mass spectrometer, whereas the lower tracing (b) gives the m/e 87 ion current which is characteristic of esters. Peaks C_7-C_{12} were identified as the aldehyde ester fragments, while peaks C_{15}-C_{22} were the aldehyde fragments. Peak C_{27} represented the unreacted ester. Minor peaks appearing near peaks C_9, C_{10}, C_{18}, C_{19}, and C_{20} were not identified. An example of the GC/MS identification of a pair of fragments from the C_{27} ester is shown in Figure 2A for the C_8 aldehyde ester and Figure 2B for the C_{19} aldehyde. Peak C_8 showed the characteristic m/e

74, m/e 87, and M-31 fragments for methyl esters (6). Peak C_{19} showed the characteristic m/e 68, m/e 82, m/e 92, M-46, M-44, and M-18 fragments for fatty aldehydes (7). Such a fragment pair indicates the presence of the Δ^8-$C_{27:1}$ isomer. Other peaks were similarly paired (aldehyde ester-aldehyde) for the isomers: C_7-C_{20} for Δ^7-$C_{27:1}$; C_9-C_{18} for Δ^9-$C_{27:1}$; C_{10}-C_{17} for Δ^{10}-$C_{27:1}$; C_{11}-C_{16} for Δ^{11}-$C_{27:1}$; C_{12}-C_{15} for Δ^{12}-$C_{27:1}$. The corresponding esters for the C_{21} and C_{22} aldehydes were not detected by GLC probably due to the small sample size and high volatility. The peaks for the aldehyde ester-aldehyde pair of C_{12}-C_{15} overlapped on GLC. Quantitation of the relative abundance of these isomers was done by GLC of the fatty aldehyde products, and the results are shown in Table I. The relative mass distribution of the unsaturated acids is also given in this table. Isomeric mixtures were relatively simple for C_{22}-C_{24} esters. In general, the odd-numbered esters showed more complex isomeric mixtures than the even-numbered esters with the C_{27} ester being the most complex since eight isomers were detected. However, the C_{26} ester which contained four prominent isomers of Δ^5-, Δ^7-, Δ^9-, and Δ^{10}-$C_{26:1}$ was also a complex mixture. In the even-numbered series, the most abundant isomers were (in order of increasing chain length Δ^4-$C_{22:1}$, Δ^5-$C_{24:1}$, Δ^7-$C_{26:1}$, Δ^9-$C_{28:1}$, Δ^{11}-$C_{30:1}$, and Δ^{13}-$C_{32:1}$.

DISCUSSION

To our knowledge, this is the first successful attempt to quantitate the isomeric abundance of such a complex mixture of monounsaturated C_{15}-C_{32} fatty acids in mycobacteria. The results revealed a definite pattern of isomeric distribution. The most abundant even-numbered long chain fatty acid isomers were C_{22}, Δ^4; C_{24}, Δ^5; C_{26}, Δ^7 and Δ^9; C_{28}, Δ^9; C_{30}, Δ^{11} and Δ^{13}; C_{32}, Δ^{13} and Δ^{15}. A specific desaturase system appears to exist which introduces a double bond at the Δ^9 position of the saturated C_{15}-C_{21} fatty acids. Since the corresponding elongation products are not present, these monounsaturated fatty acids do not appear to elongate in *M. tuberculosis*. The pattern of isomeric distribution as shown in Table I suggests the existence of a desaturase system which introduces a double bond into the Δ^5 position of a C_{24} fatty acid. The Δ^5-$C_{24:1}$ fatty acid product could elongate to form the corresponding Δ^{13}-$C_{32:1}$ acid. These unsaturated fatty acids may be precursors of mycolic acids. The position of the double bond in these monounsaturated C_{24}-C_{32} fatty acids

TABLE I

Relative Distribution and Isomeric Abundance of Monounsaturated Long Chain Fatty Acids of *Mycobacterium tuberculosis*

Mono-unsaturated fatty acid	Relative mass distribution	Relative mass abundance of isomer[a]												
		Δ^3	Δ^4	Δ^5	Δ^6	Δ^7	Δ^8	Δ^9	Δ^{10}	Δ^{11}	Δ^{12}	Δ^{13}	Δ^{14}	Δ^{15}
C_{22}	5	8.8	100	6.3	...	...	...	...	...	...	...	...	...	...
C_{24}	20	...	...	100	6.8	5.8	3.7	...	...	...	...	...	...	...
C_{26}	100	...	...	23.6	0.9	100	9.1	77.3	16.4	(1)[b]	9.0	...	...	...
C_{28}	78	...	...	...	...	13.8	2.4	100	7.4	15.1	11.0	...	...	...
C_{30}	22	...	...	...	...	...	...	7.4	trace	100	9.5	47.4	...	...
C_{32}	18	...	...	...	...	...	...	...	...	9.6	1.0	100	trace	61.3
C_{23}	3	...	28.7	100	19.5	3.7	...	...	...	...	...	...	...	...
C_{25}	8	...	...	100	70.2	68.9	26.7	23.6	1.9	...	...	...	...	...
C_{27}	35	...	...	3.7	3.7	32.2	54.8	100	38.9	8.9	(3)[b]	...	...	...
C_{29}	9	...	...	...	...	...	3.9	70.3	100	69.5	65.5	(10)[b]	25.0	...
C_{31}	5	...	...	...	...	...	...	...	8.8	62.5	100	96.3	66.3	73.8

[a] Quantitated by gas chromatography of the aldehyde fragment after ozonolysis of the purified ester.
[b] An approximation due to overlapping of the aldehyde and ester peaks on gas chromatography.

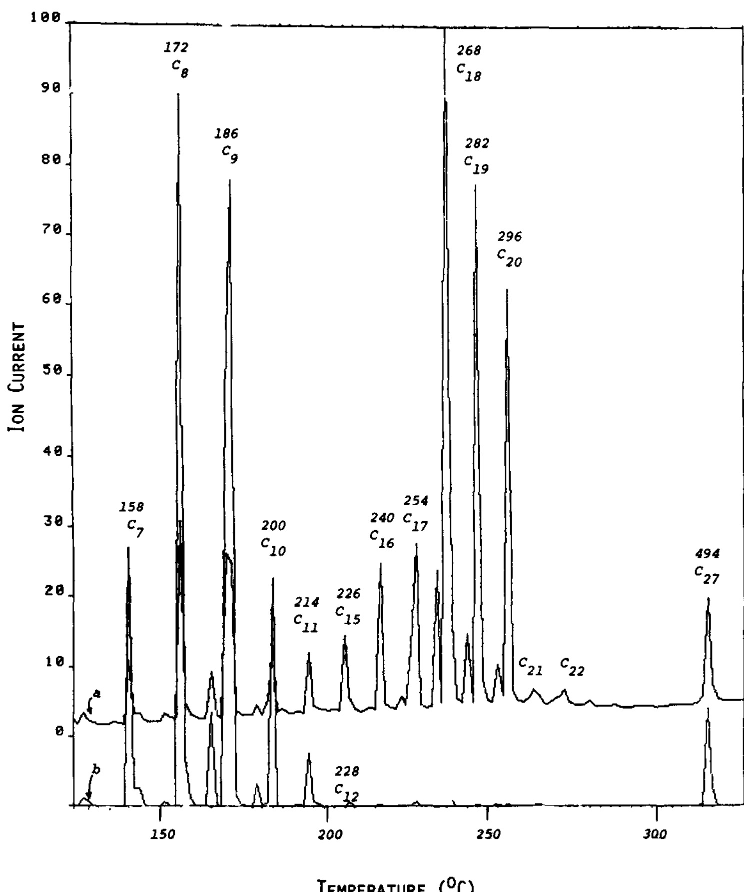

FIG. 1. Computer-generated GLC tracing of ozonized monounsaturated C_{27} ester in GC/MS analysis. Peaks C_{15}-C_{22} were aldehydes shown by the plot (a) of total ion current. Peaks C_7-C_{12} were aldehyde esters shown by the plot (b) of ion current of diagnostic ester fragment m/e 87. The chain lengths and molecular weights of the peaks are indicated.

and the position of the cyclopropane ring in the α-mycolic acids (8) relative to the alkyl terminal are identical (ω-19).

It is interesting to note that the H37Ra strain contains the entire range of odd-numbered series of unsaturated fatty acids. The significance of such a series remains unknown. The unsaturated C_{25}-C_{31} fatty acids (odd-numbered series) have patterns of isomeric distribution that are clearly distinct and different from those of the even-numbered series. The odd-numbered series tend to have greater distribution of prominent isomers with double bond positions that run consecutively (for $C_{25:1}$; Δ^5, Δ^6, Δ^7, Δ^8, and Δ^9), whereas the even-numbered series contain lower distribution of prominent isomers with double bond positions that are either singular, as in the case of Δ^5-$C_{24:1}$ and Δ^9-$C_{28:1}$, or occur in alternating series of odd-numbered Δ positions.

There are numerous examples of desaturation of C_{16} and C_{18} fatty acids to form the Δ^5, Δ^9, and Δ^{10} isomers in bacterial systems (9-11). The Δ^9 and Δ^{10} isomers of $C_{16:1}$ and $C_{18:1}$ are found together in *M. phlei* (3,12). Our results differ from those obtained by Hung and Walker (3) for *M. smegmatis* and *M. bovis* BCG. Their results suggested that the monounsaturated C_{20}, C_{22}, C_{24}, and C_{26} fatty acids are derived from the Δ^9-$C_{18:1}$ fatty acid by elongation. Our results suggest that the monounsaturated C_{15}-C_{21} fatty acids do not elongate and that the monounsaturated C_{22}-C_{26} fatty acids are synthesized by the action of desaturase systems on the corresponding saturated fatty acids. Our results are more in line with those of Asselineau et al. (4) who isolated a series of monounsaturated fatty acids from *M. phlei* with the size ranging from C_{20} to C_{27}. These fatty acids included the normal $C_{22:1}$,

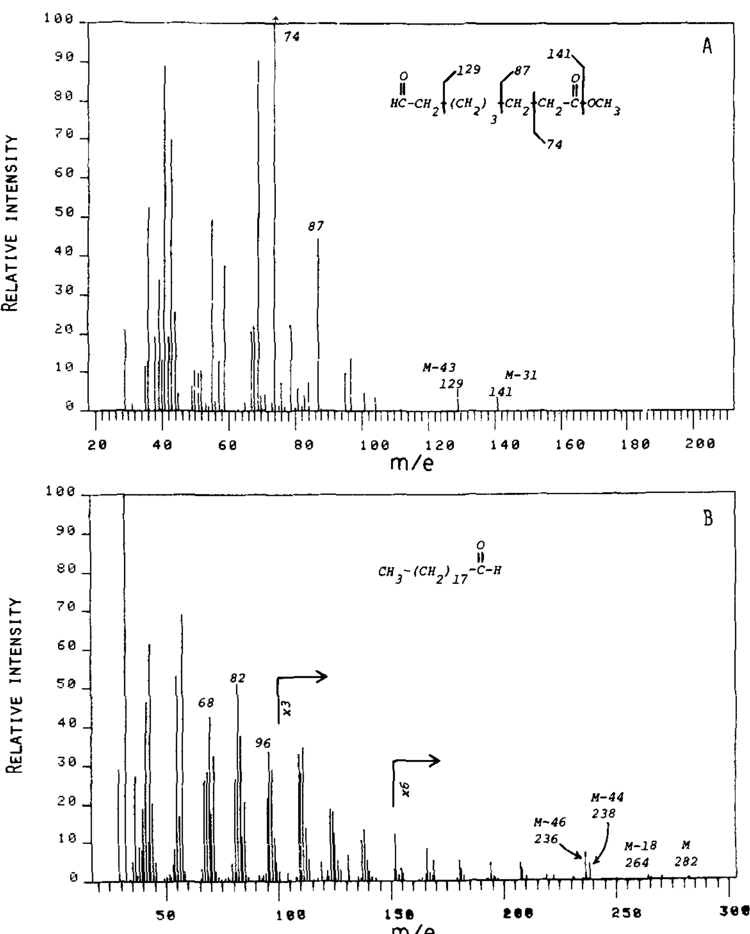

FIG. 2. Mass spectra from GC/MS analysis of (A) C_8 aldehyde ester and (B) C_{19} aldehyde fragments from ozonized monounsaturated C_{27} esters.

$C_{24:1}$, and $C_{26:1}$ as well as branch $C_{25:1}$ and $C_{27:1}$ fatty acids all of which contained a single double bond at the Δ^5 position. They also found Δ^4-$C_{20:1}$, Δ^6-$C_{22:1}$, and Δ^8-$C_{26:1}$ fatty acids. Our present studies show that *M. tuberculosis* also contains a similar series of monounsaturated long chain fatty acids.

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Cholesterol and Phospholipid Composition of Erythroblasts Isolated from Mouse Spleen after Rauscher Leukemia Virus Infection

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ABSTRACT

Erythroblasts (more than 95% pure) were isolated from MuLV-R infected mouse spleen. The cholesterol, phospholipid, and fatty acid composition of the diacyl and plasma logen derivatives of the choline and ethanolamine phosphoglycerides of mouse erythroblasts, red blood cells enriched with reticulocytes, and mature erythrocytes have been compared. It was demonstrated that during maturation of the mouse red blood cells, the following relative changes in the lipid composition occur: (a) The cholesterol/phospholipid molar ratio increases from 0.35 to 0.77. (b) The sphingomyelin concentration increases from 3.0 to 12.1 and the phosphatidylserine concentration from 5.3 to 11.2 as percentage of the total phospholipid. (c) Phosphatidylcholine decreases from 52.0 to 46.2% and phosphatidylinositol from 6.7 to 3.1%, respectively. (d) The percentage of linoleic acid in the phosphatidylcholine fraction increases more than two fold and is predominantly found at the 2-position. However, molecules containing linoleic acid at the 1-position are found to be more abundant in erythroblasts than in mature erythrocytes. (e) Phosphatidylethanolamine has been found to contain a high percentage of arachidonic acid, which was mainly found at the 2-position. The percentage of arachidonic acid at the 1-position increases during maturation, while the percentage at the 2-position decreases. (f) The plasmalogen derivatives of the choline phosphoglycerides were found to be almost absent in mouse red blood cells (<0.5%), while the ethanolamine plasmalogen phosphoglycerides represented about 8% of the phospholipids.

INTRODUCTION

Rauscher Murine Leukemia Virus (MuLV-R) infection in BALB/c mice can be used as a model to study the lipid changes in the red blood cell during maturation. During the infection, an erythroblastosis develops in the spleen, while an increased number of normoblasts and reticulocytes appear in the peripheral blood (1), accompanied by a severe anemia.

After 21 days of MuLV-R infection, it is possible to isolate a more than 95% pure preparation of erythroblasts from the infected mouse spleens and to analyze the lipids. These data can be compared with the lipid analyses on red blood cells enriched with reticulocytes and normoblasts derived from the peripheral blood of the infected mice. Erythrocytes from uninfected mice can then be used in this model as mature red blood cells.

For different animal species, the lipid composition of the erythrocyte membrane has been well established (2,3). No changes in the distribution of the different phospholipid classes on varying stages of maturity of the erythrocyte membrane have been found (4-7). Various authors (7-10) agree that with increasing age the total lipid, cholesterol, and phospholipid contents of the cell decrease.

On the other hand, a significant shift in the fatty acid patterns has been noticed, such as an increase in linoleic acid and a simultaneous decrease in arachidonic acid and other long chain acids during the aging process (5,7).

Mature erythrocytes have been shown to contain high concentrations of plasmalogens (alk-1-enylacylphosphoglycerides) mainly found in the ethanolamine containing phosphoglycerides (11-15).

Besides that, lipids form a fundamental structural and functional unit in biomembranes (3), some of them (e.g., linoleic acid) have been found to stimulate cell growth (16), whereas others (e.g., sphingolipids) have been demonstrated to stimulate erythropoiesis (17).

Herein we present the cholesterol and phospholipid composition of immature and mature red blood cells of the mouse.

MATERIALS AND METHODS

Animals

Female BALB/c mice, 6-8 weeks old, received 0.2 ml (4 mg) of a purified MuLV-R preparation intraperitioneally (18). No special diet was given; food (standard laboratory mouse food, Hope Farms, Woerden, Holland) and water were supplied ad libitum.

At 21-26 days after inoculation, the mice were sacrificed by prolonged ether anesthesia. The spleens were collected after inspection of their size, the main parameter in infection (19),

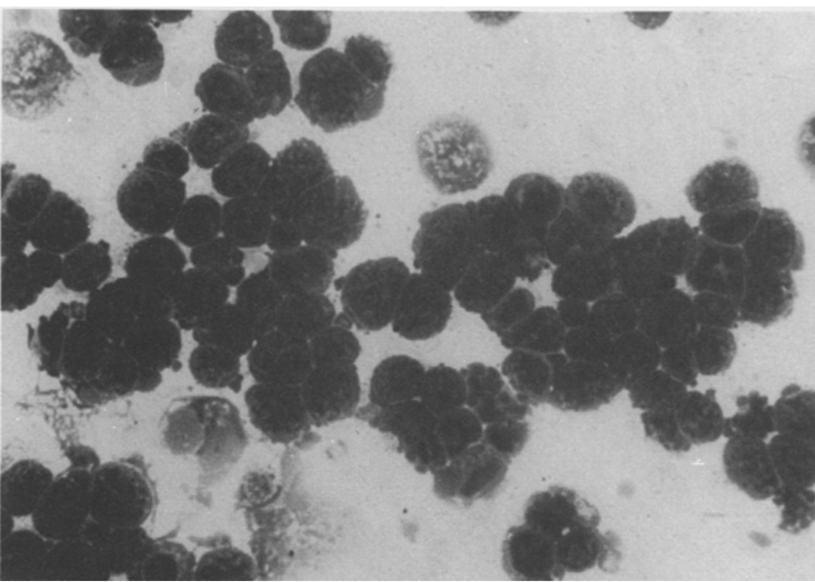

FIG. 1. A 95-99% pure fraction of mouse erythroblasts ("Rauscher cells") May Grünwald-Giemsa staining (magn. 800x).

and used for the isolation of erythroblasts ("Rauscher cells").

Isolation of Rauscher Cells

Spleens of 20 mice were minced at room temperature in HBSS (Hanks balanced salt solution) and homogenized. The suspension was filtered through four layers of nylon gauze. Two ml of the homogenous cell suspension, consisting of Rauscher cells and erythrocytes, were layered on 20 ml of fetal calf serum (Flow Laboratories, Irvine, Scotland) in a centrifuge tube with a diameter of 2 cm. The Rauscher cells were allowed to settle during 30 min while the erythrocytes stayed on top of the fetal calf serum. Prolonged sedimentation gives rise to increased contamination with red blood cells.

After aspiration of the fetal calf serum, the sediment consisting of Rauscher cells and a few red blood cells was taken up in 10 ml of fetal calf serum and centrifuged at 400 x g for 5 min. The supernatant and the few red cells on top of the pellet were removed by aspiration, and the Rauscher cells were resuspended in fetal calf serum. This suspension was layered on a discontinuous gradient composed of 7 ml of lymphoprep (Nyegaard & Co. d = 1.077 ± 0.001 g/ml) on top of 1 ml 35% (w/w) of albumin (Sigma, St. Louis, MO) in Tris-HCl buffer (pH 7.2, 300 m.osmol.).

After centrifugation for 30 min at 1250 x g, the Rauscher cells remained on top of the lymphoprep, while more mature erythroid precursor cells and erythrocytes which were still present sedimented into the albumin layer. The Rauscher cells were taken up in HBSS and centrifuged for 5 min at 400 x g. This was repeated twice, and subsequently the cells were collected at the bottom of the centrifuge tube.

May Grünwald-Giemsa staining was used after fixation with methanol. Subsequently the cells were examined by light microscopy.

Rauscher cells are recognized by their size (about three times the diameter of erythrocytes), their loose chromatin structure of the nucleus with large blue nucleoli and pink reddish cytoplasma.

Rauscher cells have a distinct appearance from the contaminating cells consisting of lymphocytes and normal erythroid and myeloid precursor cells.

Based on morphological criteria, only cell preparations containing 95-99% pure fractions of erythroblasts ("Rauscher cells") were used for analytical studies (fig. 1).

This method was developed in our laboratory by E. van 't Hull (unpublished results).

Blood Samples

Fresh samples of blood were obtained from ether anesthetized BALB/c mice by puncture from the vena cava inferior using heparin (10 mg/ml) as an anticoagulant. The blood from about 50 animals was pooled, representing one sample. The cells were spun down in a clinical centrifuge for 15 min. The plasma and the

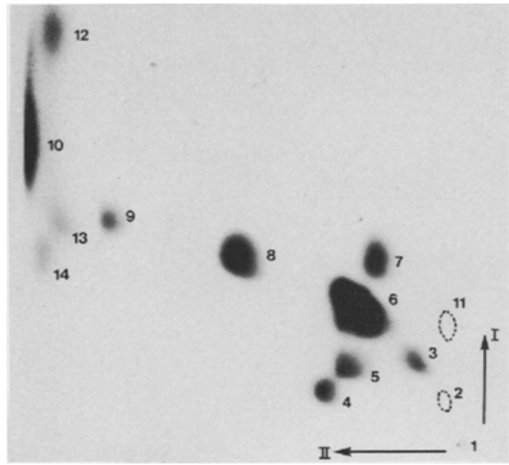

FIG. 2. Two-dimensional thin layer chromatogram of the major phospholipids of "Rauscher cells" after iodine staining. Solvent system I: chloroform-methanol-ammonia-water (112:51:4.5:4.5, by vol). Solvent system II: chloroform-methanol-glacial acetic acid-water (112:37.5:12:4.4, by vol). 1. origin; 2. lysolecithin; 3. sphingomyelin; 4. phosphatidylserine; 5. phosphatidylinositol; 6. diacylphosphatidylcholine; 7. lysophosphatidylethanolamine derived from ethanolamine plasmalogen phosphoglycerides; 8. diacylphosphatidylethanolamine; 9. cardiolipin; 10. neutral lipids; 11. lysophosphatidylcholine derived from choline plasmalogen phosphoglycerides; 12, 13, and 14 unidentified lipids.

buffy coat were removed and the cells subsequently washed three times with 0.9% NaCl.

Lipid Analyses and Thin Layer Chromatography

Lipid extraction of cells and plasma with 19 volumes of chloroform-methanol (2:1, v/v) was performed according the method of Folch et al. (20). Methanol was added first with stirring followed by addition of chloroform.

To estimate the percentage distribution of the individual phospholipids, the total lipid extract was separated by two-dimensional thin layer chromatography (TLC). The thin layer plates (20 x 20 cm) were covered with a 0.5 mm thick layer consisting of a mixture of 0.9 g magnesium silicate (Woelm TLC, ICN, Eschwege, Germany) and 45 g Silica Gel HR (Merck, Darmstadt, Germany) as described by Broekhuyse (21).

After developing the chromatograms in the first dimension using chloroform-methanol-ammonia-water (112:51:4.5:4.5, by vol), the thin layer plates were sprayed with a mixture of 0.1 M acetic acid and 0.005 M mercuric chloride to convert the plasmalogens into their lyso derivatives according to the method of Owens et al. (22). After drying the plates at room temperature for 30 min, the chromatograms were developed in the second dimension using chloroform-methanol-glacial acetic acid-water (112:37.5:12:4.5, by vol).

Lipid spots were visualized with iodine, and the phospholipids were scraped off the plates. Lipid phosphorus was determined as described by Broekhuyse (21). Reference substances (Supelco Inc. Bellfonte, PA) were chromatographed simultaneously on a separate plate to identify the lipids. Cholesterol was estimated according to Ferro and Ham (23).

Fatty Acid Analysis

The phospholipids used for fatty acid analyses were separated by one-dimensional TLC using thin layer plates (20 x 20 cm) covered with a 0.5 mm thick layer of Silica Gel H (Merck, Darmstadt, Germany). The solvent system used was chloroform-methanol-ammonia-water (70:30:2:3, by vol). After drying the plates in a N_2 atmosphere, the plates were subsequently acidified in a tank filled with acetic acid vapors and after 1 min sprayed with

TABLE I

Cholesterol and Phospholipid Composition of Erythroblasts ("Rauscher cells")[a], Red Blood Cells from MuLV-R Infected Mice[b], and Mature Erythrocytes from Control Mice[c]

	Total cholesterol (mg x 10^{-10}/cell) (4)[d]	Total phospholipid (mg x 10^{-10}/cell) (4)
Erythroblasts	18.9 ± 1.6[e]	137.5 ± 12.5
MuLV-R r.b. cells	1.2 ± 0.1	4.0 ± 0.5
Control erythrocytes	0.7 ± 0.1	1.7 ± 0.1

[a]>95% Erythroblasts, <5% lymphocytes, and normal erythroid and myeloid precursor cells.
[b]Hematocryte 30-35%, 18-25% reticulocytes.
[c]Hematocryte 50-55%, 2-5% reticulocytes.
[d]Number of samples, each sample analyzed in duplicate.
[e]Mean ± standard error.

TABLE II

Percentage Distribution of the Major Phospholipids in Erythroblasts ("Rauscher cells")[a], Red Blood Cells from MuLV-R Infected Mice[b], and Mature Erythrocytes from Control Mice[c]

	Erythroblasts ("Rauscher cells") (7)[d]	MuLV-R red bloodcells (11)	Control erythrocytes (7)
Lysolecithin	2.4 ± 0.5[e]	3.2 ± 0.3	3.4 ± 0.4
Sphingomyelin	3.0 ± 0.3	9.9 ± 0.2	12.1 ± 0.3
Phosphatidylinositol	6.7 ± 0.4	3.7 ± 0.3	3.1 ± 0.3
Phosphatidylserine	5.3 ± 0.2	10.1 ± 0.5	11.2 ± 0.3
Phosphatidylcholine	52.0 ± 0.6	46.0 ± 0.8	46.2 ± 0.6
Choline-plasmalogens	0.3 ± 0.2	0.1 ± 0.1	---
Phosphatidylethanolamine	18.0 ± 0.5	13.4 ± 0.5	15.2 ± 0.6
Ethanolamine plasmalogens	7.6 ± 0.5	9.4 ± 0.8	8.0 ± 0.6
Cardiolipin	3.1 ± 0.2	0.5 ± 0.1	---
Others	1.6 ± 0.9	3.7 ± 1.2	0.8 ± 0.8
Spingo/PC	0.06 ± 0.01	0.22 ± 0.04	0.26 ± 0.06
Chol./P. lipid	0.35 ± 0.03	0.61 ± 0.07	0.77 ± 0.10

[a]>95% Erythroblasts, <5% lymphocytes, and normal erythroid and myeloid precursor cells.
[b]Hematocryte 30-35%, 18-25% reticulocytes.
[c]Hematocryte 50-55%, 2-5% reticulocytes.
[d]Number of samples, each sample analyzed in duplicate.
[e]Mean ± standard error.

TABLE III

Cholesterol and Phospholipid Content of Plasma of MuLV-R Infected Mice and Control Mice

	MuLV-R plasma (3)[a]	Control plasma (4)
Total cholesterol (mg/100 ml)	89 ± 14[b]	118 ± 14
Total phospholipid (mg/100 ml)	144 ± 10	191 ± 41
Phospholipid distribution (%)		
Lysolecithin	21.5 ± 0.2	19.3 ± 2.2
Sphingomyelin	6.8 ± 0.5	7.5 ± 1.0
Phosphatidylinositol Phosphatidylserine	1.3 ± 0.5	2.4 ± 1.3
Phosphatidylcholine	68.4 ± 0.1	68.4 ± 1.6
Phosphatidylethanolamine	2.1 ± 0.2	2.5 ± 0.8

[a]Number of samples, each sample analyzed in duplicate.
[b]Mean ± standard error.

a mixture of 0.1 M acetic acid and 0.005 M mercuric chloride. After drying the plates for 30 min at room temperature in a CO_2 atmosphere, the plates were sprayed with a 0.01% Rhodamine-6G solution in water to visualize the phospholipids. The phosphatidylcholine (PC) and phosphatidylethanolamine (PE) spots (including the lyso derivatives of their respective plasmalogens) were scraped off the plates and eluted with 100 ml of a mixture of chloroform-methanol (1:1, v/v).

The diacylphospholipids were clearly separated from their lyso derivates using the same separation procedure.

The diacylphospholipids were eluted with a mixture of chloroform-methanol (1:1, v/v), and subsequently the total fatty acid composition as well as the fatty acid composition at the 1- and 2-position of PC and PE after phospholipase A_2 degradation were determined. About 10 mg of PC or PE was dissolved in 2 ml of freshly distilled ether; 2 mg of snake venom from *Crotalus adamanteus* (Light and Co., England) was suspended in 0.25 ml 0.1 M borate buffer (pH 7.2) and 0.25 ml of 0.005 M $CaCl_2$ were added. The mixture was shaken for 2 hr at room temperature. Complete degradation was achieved.

TABLE IV

Fatty Acid Composition at the 1- and 2-Position of Phosphatidylcholine[a] of Erythroblasts[b], MuLV-R Infected Red Blood Cells[c], and Control Erythrocytes[d] of the Mouse

(3)[e]	14:0	16:0	16:1	18:0	18:1	18:2	20:3	20:4	Others[f]
1-Position									
R-cells	0.9 ± 0.2[g]	48.6 ± 3.7	2.8 ± 0.7	11.9 ± 0.7	26.6 ± 3.1	6.6 ± 0.2	+	2.2 ± 0.1	0.4 ± 0.2
MuLV-R	0.2 ± 0.2	63.8 ± 0.8	+	22.9 ± 1.6	9.8 ± 0.9	2.4 ± 0.3	+	0.3 ± 0.3	0.8 ± 0.8
Control	+	64.6 ± 2.7	0.3 ± 0.3	26.3 ± 3.2	6.8 ± 0.7	2.0 ± 0.3	---	---	---
2-Position									
R-cells	0.8 ± 0.1	36.4 ± 0.3	2.8 ± 0.6	1.0 ± 0.4	20.9 ± 1.0	11.0 ± 3.0	+	23.0 ± 0.1	4.3 ± 0.1
MuLV-R	0.5 ± 0.5	36.2 ± 0.1	0.7 ± 0.1	2.0 ± 0.2	22.5 ± 1.5	20.6 ± 3.7	1.0 ± 0.4	14.3 ± 3.3	2.7 ± 1.7
Control	0.3 ± 0.3	36.0 ± 3.9	1.6 ± 0.5	2.6 ± 0.3	17.7 ± 0.9	26.4 ± 1.5	1.5	8.5 ± 1.3	5.7 ± 1.8

[a]Could contain some alkylacyl PC.
[b]>95% Erythroblasts, <5% lymphocytes, and normal erythroid and myeloid precursor cells.
[c]Hematocryte 30-35%, 18-25% reticulocytes.
[d]Hematocryte 50-55%, 2-5% reticulocytes.
[e]Number of samples, each sample analyzed in duplicate.
[f]Include 20:5, 22:4, 22:5, 22:6, 24:0, and 24:1.
[g]Mean ± standard error.

TABLE V

Fatty Acid Composition of the 1- and 2-Position of Phosphatidylethanolamine[a] of Erythroblasts ("Rauscher cells")[b], MuLV-R Infected Red Blood Cells[c], and control Erythrocytes[d] of the Mouse

(3)[e]	14:0	16:0	16:1	18:0	18:1	18:2	20:3	20:4	Others[f]
1-Position									
R. cells	+	33.1 ± 1.9[g]	1.3	23.4 ± 1.8	32.5 ± 2.1	4.2 ± 0.3	---	1.1 ± 1.0	4.5 ± 4.5
MuLV-R	+	24.3 ± 2.5	2.1 ± 0.5	13.5 ± 0.3	36.4 ± 2.9	6.3 ± 0.3	0.6 ± 0.3	7.4 ± 0.1	9.4 ± 2.1
Control	1.7 ± 0.2	23.0 ± 1.7	1.8 ± 0.2	15.3 ± 2.2	36.8 ± 2.8	7.3 ± 0.4	0.5 ± 0.5	9.9 ± 0.8	3.7 ± 2.2
2-Position									
R. cells	+	15.7 ± 0.1	2.6 ± 0.4	5.0 ± 0.4	11.6 ± 2.2	3.4 ± 0.3	+	48.2 ± 1.0	13.8 ± 2.7
MuLV-R	0.8 ± 0.8	12.8 ± 1.6	2.9 ± 0.2	4.7 ± 0.4	25.3 ± 2.9	6.9 ± 1.3	0.3 ± 0.3	31.9 ± 1.8	14.4 ± 4.2
Control	2.7 ± 1.0	16.4 ± 0.3	3.1 ± 1.0	5.8 ± 0.1	25.7 ± 2.0	8.2 ± 1.2	1.7	26.8 ± 0.4	9.9 ± 1.6

[a]Could contain some alkylacyl PE.
[b]>95% Erythroblasts, <5% lymphocytes, and normal erythroid and myeloid precursor cells.
[c]Hematocryte 30-35%, 18-25% reticulocytes.
[d]Hematocrytes 50-55%, 2-5% reticulocytes.
[e]Number of samples, each sample analyzed in duplicate.
[f]Include 20:5, 22:4, 22:5, 22:6, 24:0, and 24.1.
[g]Mean ± standard error.

TABLE VI

Fatty Acid Composition of the Ethanolamine Plasmalogens of Erythroblasts ("Rauscher cells")[a], MuLV-R Infected Red Blood Cells[b], and Control Erythrocytes[c] of the Mouse

(3)[d]	16:0	16:1	18:0	18:1	18:2	20:3	20:4	22:4	Others[e]
R. cells	10.2 ± 0.1[f]	1.4	2.6 ± 0.1	5.3 ± 0.1	3.1 ± 0.5	+	48.1 ± 4.2	24.4 ± 3.9	5.0 ± 1.1
MuLV-R	4.2 ± 0.3	0.8 ± 0.1	1.5 ± 0.1	9.6 ± 0.4	3.1 ± 0.4	---	52.0 ± 3.1	22.4 ± 3.4	6.4 ± 0.8
Control	8.2 ± 2.9	3.4 ± 1.8	2.0 ± 0.6	9.3 ± 0.4	3.4 ± 0.3	---	25.1 ± 0.6	24.9 ± 3.0	22.9 ± 2.6[g]

[a]>95% Erythroblasts, <5% lymphocytes, and normal erythroid and myeloid precursor cells.
[b]Hematocryte 30-35%, 18-25% reticulocytes.
[c]Hematocryte 50-55%, 2-5% reticulocytes.
[d]Number of samples, each sample analyzed in duplicate.
[e]Include 20:5, 22:5, 22:6, 24:0, and 24:1.
[f]Mean ± standard error.
[g]Include 9.2 ± 0.6% as 20:5.

Separation of the fatty acids and the lyso compounds was carried out using the above-mentioned solvent system.

The fatty acids were released from the phospholipids by saponification with 15 ml methanolic HCl (26 g HCL/l) for 2 hr at 70 C. After transesterification, the methyl esters were extracted with pentane and separated by gas chromatography. In all analyses, a N_2 atmosphere was used to prevent oxidation.

Gas liquid chromatography (GLC) was carried out in a Hewlett-Packard instrument equipped with a flame ionization detector and a 10% polyethylene glycol succinate column at 170 C.

RESULTS

Figure 2 shows an example of the consistently good separations of the major phospholipids by two-dimensional TLC as described in "Materials and Methods."

The plasmalogen derivatives of the choline phospholgycerides and lysophosphatidylethanolamine were barely detectable.

Table I shows that distinct differences have been found between the cholesterol and phospholipid content of very young cells (Rauscher cells) and mature erythrocytes.

Table II shows that phosphatidylcholine represents the major phospholipid class in erythroblasts as well as erythrocytes. The percentage distribution of the difference phospholipids in both cell types is quite distinct from each other. Especially the relatively low sphingomyelin and phosphatidylinositol content of mouse erythroblasts compared to mouse erythrocytes are worth mentioning.

Table III presents the cholesterol and phospholipid content of plasma from control and leukemic (MuLV-R) mice. No great differences were found except for a lower cholesterol and phospholipid content of leukemic plasma.

Tables IV and V present the fatty acid composition of phosphatidylcholine and phosphatidylethanolamine of erythroblasts, MuLV-R infected red blood cells, and mature erythrocytes. It is demonstrated that diacyl-GPC and diacyl-GPE have distinct fatty acid patterns.

Diacyl-GPC and Diacyl-GPE

Palmitic acid (16:0) is the major fatty acid in diacyl-GPC and is preferentially found at the 1-position in diacyl-GPC and diacyl-GPE.

Stearic acid (18:0) increases in diacyl-GPC during maturation, but decreases in diacyl-GPE and is almost exclusively found at the 1-position. The concentration at the 2-position is low and rather constant.

Oleic acid (18:1) decreases during maturation in diacyl-GPC. This reduction is most pronounced at the 1-position. However, in diacyl-GPE, an increase of oleic acid at the 2-position is found if we compare immature and mature cells.

The concentration of linoleic acid (18:2) at the 2-position of diacyl-GPC increases 2.5 times but decreases at the 1-position during maturation of the red blood cell.

Arachidonic acid (20:4) decreases in diacyl-GPC during maturation and is located almost exclusively at the 2-position.

Diacyl-GPE is characterized by a high arachidonic acid content, with a slight decrease in the overall composition during maturation.

While in erythroblasts this acid is almost exclusively found at the 2-position, during maturation the reverse species containing arachidonic acid at the 1-position increase at the expense of the opposite species.

Ethanolamine Plasmalogen Phosphoglycerides

The fatty acids given in Table VI represent the fatty acid composition of the 2-position of the molecules.

The plasmalogens are characterized by a low concentration of palmitic and stearic acid, a low oleic and linoleic acid content, but very high concentrations of polyunsaturated long chain fatty acids, of which arachidonic acid is most abundant.

The plasmalogen fatty acid compositions show good agreement with the fatty acid distribution at the 2-position of diacyl-GPE, though some distinct differences have been found, i.e., the higher concentration of 22:4.

DISCUSSION

The analyses of red blood cells, as described in this paper, demonstrate that during maturation from erythroblast to erythrocyte, substantial changes in the lipid composition take place. Our results agree with those of other investigators (4-6) who demonstrated that with increasing age, there are decreases in the total lipid, cholesterol, and phospholipid content of the cells. However, our data are in contrast with the results of Westerman (4) and Van Gastel (5) who suggested that the distribution of phospholipid classes is unchanged and lipid loss is uniform with aging. Also, no large variations in the phospholipid composition as a function of cell age were observed by Winterbourn and Batt (6).

The phospholipids of the mouse erythroblasts, however, show a drastic change in their phospholipid percentage distribution compared to the erythrocytes. The most important changes are the very low sphingomyelin and cholesterol content of the erythroblasts compared to the mature erythrocytes.

The sum of the choline-containing phospholipids is found to be rather constant in the different cells, and a reciprocal relationship is found between sphingomyelin and lecithin, as was also demonstrated in the erythrocyte membranes of different animal species (2,3).

Differences in the sphingomyelin/lecithin ratio correlate with differences in the permeability of erythrocyte membranes from different animals (3). This ratio and the cholesterol/phospholipid molar ratio are two important factors in determining the fluidity, i.e., microviscosity of a membrane (24,25).

For plasma membranes isolated from Rauscher cells, almost the same values were found, e.g., 0.42 ± 0.08 for the cholesterol/phospholipid ratio and 0.07 ± 0.04 for the sphingomyelin/lecithin molar ratio (unpublished results). Model studies in our laboratory with liposomes of well choosen lipid composition confirmed that cholesterol and sphingomyelin contribute very much to a higher microviscosity, but also showed that within a certain class of phospholipids, e.g., lecithin, the fatty ac-d composition is very important in this respect (A. Montfoort et al., paper in progress).

Leukemic cells havy also been found to be deficient in their membrane cholesterol and sphingomyelin compared to control cells (25). The lipid composition of Rauscher cells agrees with these data published for leukemic cells.

Whether or not these data are a reflection of cell immaturity or the leukemic state, particularly as a consequence of the viral-induced transformation, is still obscure.

In Rh-erythroblastosis (26), the sphingomyelin/lecithin molar ratio was found to be normal. This study was similar to the present report in that a relatively pure erythroblast preparation was analyzed. It is worth mentioning that during maturation phosphatidylinositol decreases with about 50%, while on the other hand the phosphatidylserine concentration is more than doubled. Similar results were found in analyzing the plasma membranes of the proerythroblasts, where phosphatidylserine represented 5.1 ± 1.8% and phosphatidylinositol 4.9 ± 1.3%, respectively.

Phosphatidylserine is known to play a role in membrane transport processes as a cofactor for ATPases. In addition to the structural role, cholesterol also has a regulatory function in abnormal cell growth in leukemia (25). The plasma cholesterol concentration was suggested to play an important role in its regulation. Our

data on the lipid composition of plasma of MuLV-R infected mice agree with the results of other investigators, who studied human leukemic plasma, and where a reduced cholesterol concentration was found.

It has been reported that during aging of the erythrocyte the linoleic acid (18:2) content of the phospholipids increases (5,7) which is confirmed in this study. In pathological erythrocyte populations with increased number of young cells and in fetal erythrocytes, a low level of linoleate has been found (26-29).

It should be noted that our values for ethanolamine-plasmalogen derivatives in mouse red blood cells are lower than those reported by other investigators for human erythrocytes (13-15). In this study, MuLV-R infection in BALB/C mice has been used as a model for lipid changes during red cell maturation. These results are the first comparative analyses of almost pure preparations of immature and mature red blood cells of the mouse, showing aspects of immaturity mixed with features of the leukemic state.

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Hypercholesterolemia in Rats Fed Cholesterol in Agar Gel Diets

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ABSTRACT

Female (Exp. I) or male (Exp. II) weanling rats were fed diets containing either 2% Solka-Floc or 2% agar for 28-day periods. Some groups received 1% cholesterol, either added in crystalline form or first dispersed in the oil portion of the diet, and some agar groups received their diet in a gelled form. Feces were collected for a 3-day period after 2 weeks (Exp. II) or during the fourth week (Exp. I) of experimentation. Serum and liver cholesterol, total liver lipids, fecal lipids, and fecal sterols were determined. The results indicated that cholesterol feeding increased serum cholesterol, total liver, and fecal lipids, liver cholesterol, and fecal sterols. Substitution of agar for Solka-Floc in dry (nongelled) diets further increased total liver lipids (Exp. I), but had no significant effect upon any other measured parameter. Gelling of 1% cholesterol agar diets, in contrast to the 1% cholesterol dry agar diet, resulted in reduced liver cholesterol in both experiments. Gelling significantly increased fecal sterols after 2 weeks feeding (Exp. II), but no significant differences were observed after 4 weeks feeding (Exp. I) when compared to 1% cholesterol-fed groups. Small, nonsignificant increases of liver cholesterol and total liver lipids with similar reduction of fecal sterols resulted from dispersing the cholesterol in the oil portion of the diet prior to mixing. The results indicate that (a) inclusion of 2% agar in rat diets and (b) dispersing cholesterol in oil had little effect upon serum, liver, or fecal lipids in cholesterol-fed rats. However, gelling the agar diets reduced liver cholesterol, possibly by initial reduction of dietary cholesterol absorption.

INTRODUCTION

Rogers and Harper (1) demonstrated greater food intake and weight gain for rats fed amino acid diets in an agar gel (50% water) compared to the same diets fed as a dry powder. They attributed the increased growth to the agar gel's reduction of severe osmotic effects of low molecular weight amino acids in the gastrointestinal tract. The agar gel diet regimen has been subsequently utilized by many investigators studying amino acid metabolism in several species of animals.

Such a feeding system has potential for use in studies involving highly labile nutrients, such as unsaturated lipids or vitamins. Agar gel diets should physically protect dietary constituents from light and oxidation; protection not afforded by powdered diets. If necessary, the diets could be stored, refrigerated, or frozen under anaerobic conditions, and could be fed in the animals dark cycle to minimize oxidation.

Prior to use of agar gel diets for lipid studies, knowledge of the effects of agar, an indigestible polysaccharide, upon absorption of lipids such as cholesterol is essential. Binding of various types of dietary indigestible fibers with cholesterol, bile acids, and bile salts have been reported (2). Such binding can produce a general hypolipidemic and antiatherogenic effect in experimental animals. Only a few studies (3-7) have included agar as a test fiber, and in none was the effect of gelling on hypercholesterolemia investigated.

The purpose of this investigation was to test the effect of feeding 1% cholesterol in 2% agar gel diets on serum and liver cholesterol, total liver lipids, fecal sterols, and fecal lipids in the rat. In addition, these parameters are measured in animals receiving diets where cholesterol is incorporated into the diet in a crystalline form or dispersed in the oil portion of the diet prior to incorporation. Mixing cholesterol with the oil portion of the diet prior to diet formulation should be more favorable for maximal body retention of cholesterol by the rat (8).

EXPERIMENTAL PROCEDURE

Animals and Diets

Female (Exp. I) or male (Exp. II) weanling Sprague-Dawley rats were housed in individual stainless steel cages in a temperature-controlled room. The lights were set for 12 hr of light and 12 hr of dark. The rats were adapted to the laboratory and fed a basal diet (Table I) for 3 days prior to the experimental period. The various experimental diets were fed for 28 days. on days 0, 7, 14, and 21 between 0800 and 1000 hours, some animals were anesthesized with ether, and blood was removed from the heart with a syringe inserted on the left side of the sternum caudal to the last rib. After 28 days, heart punctures were performed on all animals, the livers were removed, rinsed, blotted dry, and frozen at 0 C until analysis. Whole blood was centrifuged, and serum was

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decanted and frozen. Feces were collected for a 3-day period after 2 weeks (Exp. II) or during the fourth week (Exp. I) of experimentation. The feces were dried, cleaned, weighed, and frozen until analysis.

Gelled diets were made by heating water to 100 C, removing the heat, and vigorously mixing the water with an equal weight of dry ingredients. The order of addition of ingredients was agar dispersed in sucrose, casein, corn starch (with or without crystalline cholesterol), cottonseed oil (with or without dispersed cholesterol), mineral mix, and, after sufficient cooling, the vitamin mix. Agar diets contained 2% agar (d.b.) substituted for the Solka-Floc while 1% cholesterol was substituted for 0.5% starch and 0.5% sucrose in cholesterol-containing diets. After the diets were evenly blended, they were poured into plastic tubs with air-tight covers and allowed to cool before the covers were replaced. All diets were then refrigerated until use.

Some experimental groups received cholesterol dispersed in cottonseed oil. This was accomplished by first dissolving the crystalline cholesterol in absolute ether. This mixture was added to warmed cottonseed oil, and the ether was dissipated by bubbling nitrogen through the stirred oil. Heat was applied (not exceeding 60 C) to speed ether removal. The resulting paste or gel-like mixture was added to the other diet ingredients as noted above.

Diet and water were provided ad libitum. Food cups containing the dry diets were filled every other day. The agar gel diets were fed daily. Food intakes and weight gains were recorded. Nonconsumed gel diet was dried overnight at 105 C to determine accurately food intakes of the gel diet groups. Feed efficiencies were calculated.

TABLE I

Basal Diet Composition

Ingredient[a]	Parts
Corn starch	43.3
Sucrose	21.7
Casein	22.0
Cottonseed oil	6.0
Mineral mix[b]	4.0
Vitamin mix[c]	1.0
Nonnutritive fiber[d]	2.0
	100.0

[a]All ingredients (unless noted) were prepared by Teklad Test Diets, Madison, WI.

[b]USP XVII % composition: calcium carbonate 38.1400; cobalt chloride .0023; cupric sulfate .0477; ferrous sulfate 2.700; magnesium sulfate 5.7300; manganese sulfate .4010; potassium iodide .0790; potassium phosphate monobasic 38.9000; sodium chloride 13.9300; zinc sulfate .0548.

[c]No.40060 from Teklad Test Diets. Composition (mg/kg dry diet): p-amino benzoic acid 110.229; ascorbic acid 1017.520; biotin .441; B_{12} 29.7619; calcium pantothenate 66.137; choline dihydrogen citrate 3715.123; folic acid 1.984; i-inositol 110.229; menadione 49.603; nicotinic acid 99.206; pyridoxine HCl 22.045; riboflavin 22.045; thiamine HCl 22.045. Units/kg dry diet: vitamin A (dry) 6000.0 RE; vitamin D_2 (dry) 2204.585 IU; vitamin E acetate 121.252 IU.

[d]Solka-Floc, Brown Company, Berlin, NH.

EXPERIMENTAL DESIGN

Experiment I

Twenty-eight female rats were divided equally into four groups. Group A received a dry basal diet (see Table I). Groups B-D had 1% cholesterol (d.b.) added to their diets in the crystalline form. Group B was fed a dry 1% cholesterol diet. For Groups C and D, agar (Bacto-Agar, Difco, Detroit, MI) was substituted for Solka-Floc; Group C received a gelled diet while Group D received a powdered diet.

Experiment II

In this experiment, 60 male rats were divided equally into six groups. Group A, the dry control, received the basal diet (Table I). Groups B and C received 1% cholesterol powdered diets; the cholesterol in Group B was added in crystalline form, while Group C received cholesterol dispersed in oil. Groups D-F received gelled diets; Group D, a gel control diet, containing no cholesterol, while Groups E and F received 1% cholesterol in crystalline and dispersed forms, respectively.

Analytical Methods

Total serum cholesterol was determined colorimetrically with a stable Liebermann-Burchard cholesterol reagent (14.5% sulfuric acid, 41% acetic acid, and 43% acetic anhydride, Hycel Inc., Houston, TX). Total liver and fecal lipids were determined gravimetrically after extraction with a chloroform-methanol mixture (2:1), using the basic procedure described by Folch et al. (9). The dried lipid extracts were taken up in acetone-ethanol (1:1), and liver cholesterol and fecal sterols were determined by a ferric chloride colorimetric method of Zlatkis et al. (10). Ferric chloride reaction-positive fecal materials would include many 3-β hydroxy sterols besides cholesterol. Although the color is largely due to cholesterol (the relative colorimetric yield of coprostanol, for example, is only 18% that of cholesterol), the term fecal sterols is used instead of fecal cholesterol and refers to neutral sterols remaining in the lower chloroform phase after

TABLE II

Influence of Cholesterol (1%), Agar (2%), and the Gelling of Diets upon Serum, Liver, and Fecal Lipids (experiment No. 1)

	Group A control dry diet (Solka-Floc)	Group B 1% crystalline chol dry diet (Solka-Floc)	Group C 1% crystalline chol gel diet (agar)	Group D 1% crystalline chol dry diet (agar)
Weight gain, g	100 ± 5[ab]	110 ± 12	106 ± 11	105 ± 6
Food intake, g	328 ± 15[A]	345 ± 21[A]	295 ± 32[B]	327 ± 14[A]
Feed efficiency[c]	0.304 ± 0.024[A]	0.316 ± 0.033[A]	0.360 ± 0.016[B]	0.319 ± 0.022[A]
Liver wt as a % of body weight	4.6 ± 0.7	5.0 ± 0.5	4.3 ± 0.6	4.9 ± 0.4
Final serum cholesterol, mg/100 ml	112 ± 15[A]	156 ± 22[B]	145 ± 31[B]	144 ± 17[B]
Liver cholesterol, mg/g fresh liver	3.4 ± 0.2[A]	10.9 ± 2.5[C]	6.9 ± 1.5[B]	10.3 ± 1.8[C]
Liver lipids, %	3.9 ± 0.2[A]	4.9 ± 0.8[B]	4.8 ± 0.6[B]	5.9 ± 0.4[C]
Dry fecal weight, g/day[d]	0.9 ± 0.4	1.5 ± 0.4	1.5 ± 0.5	1.3 ± 0.4
Fecal lipids, %[d]	2.8 ± 0.7[A]	9.6 ± 1.4[B]	8.5 ± 0.9[B]	9.6 ± 0.6[B]
Fecal sterols, mg/g dry[de]	3.5 ± 2.0[A]	41.4 ± 6.9[B]	42.7 ± 6.7[B]	41.6 ± 4.2[B]
Daily fecal sterols, mg/day[de]	3.1 ± 1.7[A]	60.5 ± 18.5[B]	61.1 ± 24.3[B]	53.3 ± 16.8[B]

[a]Mean ± SD for seven rats.
[b]Values in the same line not sharing a common superscript letter are significantly different ($P<0.05$).
[c]Weight gain (g) ÷ food intake for 28 days (g).
[d]Three-day collection period in final week of experiment.
[e]$FeCl_3$ reaction-positive sterols.

Folch separation. Interference from bilirubin in fecal samples is minimized by utilizing the iron reaction rather than the Liebermann-Burchard reaction (11), and by using absorbance at 560 nm (10).

All data were subjected to standard analysis of variance and LSD tests (12).

RESULTS

Experiment I

The effect of Solka-Floc diets or agar upon serum, liver, and fecal lipid levels in cholesterol-fed rats was studied in this experiment. Both Solka-Floc diets and one of the agar diets were prepared as dry powders. The other agar diet was prepared in the gelled form. The results of this experiment are found in Table II. Although no differences in weight gain between groups were observed, the group fed the agar gel diet (Group C) consumed less diet and showed improved feed efficiency compared to the other groups. Serum cholesterol analysis after 28 days on experimental diets indicated all groups fed 1% cholesterol had significantly increased ($P<0.05$) serum cholesterol. Cholesterol analysis of serum obtained via heart punctures of two animals per group on day 7, 14, 21 (not shown) also revealed increased (nonsignificant) blood cholesterols in Groups B-D compared to the control Group A. Cholesterol feeding increased liver lipids, liver cholesterol, fecal lipids, and fecal sterols. In addition, the cholesterol-fed dry diet groups (Groups B and D) had significantly higher liver cholesterol concentrations (shown) and total liver cholesterol contents (not shown) than the cholesterol-fed agar gel diet group (Group C).

Experiment II

The results of Experiment II are found in Table III. In this experiment, serum, liver, and fecal lipid levels were determined in groups of rats fed 2% dry Solka-Floc or 2% agar gel diets. Within fiber type, groups received control, 1% crystalline, or 1% dispersed cholesterol diets. Feed efficiencies of all agar gel diets were increased as compared to the corresponding Solka-Floc diets. Liver weights (as % of body weight) increased in cholesterol-fed rats. Small, but significant, increases in serum cholesterol were found in all groups compared to the Solka-Floc control diet group (Group A). Serum cholesterol for Group D was not significantly lower than Groups E and F after 4 weeks, but was less ($P<0.05$) after 21 days (not shown). Liver lipids and cholesterol and fecal lipids and sterols were elevated in cholesterol-fed groups. Agar gel cholesterol-fed groups had higher fecal sterols and lower liver cholesterol than the corresponding Solka-Floc cholesterol-fed groups.

DISCUSSION

Agar is a polymer of D-galactose residues and 3,6 anhydro-L-galactose residues with a half-ester sulfate on approximately every 10th galactose residue, while Solka-Floc is 85% wood alpha cellulose with 15% nonglucose hemicellu-

TABLE III

Influence of Dispersing Cholesterol and Gelling Diets upon Serum, Liver, and Fecal Lipids (Experiment No. 2)

	Group A control dry diet (Solka-Floc)	Group B 1% crystalline chol dry diet (Solka-Floc)	Group C 1% dispersed chol dry diet (Solka-Floc)	Group D control gel diet (agar)	Group E 1% crystalline chol gel diet (agar)	Group F 1% dispersed chol gel diet (agar)
Weight gain, g	194 ± 11[a,b]	199 ± 16	196 ± 11	203 ± 10	195 ± 11	208 ± 10
Food intakes, g	409 ± 18	412 ± 29	403 ± 23	405 ± 21	394 ± 19	407 ± 20
Feed efficiency	0.475 ± 0.021^{A}	0.483 ± 0.019^{A}	0.487 ± 0.014^{AB}	0.502 ± 0.022^{CD}	0.496 ± 0.012^{BC}	0.511 ± 0.0216^{D}
Liver weight as a % of body weight	4.9 ± 0.4^{A}	5.8 ± 0.5^{BC}	5.9 ± 0.4^{C}	4.9 ± 0.2^{A}	5.4 ± 0.3^{B}	5.7 ± 0.3^{BC}
Final serum cholesterol mg/100 ml[c]	90 ± 8^{A}	111 ± 16^{B}	116 ± 13^{B}	110 ± 11^{B}	113 ± 12^{B}	110 ± 12^{B}
Liver lipids, %	3.8 ± 0.2^{A}	7.9 ± 0.9^{B}	8.2 ± 0.7^{B}	3.6 ± 0.2^{A}	7.1 ± 0.5^{B}	7.6 ± 1.5^{B}
Liver cholesterol, mg/g fresh liver	3.0 ± 0.7^{A}	5.9 ± 0.9^{CD}	6.7 ± 1.0^{D}	3.3 ± 0.7^{A}	4.9 ± 0.9^{B}	5.3 ± 1.1^{BC}
Dry fecal weight, g/day[d]	0.49 ± 0.08^{A}	0.61 ± 0.08^{BC}	0.62 ± 0.11^{BC}	0.54 ± 0.11^{AB}	0.65 ± 0.14^{C}	0.63 ± 0.06^{BC}
Fecal lipids, %[d]	3.5 ± 1.1^{A}	11.6 ± 2.5^{B}	10.7 ± 3.1^{B}	2.1 ± 0.7^{A}	10.1 ± 3.4^{B}	11.3 ± 1.9^{B}
Fecal sterols, mg/g dry feces[d,e]	4.9 ± 3.6^{A}	36.4 ± 14.7^{B}	34.6 ± 8.6^{B}	3.0 ± 2.6^{A}	61.8 ± 19.5^{C}	56.6 ± 16.7^{C}
Daily fecal, sterols, mg/day[d,e]	2.3 ± 1.8^{A}	21.9 ± 8.0^{B}	21.1 ± 8.0^{B}	1.8 ± 1.5^{A}	39.2 ± 12.9^{C}	36.2 ± 12.2^{C}

[a]Mean ± SD for ten rats.

[b]Values in the same line not sharing a common superscript capital letter are significantly different ($P<0.05$).

[c]After 28 days on diet.

[d]Three-day collection period after 2 weeks on diet.

[e]$FeCl_3$ reaction-positive sterols.

lose (13). Agar, due to its gel forming properties and resistance to bacterial attack, is commonly utilized as a solid support for microbial growth while Solka-Floc is a common fiber used in animal feeds.

The rates of weight gain for female rats in the first experiment and for males in the second were at least as good as those reported for typical laboratory rats (14). Small, but significant, improvement in feed efficiency was observed on most of the agar gel diets as compared to the corresponding Solka-Floc diet groups. Rogers and Harper (1) reported that the increased rate of weight gain in agar gel diets compared to dry diets was primarily due to increased food intake. In the present work, less food intake was observed with female animals and no significant change was observed for the males. The growth and feed efficiency-promoting characteristics of agar gelling are of interest and merit further study.

As reported by Bartov et al. (15), the cholesterol-fed female rats attained higher serum cholesterol levels than the males. Solka-Floc control groups (Group A, Exp. I & II) had lower serum cholesterol levels than all of the cholesterol-fed groups after 28 days on diet. In Experiment II, only moderate increases of serum cholesterol were found. The agar gel control diet group (group D) had elevated serum cholesterol after 4 weeks but not after 3 weeks, compared to the Solka-Floc control. The 4-week data agree with previous reports (3,4) of nonsignificant increases in plasma cholesterol levels due to 5% agar feeding to cholesterol-fed rats. However, the 3-week serum analyses in Experiment II do not. This discrepancy may be due to the variable nature of serum cholesterol levels.

Kiriyama et al. (3) reported reduced cholesterol absorption in 5% agar, cholesterol-fed rats, but found, as did Wells and Ershoff (4), small increases in serum and liver cholesterol levels. Tsai et al. (5) reported elevated liver cholesterol in 5% agar cholesterol-fed rats in one of three experiments. They found no significant changes in serum, carcass, or aortic cholesterol levels but concluded that feeding agar probably resulted in an expansion of the whole body pool. Recently, Kelley and Tsai (6) found that 5% dietary agar decreased cholesterol absorption. Mathe' et al. (7) found decreased fecal excretion, an increased intestinal absorption coefficient, and decreased total body cholesterol synthesis in 2.3% agar-fed versus 2.3% cellulose-fed rats. All groups in that work received less than 0.5% dietary cholesterol. These studies (3-7) point to variable tissue cholesterol alterations in rats fed greater than 2% agar in dry diets. Increases in excretion of endogenous fecal nitrogen and corresponding decreases in apparent protein digestiblility and net protein utilization with 3% agar diets have also been reported (16).

In the present studies, cholesterol feeding increased liver and fecal lipids, liver cholesterol, and fecal sterols. Agar substitution for Solka-Floc in the dry cholesterol diets (Exp. I) resulted in no differences in liver cholesterol, fecal lipids, or fecal sterols, although liver lipids were elevalted. However, gelling the agar diet significantly reduced liver cholesterol but had no significant effect on fecal sterol excretion as compared to the other cholesterol-fed groups.

In the second experiment, one finds not only reduced liver cholesterol, but also increased fecal sterols in agar gel cholesterol-fed groups in comparison to Solka-Floc cholesterol-fed groups. Fecal sterols were obtained from 2-week data in this experiment. These 2-week data support the conclusion that the physical gelling of agar diets reduces dietary cholesterol absorption, although a role of agar gelling in increasing endogenous sterol losses can not be ruled out. Neither can an effect upon liver cholesterol synthesis be ruled out. The fact that no significant changes in fecal sterols were found in the first experiment could be explained by a differential sex response to gel diets or, more likely, an adaptation to gel diet (an increased cholesterol absorption capability) by the fourth week of feeding.

Kiriyama et al. (3) calculated cholesterol absorption by subtracting daily "corrected" fecal sterol excretion from daily cholesterol intake. Fecal sterol excretion was corrected by subtracting the fecal excretion of Liebermann-Burchard reaction-positive materials of a group receiving no dietary cholesterol from the fecal excretion of cholesterol-fed groups. This manipulation has inherent difficulties due to the variable nature of fecal sterol excretion and the fact that specific sterols vary greatly in color production with chromophores. Given the nonspecifity of such calculations, we have estimated apparent cholesterol absorptions for all cholesterol-fed groups and have found that significantly less cholesterol was absorbed from the gel diets (Groups E and F) in Experiment II than from the corresponding dry diets (Groups B and C). No differences in apparent cholesterol absorption were detected in Experiment I. These data manipulations lend some support for the conclusion that physical gelling of agar diets reduces dietary cholesterol absorption, at least after 2 weeks experimentation.

One of the objectives in the second experiment was to compare the hypercholesterolemic

effect of cholesterol added to diet in a crystalline form to that when cholesterol is dispersed in the oil portion of the diet prior to diet mixing. Small, nonsignificant increases of liver cholesterol and liver lipids with similar reduction of fecal sterols resulted from dispersing the cholesterol in the oil prior to mixing. No trends were found from serum cholesterol or fecal lipids. It would appear that intestinal uptake of cholesterol from "dispersed" cholesterol diets is slightly greater than from crystalline cholesterol diets. However, the difference is small and it has less of an effect upon cholesterol uptake than gelling of diets with agar. In a series of experiments, Kritchevsky et al. (17) found nonspecific changes in serum and liver cholesterol but did find increased atheromata in rabbits fed 2% cholesterol dissolved in oil rather than suspended in oil. They attributed the increased atherogenicity to the high level of free fatty acids resulting from hydrolysis of corn oil during the "dissolving" process (200 C for 15 min). The current study utilized both lower temperatures (60 C) and nitrogen to prevent fat hydrolysis.

The results of this study indicate significantly decreased liver cholesterol when 1% cholesterol agar diets are gelled. The data suggest that the physical gelling reduces cholesterol absorption, but adaptation may occur by the fourth week of feeding. Increases in feed efficiency in gel groups were also observed. Despite these and other changes, it would appear that an agar gel feeding system can be utilized for lipid studies as long as all groups receive their diets in a gel form. In addition, for the rat at least, it makes little difference whether cholesterol is added to the diet in a crystalline form or dispersed in oil.

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Models for Lipid Organization in Cholesterol-Phospholipid Bilayers Including Cholesterol Dimer Formation

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ABSTRACT

Three new structural models, which account for abrupt changes in physical properties observed at several molar concentrations of cholesterol in phospholipid bilayers, are described. Cholesterol monomers, each surrounded by its own envelope of unshared acyl hydrocarbon chains of the phospholipid, can accommodate 22% cholesterol. Cholesterol dimers, each surrounded by its own envelope of unshared acyl hydrocarbon chains, can accommodate 31% cholesterol. When surrounded by shared acyl hydrocarbon chains, cholesterol dimers can accommodate about 47% cholesterol. At greater concentrations, cholesterol aggregation occurs, the system is unstable, and cholesterol forms a separate phase.

INTRODUCTION

Cholesterol is a major lipid component of membranes. The membranes of the red blood cell and myelin nerve sheath contain about 40 mole percent cholesterol. Despite its prevalence, the role and disposition of cholesterol in membranes are not fully understood. Cholesterol fluidizes a bilayer in the gel (crystalline state) and rigidifies the hydrocarbon chains when the phospholipid is in a liquid crystalline state. Furthermore, cholesterol reduces the permeability of a phospholipid bilayer. The interaction of cholesterol with phospholipid in bilayers is fundamentally important to the understanding of membrane functions. The purpose of this paper is twofold: to propose three new models for phospholipid-cholesterol interactions over the entire span of attainable concentrations in a bilayer and in so doing to encourage experimentation for deciding the conditions and range of validity of four models for these interactions.

Most of the cholesterol lies within the hydrocarbon region of a phospholipid bilayer, oriented approximately perpendicularly to the bilayer surface with its β-OH group hydrogen bonded near the aqueous interface, perhaps to a carbonyl oxygen of an acyl hydrocarbon chain (1-3). Nuclear magnetic resonance studies have determined that the fused rings of cholesterol are more restricted in their motion than the hydrocarbon tail, which displays motional properties similar to the ends of the acyl hydrocarbon chains (4,5). Above the gel transition temperature in the liquid crystalline state, membranes and bilayers are fluid-like with rapid diffusion of most components.

The widely accepted model for cholesterol interaction in phospholipid membranes is that of Engelman and Rothman (6) (hereafter ER), wherein each cholesterol is disordered about its long axis and completely surrounded by acyl hydrocarbon chains, while at the same time the maximum number of acyl chains remains in the gel phase out of contact with cholesterol. ER then built a model in which each cholesterol is surrounded by seven acyl chains, almost all of which are shared by two separate cholesterol molecules. Two phases, a mixed cholesterol-phospholipid and a pure phospholipid phase, exist up to 35% cholesterol (all percentages in this paper are mole percents), whereafter some association of cholesterol molecules must, of necessity, take place. By this model, ER accounted for the disappearance at 30-33% cholesterol of the sharp 4.15 Å wide angle X-ray diffraction line, due to hexagonal gel phase packing of hydrocarbon chains, in a lamellar phase of dipalmitoyl phosphatidylcholine below the phase transition temperature. The ER model invokes phase separation even at low concentrations of cholesterol, does not apply to systems with greater than 35% cholesterol, and does not account for breaks observed in physical properties near 22% cholesterol to be discussed below.

It is possible to account for transitions over the entire range of cholesterol content in phospholipid bilayers by considering structural models which possess two new features: unshared acyl hydrocarbon chains and cholesterol dimer formation. Three new models are proposed and developed in the same style that ER used for their model.

RESULTS AND DISCUSSION

If both monomers and dimers of cholesterol are considered together with the question whether phospholipid chains are in contact with only one monomer or dimer or are shared by a pair of them, four structural models are

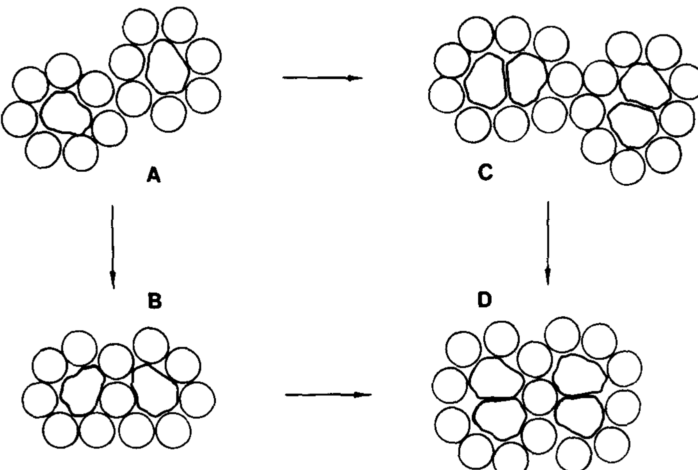

FIG. 1. Representation of models for packing in the hydrocarbon region of cholesterol-phospholipid bilayers. The view is perpendicular to the bilayer plane with the irregular shapes cholesterol molecules and the circles phospholipid acyl hydrocarbon chains both viewed down their long axes. Each model is depicted at the composition where the maximum amount of cholesterol can be incorporated: model A, 22% cholesterol; B, 35%; C, 31%; and D, 47%. At lesser amounts of cholesterol, more phospholipid occurs among cholesterol monomers in model A and cholesterol dimers in model C. In models B and D, where the acyl hydrocarbon chains are shared, excess lipid appears as a second phase. Horizontal arrows refer to dimerization of cholesterol and vertical arrows to sharing of acyl hydrocarbon chains between cholesterol monomers at the left and cholesterol dimers at the right.

conceivable in a phospholipid bilayer. (A) Cholesterol exists as monomers and is surrounded by its own envelope of unshared phospholipid chains. (B) Cholesterol exists as monomers and is surrounded by phospholipid chains which are shared with another cholesterol monomer. This is the ER model. (C) Cholesterol forms dimers and each dimer is surrounded by its own envelope of unshared phospholipid chains. (D) Cholesterol forms dimers and each dimer is surrounded by shared chains as in B. Models A, C, and D are described here for the first time. Representations of the four models are shown in Figure 1.

It is possible to estimate the maximum mole percentages of cholesterol allowed in each of the four models. Following ER, we note that to surround a single cholesterol molecule, seven hydrocarbon chains or an average of 3.5 phospholipid molecules are required. Thus, in model A, 100/4.5 = 22% is the maximum amount of cholesterol that can be accommodated. In model B where acyl chains are to be shared between two cholesterol monomers, ER determined that an average of almost 3.8 chains is required. Their calculation then yields a maximum of 35% cholesterol that may exist in the structure of model B. By building models in the same fashion as ER, nine chains surround the cholesterol dimer leading to accommodation of up to about 31% cholesterol in model C. When these nine chains are shared by 2 dimers as in model D, the model can accommodate ca. 1.13 phospholipids per cholesterol molecule, corresponding to 47% cholesterol. Therefore, the maximum mole percentages of cholesterol allowed in each of the four models, A, B, C, and D are 22%, 35%, 31%, and 47%, respectively. Allowance for inefficiencies in packing may reduce all values except the second by 1 to 2%.

One of the novel features of two of the models proposed here is formation of cholesterol dimers. The favoring of some cholesterol-cholesterol over cholesterol-acyl hydrocarbon chain interactions is supported by X-ray crystal structures of two cholesteryl esters. In cholesteryl myristate (7) and cholesteryl-17-bromoheptodecanoate (8,9), the steroid fused rings and hydrocarbon chains separate into alternating regions. The steroid fused rings appear in double layers such that the planar or α-faces are in contact only with other α-faces and similarly for the β-faces. It has been pointed out how a cholesterol dimer may be formed by van der Waals' interactions between two β-faces (7). β-Face interactions also occur between pairs of molecules in the structure of cholesterol monohydrate (10). A dimer involving the planar α-faces is also easy to visualize and permits closer contacts. In all these structures, the cholesterol β-OH group is not involved in dimer formation and would remain free to hydrogen bond as in the monomer. Specific

attractive interactions have also been suggested to occur between monomers in cholesterol micelles (11,12).

The four models described above suggest that phase boundaries occur at about 22%, 31-35%, and 47% cholesterol. Discontinuities or breaks in observed physical properties might then be expected to occur in phospholipid bilayers at or near these three compositions. Discontinuities or breaks in physical properties have indeed been reported to occur almost exclusively in these three regions of cholesterol molar concentrations. Some examples from the literature for each region are now mentioned.

The maximum amount of cholesterol that has been found to be stable without crystallizing out of lecithin bilayers corresponds to an approximate 1:1 ratio of the two kinds of lipid (13), close to the 47% cholesterol of model D. [One example of a 2:1 cholesterol to phospholipid complex proved unstable over time (14).] The acyl hydrocarbon chains achieve a maximum of nearly complete order at about 50% cholesterol (15,16). Also, at this composition, the glucose permeability of egg lecithin liposomes decreases to zero (17). These results are consistent with a transition from a phase of structure D to one in which cholesterol forms a separate phase. In structure D, all the acyl chains of the phospholipids are next to a rigid cholesterol so that maximum ordering of the hydrocarbon chains is achieved. Kink diffusion along the chains, which may be involved in glucose transport, is at a minimum in this phase, virtually eliminating glucose permeability. Thus, the cholesterol dimers with shared acyl hydrocarbon chains of model D with its implied ordering and reduced fluidity account naturally for the upper limit of stability and impermeability of cholesterol containing phospholipid bilayers. Model D, in conjunction with models B and C, provides a picture of the lipid interactions in the erythrocyte and myelin sheath membranes, each of which contain about 40% cholesterol.

An abrupt change has been observed in a variety of physical properties in bilayers at about 22% cholesterol. The band pattern in freeze-fracture electron microscopy is lost at about 20% cholesterol (18). The solidus curve in a phase diagram determined by a spin label in electron spin resonance spectroscopy shows a break from 20-25% cholesterol (19,20). A component in heat absorption determined calorimetrically disappears at cholesterol concentrations above 20% (21). The preceding three studies were performed on lecithins with saturated hydrocarbons chains. In a hydrodynamic study of egg lecithin vesicles, it was found that the number of phosphatidylcholine molecules per vesicle increases abruptly at 22% cholesterol, and at the identical composition, a maximum occurs in the frictional coefficient and the total and bound water per vesicle (22). Finally, the results of a wide angle X-ray scattering study suggest that a maximum in the thickness of the polar part of a partially unsaturated lecithin occurs close to 20% cholesterol (23). The structure represented by model A accommodates at most 22% cholesterol and the transition to another structure can account for the observed abrupt changes in physical properties.

A variety of experiments indicate that a phase boundary occurs in the region of 30-35% cholesterol. The wide angle X-ray result of ER was mentioned above. In differential scanning calorimetry, increasing concentrations of cholesterol cause a linear decrease in the heat of the gel to liquid crystalline transition until it vanishes near 33% cholesterol (24). Both of the above experiments were performed on phospholipids with saturated acyl chains. Proton magnetic resonance studies with egg lecithin vesicles with a partially unsaturated chain reveal discontinuities at about 30% in chemical shift, outside/inside mole ratios (25), and linewidths (26). In a hydrodynamic study of egg lecithin vesicles, the diffusion coefficient and the effective specific volume appear to change sharply at about 35% cholesterol, and the sedimentation coefficient undergoes an abrupt change with a transition midpoint at 32% cholesterol (22). Note that this study is the same as that referred to above and that, in this single system, abrupt changes occur at 22%, 32-35%, and near 50% cholesterol.

Within the experimental and modeling uncertainties, both models B and C with compositional limits at 35% and 31% cholesterol, respectively, can account for the results quoted in the previous paragraph. As indicated in Figure 1, both models B and C lead logically to model D, which accounts for the maximum stable 50% incorporation of cholesterol into phospholipid bilayers. The Engelman-Rothman (6) model B of cholesterol monomers with shared acyl hydrocarbon chains and pure phospholipid in two different phases is a state of relatively low entropy. Model C with cholesterol dimers surrounded by unshared acyl hydrocarbon chains is a one-phase system, and separation of two phases is unnecessary but may occur in the gel state. The results of freeze-fracture electron microscopy do not support the obligatory phase separation of model B (18,20). The loss of the 4.15 Å line in the ER wide angle X-ray study may also be accounted for by model C because, at 31% cholesterol, the

hexagonal array of pure phospholipid giving rise to the line no longer exists. Depending upon conditions and systems, perhaps the structures of both models B and C occur.

Increasing concentrations of cholesterol in bilayers produce transitions from one structural model to another. Assuming for simplicity that the structures represented by models B and C do not coexist significantly, the following sequences of transitions seem possible: B → D, A → B → D, A → C → D, and C → D. Except for the first two which are equal, the sequences are listed in order of increasing equilibrium constant for cholesterol dimer formation in a phospholipid bilayer. For systems with high values of the dimer formation equilibrium constant, structures corresponding to models A and B would not occur to a significant extent. The value of the equilibrium constant may depend upon the length of the acyl hydrocarbon chains, their degree of unsaturation, their distribution within a single phospholipid molecule, and the collection of molecules in the bilayer. These features may be important in determining the range of applicability of the models. The sequence (A →) C → D would appear capable of explaining virtually all the results mentioned in this paper. In this sequence at low concentrations, monomeric cholesterol is surrounded by unshared acyl hydrocarbon chains. At about 22% cholesterol, dimerization of cholesterol becomes important and, at about 31% cholesterol, the dimers begin to share acyl hydrocarbon chains. If the equilibrium constant for cholesterol dimerization is high, dimerization is complete at lower concentrations and the transition near 22% cholesterol does not occur. Finally, at greater than about 47% cholesterol, larger cholesterol aggregates appear, the system is unstable, and cholesterol forms a separate phase.

The interplay of the structures represented by the four models was suggested by the results quoted from the literature. Because of the difficulty in determining directly the behavior of cholesterol in bilayers, a more direct verification of the dimer hypothesis is challenging. Cholesterol enriched with ^{13}C in the ring system and incorporated into vesicles yields readily measurable ^{13}C NMR peaks (27). Since protonated carbons are dominated by dipolar relaxation from the attached protons, the rotational correlation time of cholesterol about its long axis, which might be sensitive to dimer formation, may be accessible from spin lattice relaxation time (T_1) measurements.

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Acid Triacylglycerol Lipase from Bovine Thyroid Gland

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ABSTRACT

An acid lipase has been detected in bovine thyroid tissue using triolein as a substrate. The activity, probably associated with the lysosomes, displays a rather broad pH-optimum in the pH 4 to pH 6.5 range. The lipase activity can be partially purified by cosedimentation with lysosomes followed by solubilization through detergent and chromatography on Sephadex G-200 and carboxymethyl cellulose. The elution profile on Sephadex G-200 shows one peak (moleculare weight 67,000 ± 2,000). In the final CM-cellulose step, two lipase peaks (lipase L_A and lipase L_B) are found. Sulhydryl reagents (iodoacetate, iodoacetamide, and N-ethylmaleimide) as well as mercuric ions markedly reduce both enzyme activities. Calcium ions, EDTA, and heparin have no effect. Sodium fluoride and diisopropylfluorophosphate are only slightly inhibitory. Sodium chloride causes a slight increase in both lipase activities. Anionic phospholipids such as cardiolipin and phosphatidylserine are not essential for enzyme activity.

INTRODUCTION

In mammals, triacylglycerol lipase activity (EC 3.1.1.3) has been described in various tissues (1). However, very little is known about the enzymes responsible for triacylglycerol catabolism in thyroid cells.

In order to explain the stimulatory effect of cAMP on prostaglandin synthesis in thyroid (2), Haye et at. (3) suggested that, apart from a phospholipid precursor pool (phosphatidylinositol), the neutral lipids (triacylglycerols) could also serve as an arachidonic acid pool. From these triacylglycerols, arachidonate is liberated by a cAMP-dependent lipase. They also observed a high incorporation of [^{14}C]-arachidonate into diacylglycerols (4). However, the mechanism by which these diacylglycerols are formed is not known.

In this paper, the presence of triacylglycerol lipase in bovine thyroid is reported. Some enzyme properties were studied after partial purification. Finally, the subcellular localization of the enzyme has been investigated by centrifugation techniques.

MATERIALS AND METHODS

Chemicals

Diisopropylfluorophosphate (2359p), phosphatidylserine (4647h), sodium fluoride (8995p), and iodoacetamide (3441h) were purchased from Koch-Light Laboratories Ltd. Bovine albumin (fraction V powder; puriss. A-4503), triolein (T-7879), N-ethylmaleimide (E-3876), p-nitrophenyl esters of saturated fatty acids and cardiolipin (bovine heart; C-3760) were obtained from Sigma Chemical Co. Molecular weight standards for gel chromatography were from Serva (kit MSII 39064). Glycero-tri-[1-^{14}C]-oleate (specific activity 41 mCi/mmole) was acquired from the Radiochemical Centre, Amersham. Titriplex III (Art. 8418), mercuric nitrate (Art. 4419), and iodoacetate (Art. 374) from Merck, Sephadex G-200 (particle size 40-120 μ) from Pharmacia Fine Chemicals, and CM-52 carboxymethyl cellulose from Whatman Biochemicals Ltd. were used. Heparin sodium salt ($\geqslant$ 140 iu/mg) was obtained from Fluka A.G. (51550). Fluorescamine was acquired from Roche Diagnostica (Fluram). Aquasol was obtained from New England Nuclear, and Triton X-100 was obtained from Rohm and Haas. All other solvents and reagents were analytical grade and used without further purification.

Chemical Analyses

Proteins were determined as previously described (5) or fluorimetrically according to Sims and Carnegie (6) with bovine serum albumin as a standard. Cholesterol, phospholipids, and lipid-bound sialic acid were determined as described by Martin et al. (7).

Enzyme Assays

Lipase activity: Incubation conditions were essentially the same as described by Mahadevan et al. (8). However, a labeled substrate was used. A chloroform solution containing Triton X-100 (125 mg/ml), glycerol-tri-[1-^{14}C]-oleate, and carrier (10 μmoles/ml) was evaporated under a stream of nitrogen. Remaining traces of chloroform were eliminated by drying under vacuum during 1 hr at room temperature. The dry residue was dispersed ultrasonically (Braun-Sonic 300, setting 60, 3 min, 0 C) in distillled water (1 μmole substrate per 0.1 ml). During sonication, small amounts of monoacylglycerols ($<$0.5%) and diacylglycerols (0.5%) were formed. Substrate emulsions were prepared

TABLE I

Purification of Acid Triacylglycerol Lipase (EC 3.1.1.3) from Bovine Thyroid

		Protein (mg)	Total activity	Specific activity[a]	Percent recovery	Purification factor
Step 1.	Homogenate	16,223	199,400	12		
Step 2.	M+L fraction	767	69,409	90	35	7
Step 3.	Solubilization	441	68,354	155	34	13
Step 4.	Ammonium sulfate precipitation	245	61,139	250	31	20
Step 5.	Sephadex G-200	39	64,304	1,640	32	133
	Dialysis	25	42,532	1,736	21	141
Step 6.	CM-cellulose					
	Lipase L_A[b]	3	9,187	3,168	5	258
	Lipase L_B[c]	2	27,180	15,100	14	1,229

[a]nmoles 1,2-(2,3-)diacylglycerols + 1,3-diacylglycerols released/mg protein/hr.
[b]Data obtained after pooling fractions as indicated by arrows in Figure 2.
[c]Data obtained after pooling fractions as indicated by arrows in Figure 2. Experimental conditions: see Material and Methods subenzyme assay:lipase activity (substrate:triolein).

fresh on the day of use. Triolein suspension (0.1 ml containing 1 μmole [^{14}C]-triolein and 12.5 mg Triton X-100) was incubated with 0.2 ml enzyme extract and 0.2 ml 0.25 M sodium citrate buffer pH 5.0 during 45 min (unless stated otherwise). All enzyme assays were performed in screw-capped test tubes at 37 C under continuous shaking. The reactions were stopped by the addition of 1.9 ml chloroform-methanol (1:2, v/v), and the reaction products extracted according to the method of Bligh and Dyer (9). The hydrolysis products were separated from substrate by thin layer chromatography (Silica Gel G, 0.3 mm/20 x 20 cm) using petroleum benzene (boiling point range 60-80 C)-diethyl ether-acetic acid (60:40:1, v/v/v) as solvent system. The compounds were visualized with iodine vapor and the spots scraped into liquid scintillation counting vials containing 10 ml Aquasol.

Esterase assay: Chloroform solutions of p-nitrophenyl esters and Triton X-100 were mixed and evaporated to dryness under a stream of nitrogen. Traces of chloroform were further removed by drying under vacuum (1 hr at room temperature). The dried compounds were dispersed ultrasonically (Braun-Sonic 300, setting 60, 2 min, 0 C) in acetate buffer. The reaction (at 25 C) was started by the addition of enzyme protein (0.2 ml). Reaction mixture (final volume 1.0 ml) contained 2.5 mM ester, 50 mM sodium acetate buffer, and 16 mg Triton X-100. The release of p-nitrophenol was measured at 336 nm and recorded continuously with a Varian double beam spectrophotometer (model 635). A control reaction mixture containing no enzyme was used as a blank.

Marker enzymes: Most marker enzymes were determined as described earlier (10) except for monoamine oxidase (11) and acid ribonuclease (12).

Subcellular Fractionation

Differential pelleting and buoyant-density gradient centrifugation in a zonal rotor were performed as described by Hilderson et al. (10).

Purification of Acid Triacylglycerol Lipase

The purification scheme finally adapted for acid lipase consisted of six major steps.

Step 1 – Preparation of cell homogenate: Bovine thyroid glands were obtained fresh from the slaughterhouse. After anatomic preparation, the tissue was cut into small cubes and washed several times until free of blood. Portions of 100 g were homogenized in 4 volumes of 5 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose in a Waring commercial blendor (high speed, 30 sec at 4 C). Portions (25 ml) of the resulting suspension were homogenized in a Potter-Elvehjem homogenizer (Teflon pestle; 3,000 rev/min; 6 strokes).

Step 2 – Preparation of a combined mitochondrial and light mitochondrial (M+L) fraction: To remove blood cells, connective tissue, cell debris, and nuclei, the homogenate was centrifuged at 1,000 x g for 3 min in a MSE Mistral 6L centrifuge. The ensuing supernatant was aspirated and centrifuged during 10 min at 73,300 x g (6 x 100 ml angle rotor; MSE 18 centrifuge) resulting in a M+L sediment.

Step 3 – Solubilization: The M+L pellet was suspended in 10 mM sodium acetate buffer pH 5 containing 0.14 M NaCl and 1% (w/v) Triton X-100 and rehomogenized in a Potter-Elvehjem homogenizer (3,000 rev/min; 5 strokes). The resulting suspension was left overnight at -20 C. After thawing, 25-ml portions were subjected to ultrasonic disintegration in a Braun-Sonic

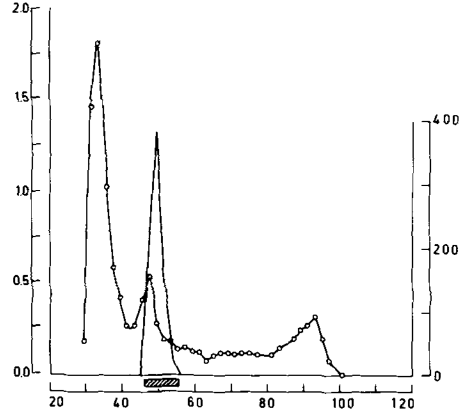

FIG. 1. Gel chromatography on Sephadex G-200. Experimental conditions: see Materials and Methods substep 5 of the purification scheme. Ordinate (left): mg protein/ml (Lowry method); (right): nanomoles diacylglycerols/hr/ml. Abscissa: fraction number (10 ml/fraction); ○—○ proteins; —— acid lipase; ▨ fractions pooled for CM-cellulose chromatography.

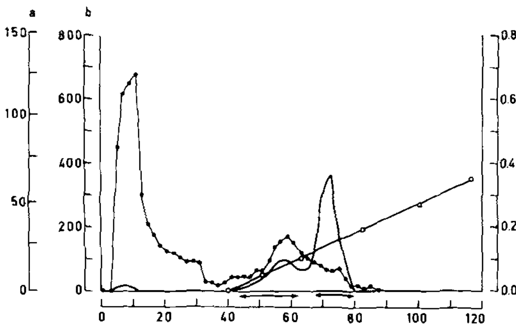

FIG. 2. Ion exchange chromatography on CM-cellulose. Experimental conditions: see Materials and Methods substep 6 of the purification scheme. Ordinate (left): (a) μg protein/ml (fluorimetric assay); (b) nanomoles diacylglycerols/ml/hr; (right): NaCl concentration (in moles/l). Abscissa: fraction number (10 ml/fraction); ●—● proteins; — acid lipase; ○—○ NaCl gradient.

300 (setting 60, 3 min). During sonication, the suspension was chilled in crushed ice. Subsequently, the extract was centrifuged during 1 hr at 144,700 x g (MSE 65 ultracentrifuge). The resulting sediment was discarded.

Step 4 – Ammonium sulfate precipitation: The extract was stirred until 50% saturation was reached. This mixture was stirred for 1 hr and centrifuged (144,700 x g, 1 hr, MSE 65 ultracentrifuge). The supernatant was discarded and the pellet dissolved in 10 mM sodium acetate buffer pH 5 containing 0.14 M NaCl and 1% Triton X-100 (total volume: 25 ml).

Step 5 – Gel chromatography: Gel chromatography was conducted on a Sephadex G-200 column (2.9 cm x 140 cm). The column was equilibrated with 10 mM sodium acetate buffer pH 5.0 containing 0.14 M NaCl and 1% Triton X-100 and eluted with the same solvent at 4 C. Fractions of 10 ml were collected at a flow rate of 10 ml per hr. The Sephadex G-200 fractions containing the acid lipase activity were combined and desalted by dialysis against 10 mM sodium acetate buffer pH 5 containing 1% Triton X-100 (48 hr, dialysis solution replaced after 24 hr). The content of the dialysis bag was centrifuged at 144,700 x g for 1 hr in a MSE 65 ultracentrifuge.

Step 6 – Carboxymethyl cellulose chromatography: The ensuing clear supernatant was loaded on a CM-52 carboxymethyl cellulose column (2 cm x 30 cm) equilibrated with 10 mM sodium acetate buffer pH 5.0 containing 1% Triton X-100. Elution was started with 400 ml of equilibrating buffer followed by sodium chloride linear gradient elution (800 ml, linear gradient in equilibrating buffer from 0-0.35 M NaCl). The flow rate was adjusted to 15 ml/hr and fractions of 10 ml were collected.

RESULTS

Purification

The results of the final purification scheme are presented in Table I. Figure 1 illustrates the results obtained by gel chromatography on Sephadex G-200 (step 5). Proteins eluted in three distinct peaks. Lipase eluted as a single peak almost coinciding with the second protein peak. Figure 2 depicts the elution behavior of protein and lipase activity in step 6 (CM-cellulose chromatography). The bulk of the proteins did not adsorb on the column, while the acid lipase could only be eluted from the column by means of a sodium chloride gradient. The lipase profile displayed two peaks: lipase L_A at 0.11 M and lipase L_B at 0.17 M NaCl. Lipase L_A did coincide with the protein peak in the gradient. For lipase L_A and lipase L_B purification factors of about 260 (5% recovery) and about 1230 (14% recovery) were achieved, respectively.

Properties of the Acid Lipase

Lipase activity in bovine thyroid is optimally active in the pH 4-6.5 range (Fig. 3). Digesting glycerol-tri-[1-^{14}C]-oleate indicated specificity for the primary esters. By thin layer chromatography, three times more 1,2-(2,3)-diacylglycerols were found than 1,3-diacylglycerols. Further deacylation of the diacylglycerols was also observed. The stoichiometric recovery of the reaction products suggested that in the experimental conditions no further degradation

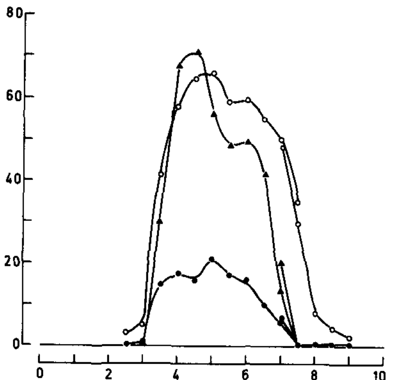

FIG. 3. Acid lipase activity in a mitochondrial fraction of bovine thyroid as a function of pH. Incubation: see Materials and Methods subenzyme assay: lipase activity, except that Tris-buffer was used in the pH 7 to 9 range. Ordinate: nanomoles reaction product formed. Abscissa: pH of incubation; ○—○ 1,2- (2,3)-diacylglycerols; ●—● 1,3-diacylglycerols; ▲—▲ monoacylglycerols.

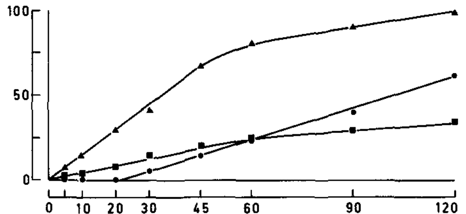

FIG. 4. Acid lipase activity in a mitochondrial fraction of bovine thyroid as a function of incubation time. Incubation: see Materials and Methods subenzyme assay:lipase activity, except that the incubation time varied from 5 up to 120 min. Ordinate: nanomoles reaction product formed. Abscissa: incubation time (min); ▲—▲ 1,2- (2,3)-diacylglycerols; ■—■ 1,3-diacylglycerols; ●—● monoacylglycerols.

of monoacylglycerols occurred. The rate of hydrolysis of glycerol-tri[1-^{14}C]-oleate was linear up to 45 min of incubation time (2.48 mg protein/ml). After a lag period of about 20 min, a progressive accumulation of glycerol monooleate was noted (Fig. 4). Acid lipase activity measured as the accumulation of 1,2-(2,3)-diacylglycerols (incubation time, 30 min) was directly proportional to the amount of enzyme added up to 1.5 mg protein.

From Sephadex G-200 column chromatography (step 5), a molecular weight of 67,000 ± 2,000 was estimated for acid triacylglycerol lipase. After heating lipase L_A and L_B preparations for 10 min at 60 C, no drop in activity occurred, while at higher temperature (5 min at 75 C) the enzyme activity was almost completely abolished. Upon storage of lipase L_A and lipase L_B preparations at +4 C, the enzyme activity was stable for at least 3 months. Freezing and thawing, however, caused a rapid denaturation (one treatment reduced the activity about 80%). In Table II, the influence of several effectors on the partially purified lipase L_A and L_B is summarized. Addition of iodoacetate (50 mM), iodoacetamide (50 mM), and N-ethylmaleimide (5 mM) decreased enzyme activities by about 60%, 20%, 45% for lipase L_A and 70%, 10%, 40%, for lipase L_B respectively. Increasing concentrations of

TABLE II

Influence of Effectors on Lipase L_A and Lipase L_B[a]

Effector	Concentration	Percent activity	
		Lipase L_A	Lipase L_B
$CaCl_2$	5 mM	109	100
EDTA	5 mM	106	113
Diisopropylfluoro-	0.1 mM	98	99
phosphate	1 mM	85	72
NaF	1 mM	85	86
NaCl	1 M	120	136
Iodoacetate	5 mM	93	102
	50 mM	42	30
Iodoacetamide	5 mM	97	103
	50 mM	81	89
N-ethylmaleimide	1 mM	102	109
	5 mM	54	62
$HgCl_2$	1 mM	92	96
	5 mM	73	83
	10 mM	57	57
Heparin	10 μg/ml	94	91
Cardiolipin	240 μg/ml	102	106
Phosphatidylserine	240 μg/ml	89	82

[a]Experimental conditions: see Material and Methods subenzyme assay:lipase activity (substrate:triolein).

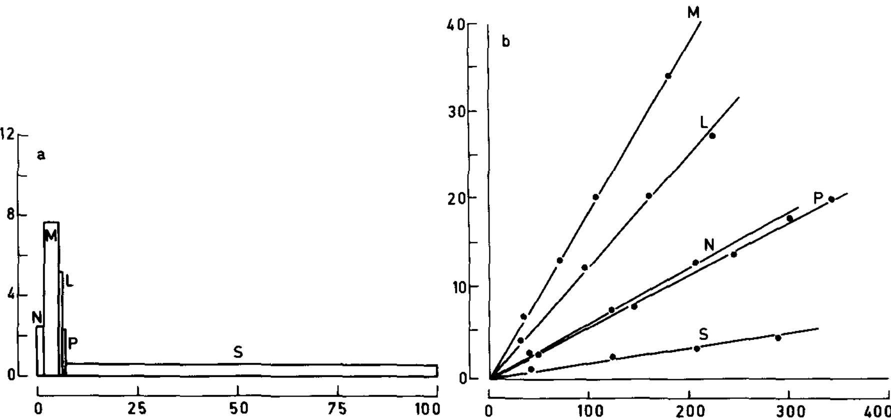

FIG. 5. Acid lipase activity in subcellular fractions. Experimental conditions: see Materials and Methods. N, nuclear fraction; M, mitochondrial fraction; L, light mitochondrial fraction; P, microsomal fraction; S, supernatant. (a) Distribution pattern after differential pelleting. Ordinate: relative specific activity. Abscissa: % total protein recovered in each fraction. (b) Lipase activity as a function of protein concentration. Ordinate: enzyme activity [nanomoles 1,2-(2,3)-diacylglycerols formed]. Abscissa: protein concentration (μg/incubation).

mercuric ions (1, 5, and 10 mM) also decreased the activities of both lipase peaks. The mercuric ion effect was more pronounced when lipase activity was assayed at pH 4 (only 20% activity remained in the presence of 10 mM Hg^{2+}). Sodium chloride (1 M) had a slightly activating effect. Heparin and albumin (0.4 mg/ml) did not affect enzyme activities. The anionic detergent sodium taurocholate (0.75 mg/ml) decreased activity by 35%. Anionic phospholipids such as phosphatidylserine and cardiolipin (240 μg/0.5 ml) did not cause any activation. Rechromatography of the purified lipase L_B on CM-cellulose, but omitting Triton X-100, resulted in a complete loss of the enzyme activity. The purified enzyme preparation also exhibits some p-nitrophenylesterase activity, as was also reported for pancreatic lipase (1). For long chain fatty acid derivatives, substantially higher velocities were noted than for the corresponding acetate derivative, a seven times higher activity being observed for p-nitrophenylcaprylate. For the myristate ester, the hydrolysis rate was still five times higher than for the acetate ester. Medium and long chain fatty esters of p-nitrophenol as well as triolein were hydrolyzed at comparable rates.

Subcellular Localization

The distribution pattern (obtained through differential pelleting) of markers and marker enzymes were extensively discussed in previous papers (10,13,14). The distribution pattern of lipase activity, as obtained by differential pelleting, is represented in Figure 5a (recovery 85 ± 11%). In each subfraction, the enzyme activity was determined within the linear range of the calibration curve as depicted in Figure 5b. The enzyme showed the highest relative specific activity (7.7) in the mitochondrial fraction. Comparison with marker enzymes of mitochondria, plasma membranes, and endoplasmic reticulum membranes suggests that a localization of acid triacylglycerol lipase in those membranes is rather unlikely. The triacylglycerol lipase follows more or less the distribution of some lysosomal markers.

Additional indication for a lysosomal localization was obtained by subjecting a M+L fraction to isopycnic gradient centrifugation in a B-XIV zonal rotor (15).

DISCUSSION

Attempts to isolate and purify intracellular triacylglycerol lipases have not been very successful mainly due to the association of lipids with the enzymes (16-19). Using a six-step purification scheme, we finally succeeded in partially purifying the acid triacylglycerol lipase activity present in bovine thyroid. For the lipase L_B activity, the highest purification factor was achieved (± 1,230; recovery 14%). The lipase L_A activity (purification factor ± 260; recovery 5%) coincides with the protein peak eluting in the gradient. At this moment, no clear-cut conclusion can be drawn regarding a possible relationship between those two lipase activities. Since recoveries of activities were relatively satisfactory, requirement for a cofactor seems less probable. This is at variance with the results obtained for rat liver lysosomal lipase

showing an absolute requirement for phospholipids (16). Anionic phospholipids such as cardiolipin and phosphatidylserine do not have any effect on thyroidal lipase. No activation occurred on adding exogenous phospholipids after each step of the purification scheme. Although a progressive depletion of endogenous phospholipids was noted (as controlled by thin layer chromatography) during the successive steps of the purification scheme, even the most purified preparation does not need exogenous phospholipids. Moreover, the recovery of enzyme activity during purification was never drastically impaired. Therefore, endogenous phospholipids probably do not function as activators of thyroidal lipase.

The enzyme activity studied may be classified as a triacylglycerol acylhydrolase (systematic name: EC 3.1.1.3) as it is capable of hydrolyzing long chain acylglycerols (triolein) at an oil-water interface (1). Moreover, it shows specificity for primary esters, producing a 1,2-(2,3)-dioleate over 1,3-dioleate rate of three. The formation of the 1,3-isomer is probably due to spontaneous isomerization through migration (1,20). The chain length specificity of the enzyme was studied by using p-nitrophenyl monoesters of different chain length (2C → 18C), maximal rate of hydrolysis being observed when using p-nitrophenylcaprylate. The enzyme cannot be classified as a lipoprotein lipase because it shows no activation by heparin, was not inhibited by 1 M NaCl, and did not require addition of serum protein cofactor in the incubation mixture. These criteria have been used by most investigators to identify lipoprotein lipase activity. However, they do not apply to the hepatic lipoprotein lipase (1).

Many hydrolases are serine enzymes being inhibited by organophosphorous compounds such as diisopropylfluorophosphate. Lipase of bovine thyroid, in analogy with the lysosomal (16) and mitochondrial (19) lipases of rat liver, is less sensitive to this reagent. However, one must be cautious in interpreting this result. Indeed, lipase of pig pancreas (purified till homogeneity) is also not inhibited by diisopropylfluorophosphate, although it has been identified as a serine enzyme (21,22).

Sulfhydryl reagents were the most effective inhibitors for thyroidal lipase, suggesting the involvement of sulfhydryl groups in the catalytic mechanism. They also inhibit other lipases (23,24). However, lipase from pig pancreas that is partially inhibited by p-chloromercuribenzoate (1) is not a sulfhydryl enzyme.

Subcellular localization studies are complicated by the drastic procedures required for homogenization of thyroid tissue. Nevertheless, the results available allow some conclusions to be drawn by reasoning through elimination (10,13,15). Thyroidal acid triacylglycerol lipase is most probably localized in the lysosomes. Moreover, lysosomal triacylglycerol lipase has been demonstrated in other mammalian tissues (1).

The full physiological significance of bovine thyroidal lipase remains uncertain. It could function by providing fatty acids for covering the energy requirements of the cell. Moreover, a function specifically related to hormone production in conceivable. As already mentioned, Haye et al. (4) suggested that arachidonate is liberated from triacylglycerols by a hormone-dependent lipase. However, there is disagreement in the literature about the arachidonate content in thyroidal triacylglycerols (4,25). Because of its lysosomal localization, low molecular weight, and small effect of sodium fluoride, the lipase reported here is probably not such an enzyme under direct hormonal control. However, the enzyme described in this paper could eventually be controlled in a more indirect way by the effect of thyroid-stimulating hormone on the behavior of lysosomes.

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Sterol Metabolism Studies in Rats: Effects of Taurodeoxycholic Acid Feeding on Sterol Metabolism

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ABSTRACT

Sterol metabolism studies using isotopic and chromatographic techniques were carried out in: (a) control rats fed stock chow + 0.1% cholesterol (control group), and (b) rats fed stock chow + 0.1% cholesterol and supplemented with 0.5% sodium taurodeoxycholate (taurodeoxycholate group). Feeding the bile acid enriched diet led to decreased acidic steroid synthesis, decreased cholesterol turnover, and cholesterol balance compared to nonsupplemented controls. There were no significant differences in fecal neutral sterol output, endogenous neutral sterol output, or cholesterol absorption between bile acid fed animals and controls. Tissue cholesterol levels (liver, plasma, and bile) in the two groups were also similar.

INTRODUCTION

We have previously reported the effects of sodium taurocholate feeding on cholesterol and bile acid metabolism in rats (1,2). These studies showed that sodium taurocholate administration at the 0.5% level increased cholesterol absorption while depressing cholesterol and bile acid synthesis. Significant amounts of deoxycholic acid, the 7α-dehydroxylation product of cholic acid, were formed during cholic acid feeding. To determine whether the metabolic effects we observed were due to taurocholate and/or taurodeoxycholate, the present investigation was carried out.

MATERIALS AND METHODS

Animals and Diet

Male Sprague-Dawley derived rats weighing between 180-240 g were purchased from Charles River Breeding Laboratories, Wilmington, MA. The animals were placed in metabolic cages and given access to food and water ad libitum. The cages allowed for quantitative feces collection and determination of food intake. The animals were fed a stock diet consisting of Rockland rat chow supplemented with 5% corn oil. The diet contained 0.30 mg/g cholesterol and 0.69 mg/g β-sitosterol. The control chow was supplemented with 0.1% cholesterol (for the control rats) and with 0.1% cholesterol + 0.5% sodium taurodeoxycholate (for the bile acid group). These materials were dissolved in ethanol, thoroughly mixed with the food, and the ethanol was allowed to evaporate.

On the first day of the experimental period, each animal was given an intraperitoneal injection of 10 μCi of DL-[2-^{14}C]mevalonolactone, and the experimental feeding period was started several hours later. The experimental and the control animals were studied simultaneously. Three 2-day fecal pools (days 10, 12, and 14) were collected, dried, and ground in a mortar for subsequent neutral sterol and bile acid analyses. Beginning on day 4 and every 2 days thereafter, blood was obtained from the tail vein for determination of plasma cholesterol concentration and plasma cholesterol specific activity. At the end of the 14-day experiment (10:00 a.m.), the rats were weighed, anesthetized with Diabutal (Diamond Laboratories, Des Moines, IA), cannulas were inserted into the common bile duct, and bile was collected for 1 hr to determine biliary cholesterol and bile acid concentrations and specific activities. A section of the liver was excised for determination of liver cholesterol concentration and specific activity as described previously (1,2).

Labeled Compounds

DL-[2-^{14}C]mevalonolactone (Amersham-Searle, Arlington Heights, IL) was dissolved in sterile isotonic saline to give a final concentration of 10 μCi/ml.

Reference Compounds

Cholesterol (U.S.P., Nutritional Biochemical Corp., Cleveland, OH) was recrystallized from ethanol. Taurodeoxycholic acid (sodium salt) was obtained from Calbiochem, In., (Oak Grove Village, IL) and found to be greater than 98% pure by thin layer chromatography (TLC) (3). 5α-Cholestane (Applied Science Laboratories, State College, PA) was used as an internal standard for the gas liquid chromatographic

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TABLE I

Weights, Food Intakes, and Fecal Outputs of Rats[a,b]

Group no.	Diet (no. of animals)	Initial weight (g)	Weight at death (g)	Food intake[c] (g/day)	Fecal output[d] (g/day)
1	Control diet (3)	181 ± 18 (169-202)	292 ± 14 (277-301)	26.3 ± 2.2 (23-30)	7.4 ± 0.8 (7.0-8.5)
2	Sodium taurodeoxycholate - 0.5% (5)	187 ± 9 (179-200)	281 ± 18 (265-312)	26.8 ± 2.0 (23.0-30.0)	8.0 ± 1.0 (7.0-9.5)

[a]Rats were fed the experimental diets for 14 days.
[b]Values reported represent average ± SD; numbers in parentheses represent the range.
[c]Values represent the average food intake from days 4-14 of the experiment.
[d]Values represent the average daily fecal outputs (three pools per animal) on days 9-14 of the experiment.

TABLE II

Representative Specific Activities of Cholesterol in Liver, Plasma, and Bile on Day 14[a]

Group No.	Animal[b]	Liver cholesterol specific activity (dpm/mg)	Plasma cholesterol specific activity (dpm/mg)	Bile cholesterol specific activity (dpm/mg)
1) Control	A	2120	2680	1880
	B	3670	3520	3560
	C	2840	3200	2600
2) Sodium Taurodeoxycholate	A	5560	6000	5500
	B	6100	6500	6060
	C	4900	4510	4360
	D	5880	6600	5520
	E	6650	6290	6950

[a]Determined by combined TLC/GLC techniques and liquid scintillation counting on day 14 of the experiment.
[b]Represents the animals in each group.

separations. 3α,7α-Dihydroxy-12-keto-5β-cholanoic acid, used as internal standard for the bile acid analyses, was synthesized according to the procedure of Fieser (4).

Thin Layer Chromatography

The TLC of the neutral and acidic steroids was carried out as previously described (5,6).

Gas Liquid Chromatography (GLC)

The methods and conditions of all gas liquid chromatographic analyses have been described in detail (5,6).

Methods for the Isolation and Quantitation of Neutral and Acidic Steroids from Feces

The methods used for extraction of the neutral and acidic steroids have been described (6,7). The material in each fraction was analyzed by GLC of the trimethylsilyl ether (TMSi) derivatives. 5α-Cholestane was used as an internal standard. The recovery of β-sitosterol was used as an index to correct for losses of neutral sterols. Since the recovery was 90% or better, no corrections were required.

Quantitation of the fecal bile acids was carried out using methods previously described (1). The specific activity of plasma cholesterol was determined 6 days prior to the collection of the fecal sample. The total radioactivity in the fecal acidic steroid fraction (dpm) divided by the plasma cholesterol specific activity (dpm/mg) 6 days earlier yielded the amount of newly synthesized bile acids in the feces. Corrections for losses were made where required using 3α,7α-dihydroxy-12-keto-5β-cholanoic acid (1).

Methods for Determination of the Specific Acitivity and Concentration of Cholesterol in Plasma, Bile, and Liver

These methods have been described in detail (1,2).

Methods for Determination of Biliary Bile Acids

Bile (0.1 ml) was placed in a tube, and 2 ml of 2N NaOH were added. Samples were autoclaved for 3 hr at 14-15 psi, cooled in ice, and 2.5 ml of water was added. Concentrated HCl was used to bring the pH to 1-2. The bile acids

were extracted twice with 5 ml of chloroform-methanol (2:1, v/v), and esterified with methanolic HCl using procedures previously described (1). The bile acid methyl esters were applied to Silica Gel H plates, and the plates were developed in benzene-acetone (60:40, v/v). The bands corresponding to monohydroxy, dihydroxy, and trihydroxy bile acids were eluted with methanol, and 5α-cholestane was added as an internal standard. Quantitation of the biliary bile acids was carried out by GLC of the methyl esters on a column of 3% SE-30 after preparation of the trimehtylsilyl ether derivatives.

Radioactivity Measurements

All radioisotope measurements were made using a Beckman LS-200 liquid scintillation system (Beckman Instruments, Fullerton, CA). The radioactivity in each sample was obtained after evaporation of solvent by the addition of 12 ml of 2,5-bis-[2-(5-tert-butylbenzoxazolyl)]-thiophene (BBOT), 4 g/liter in toluene. Each sample was corrected for quench and background.

Calculations and Statistics

Acidic steroid synthesis was calculated using the isotopic techniques described above. The neutral steroid output was determined by chromatographic techniques (combined TLC and GLC). Endogenous neutral sterol production was estimated as previously described (1,2,7). Cholesterol absorption was determined as the difference between the dietary intake of cholesterol (determined chromatographically) and the unabsorbed neutral steroid in the feces. The unabsorbed dietary neutral steroid (mg), cholesterol turnover (mg/day), and cholesterol balance (mg/day) were calculated as described below (1,2,7): Cholesterol turnover (mg/day) = daily fecal endogenous neutral steroid output (isotopic) (mg/day) + fecal acidic steroid synthesis (isotopic) (mg/day); cholesterol balance (mg/day) = cholesterol output - cholesterol input = fecal neutral sterols (mg/day) + endogenous fecal bile acids (mg/day) - cholesterol intake (mg/day). Student's t-test was used to determine significance.

RESULTS

The weight gain, daily food intakes, and fecal outputs for all the rats were similar (Table I). Tissue cholesterol levels (liver, plasma, and bile) in the taurodeoxycholate fed rats were similar compared to controls (average ± SD) (liver cholesterol - 3.0 ± 1.2 mg/g vs. 2.5 ± 0.2 mg/g; plasma cholesterol - 86 ± 15 mg/100 ml

TABLE III

Comparative Sterol Balance Data of Rats on Different Diets[a,b]

Group No.	Diet	Daily endogenous acidic steroid output (mg/day)	Daily neutral steroid output (mg/day)	Daily endogenous neutral steroid output (mg/day)	Daily cholesterol absorption (mg/day)	Cholesterol turnover (mg/day)	Cholesterol intake (mg/day)	Cholesterol balance (mg/day)
1	Control	13.4 ± 3.9[c]	15.7 ± 1.3	4.8 ± 0.9	16.2 ± 1.8	18.2 ± 4.3[c]	27.3 ± 2.3	1.8 ± 0.4[c]
		(8.2-18.7)	(13.3-17.3)	(3.9-5.8)	(14.5-19.1)	(12.1-22.9)	(25.0-31.2)	[7.1-(-2.4)]
2	Sodium taurodeoxycholate (0.5%)	4.4 ± 1.0[c]	16.6 ± 2.5	5.9 ± 1.0	17.2 ± 2.9	10.3 ± 1.8[c]	27.5 ± 2.0	(-6.5) ± 3.4[c]
		(2.3-5.5)	(11.7-20.8)	(4.4-7.8)	(13.9-22.7)	(7.0-13.3)	(25.0-31.2)	[-2.8-(-13.0)]

[a]The calculations were made using the following relationships: Acidic steroid and neutral steroid outputs were determined using methods previously described (1,2); endogenous neutral steroid output (mg/day) = total radioactivity in neutral steroid fraction (corrected for variations in fecal flow) (dpm/day) divided by the specific activity of plasma cholesterol on first day of each 2-day fecal pool (dpm/mg); unabsorbed cholesterol was determined by subtracting daily endogenous fecal neutral steroid output (using isotopic method) (mg/day) from total daily fecal neutral steroid output determined by chromatography (mg/day). Cholesterol absorbed (mg/day) = cholesterol intake (mg/day) minus daily unabsorbed cholesterol (mg/day); cholesterol turnover (mg/day) = daily fecal neutral steroid output (isotopic) (mg/day) plus fecal acidic steroid output (isotopic) (mg/day); cholesterol balance (mg/day) = cholesterol output minus cholesterol intake (mg/day).

[b]Values represent averages ± SD of 15 pools for control rats and 25 pools for the sodium taurodeoxycholate groups; numbers in parentheses represent the range.

[c]Differs significantly from controls, p<0.01.

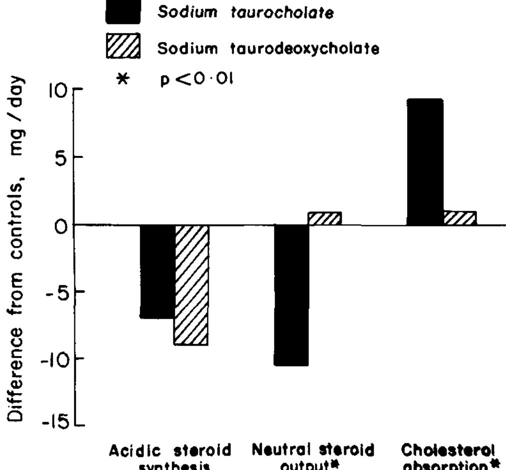

FIG. 1. Sterol metabolism data of rats given stock chow + 0.1% cholesterol + 0.5% sodium taurodeoxycholate compared with those fed stock chow + 0.1% cholesterol + sodium taurocholate (0.5%) (Ref. 1). The values are expressed as the differences between the experimental groups and the corresponding controls (mg/day).

vs. 86 ± 22 mg/100 ml; bile cholesterol - 0.17 ± 0.03 mg/ml vs. 0.15 ± 0.02 mg/ml). Biliary bile acid composition in the taurodeoxycholate fed rats revealed that taurodeoxycholate comprised 77% of these bile acids compared to 5% in the controls.

The specific activities of cholesterol in plasma, liver, and bile for the animals in each group on day 14 were similar and indicated that isotopic equilibrium of cholesterol had been achieved in these tissues during this short term metabolism study (Table II).

Sterol metabolism data are summarized in Table III. Daily cholesterol intake, fecal neutral sterols, and endogenous neutral sterols were similar in both taurodeoxycholate and control rats. Cholesterol absorption was not increased by taurodeoxycholate feeding. Daily acidic steroid synthesis was significantly reduced by taurodeoxycholate. This was accompanied by a reduction in cholesterol turnover and cholesterol balance.

DISCUSSION

The sterol balance method used in this investigation is believed to reflect the overall state of cholesterol metabolism in vivo (5,7,8). The measurements are usually made under the conditions of the metabolic steady state. This has been defined by others (8) as: (a) constant plasma cholesterol concentrations, (b) unvarying output of fecal neutral sterols, and (c) constant body weight. The rats in this experiment were in a steady state (constant plasma cholesterol concentrations and unvarying fecal neutral sterol output) except that they were allowed to gain weight. We have reported that valid comparisons of sterol balance data can be made between groups of rats treated under similar experimental conditions and allowed to gain weight. We have not made corrections for weight gain, thus resulting in a negative cholesterol balance in certain groups. Beher et al. determined that there were no significant differences in carcass cholesterol in rats fed: (a) control diet, (b) control diet + cholic acid, (c) control diet + cholic acid + cholesterol, or (d) control diet + chenodeoxycholic acid (9). The determination of absolute cholesterol synthesis would require knowledge of the amount of cholesterol (mg/day) retained in the animal during the experimental period (10). Thus, we report values for cholesterol balance rather than cholesterol synthesis since cholesterol retention was not measured and no increase in tissue cholesterol occurred in these rats. Cholesterol retention was presumably similar in both groups.

The rats ingesting the sodium taurodeoxycholate diet consumed an average of 187 mg/day of this bile acid. Apparently, this bile acid caused no adverse effects as seen by the similarities of weight gain, food intake, and fecal output over the entire experimental period. The sodium taurodeoxycholate was absorbed from the intestine of the rats and circulated in the enterohepatic circulation. It markedly altered biliary bile acid composition with taurodeoxycholic acid becoming the predominant component (77% of total). Presumably, the hepatic 7α-hydroxylase does not have sufficient capacity to convert a large portion of the administered bile acid to taurocholate (11).

Comparative sterol balance data can be obtained using the methods reported here if the specific activities of cholesterol in liver, plasma, and bile are similar. This was found to be the case at day 14 of the experiment. Previously, we have found that these specific acitvities are similar as early as day 3 after isotopic labeling (7).

Taurodeoxycholic acid did not produce elevated liver cholesterol or plasma cholesterol concentrations. This was in contrast to taurocholate feeding where both liver and plasma cholesterol concentrations were increased (1). It appears that taurodeoxycholate resembled taurochenodeoxycholate in its effect on these tissue cholesterol concentrations (1).

Comparative sterol metabolism data of

sodium taurodeoxycholate and sodium taurocholate are shown in Figure 1. Bile acid synthesis was suppressed to a similar extent with both sodium taurocholate feeding reported earlier (1), as well as with sodium taurodeoxycholate administration in the current study. Neutral sterol output was lower with taurocholate feeding but not with taurodeoxycholate compared to controls. Cholesterol absorption was significantly elevated by taurocholate but not by taurodeoxycholate. Other investigators using bile diverted, lymph fistula rats observed that cholic, but not deoxycholic acid, caused increased cholesterol absorption (12,13). The mechanism of this increase remains to be elucidated.

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The Transfer of Free Palmitic and Linoleic Acids across the Ovine Placenta

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ABSTRACT

Following the injection of ^{3}H-16:0 and ^{14}C-18:2 (n-6) into the jugular vein of the ewe, the rates of disappearance of these fatty acids from the maternal plasma and their rates of appearance in the fetal plasma have been studied. The rates of disappearance of both labeled acids from the maternal circulation were similar. The appearance of radioactivity in the fetal plasma was rapid and could be detected within 2.5 min after injection into the mother. In contrast to the similar rates of disappearance of the fatty acids from the maternal plasma, the rate of accumulation of ^{3}H-16:0 within the fetal plasma was appreciably greater than that of ^{14}C-18:2 (n-6). The absolute concentrations of the total lipid and unesterified fatty acid fractions within the fetal plasma were significantly lower than that of the maternal plasma. In comparison to that of maternal plasma, fetal plasma lipid contained an extremely low proportion of 18:2 (n-6) accompanied by an appreciable accumulation of 20:3 (n-9). On the other hand, the proportion of 20:4 (n-6) within the fetal plasma was about twice that present within the maternal plasma. The obvious inability of the placenta to be as freely permeable to the passage of 18:2 (n-6) as to 16:0 is discussed in relation to these differences in fatty acid composition between the maternal and fetal plasmas and the known poor essential fatty acid status of the developing fetus.

INTRODUCTION

Although 18:2 (n-6) may comprise up to 40% of the total fatty acids present in the plasma of the adult ruminant (1-5), its transfer across the placenta would appear to be exceptionally limited as the concentration of 18:2 (n-6) within the plasma and tissues of the newborn lamb or calf do not exceed 2-3% of the total fatty acids present (6-10). Furthermore, the tissues of the newborn ruminant animal exhibit high levels of the fatty acid 20:3 (n-9), the accumulation of which in nonruminant species is associated with an inadequate supply of 18:2 (n-6) (11,12). In the ruminant animal, as in other species, only the unesterified fatty acids of the maternal circulation contribute significantly to the supply of fatty acids to the fetus (9,13-15), and the presence of only low concentrations of 18:2 (n-6) in this fraction of the maternal plasma may partly explain the presence of such low concentrations of 18:2 (n-6) in the neonatal tissues at birth. However, the present investigations indicate that the problem in the essential fatty acid supply to the fetus may be further complicated by an inability of the placenta to be as freely permeable to the transfer of 18:2 (n-6) as to other long chain fatty acids.

EXPERIMENTAL PROCEDURE

The animals used were six 5-year-old ewes of the Cheviot breed. The animals were housed in individual pens and were fed a diet of hay and concentrates at 0700 hr and 1700 hr; water was available ad libitum. In-dwelling polyethylene and polyvinyl chloride catheters were placed in the maternal femoral artery and the fetal abdominal aorta (via the femoral artery), respectively, in each of the ewes at 118-120 days of gestation. The catheters were kept clear by twice daily injections of sterile isotonic saline. A strictly aseptic regimen was maintained throughout the entire experimental period. At least 10 days after implantation of an appropriate polyethylene catheter, 100 μCi each of 9,10-(^{3}H)-palmitic acid and 1-(^{14}C)-linoleic acid (Radiochemical Centre, Amersham, England), freshly complexed with 4 ml of sterile lamb serum (Biocult Laboratories, Paisley, Scotland), was injected into the left maternal external jugular vein. The fatty acid solutions were prepared as follows: 100 μCi of each of the labeled fatty acids were dispensed into a small beaker and the benzene removed under a flow of nitrogen. The fatty acids were then immediately redissolved in 100 μl of acetone; 4 ml of sterile lamb serum was added slowly with stirring, and the mixture placed under nitrogen until the smell of acetone could no longer be detected.

Immediately prior to, and at, 2.5, 5.0, 10, 15, and 20 min after the injection of the labeled fatty acid solution, ca. 10 ml and 5 ml of blood were withdrawn from the maternal femoral artery and the fetal femoral artery, respectively, using heparinized syringes; the blood was chilled to 4 C as rapidly as possible. Following separation of the plasma by centri-

fugation, 100 μl of the plasma was added to a liquid scintillation vial and solubilized in 1 ml of Soluene (Packard Instruments Ltd., Downers Grove, IL); 10 ml of organic scintillator (Scintimix II, Koch-Light Laboratories Ltd., Slough, England) was then added, and the ^3H and ^{14}C radioactivities determined by differential counting in a Packard 2425 liquid scintillation spectrometer. The lipid was extracted from the remainder of the maternal and fetal plasma by the method of Nelson and Freeman (16), and, following the addition of a heptadecanoic fatty acid standard, the absolute and relative amounts of the fatty acids were determined by gas liquid chromatography (GLC) of methyl ester derivatives on packed columns of EGSS-X (5,10). Separation and analysis of the major lipid fractions of the maternal and fetal plasmas were carried out on bulked samples obtained subsequently.

RESULTS

Absolute (mg/100 ml plasma) and relative (weight percentages of the total) concentrations of the major fatty acids present within the maternal and fetal plasmas are given in Table I. It is clear that the various surgical manipulations involved in obtaining the sequential blood samples were without effect on plasma lipid composition. However, there were marked differences in the absolute concentrations and relative proportions of the fatty acids between the maternal and fetal plasmas. Thus, the concentration of total fatty acids circulating within the fetus was only about one-third of that in the maternal circulation, and striking differences were observed between the relative proportions of polyunsaturated fatty acids in the maternal and fetal plasmas. In the maternal plasma, 18:2 (n-6) accounted for some 24% of the total fatty acids present, whereas the proportion of 18:2 (n-6) in the plasma of the fetus was only some 3% of the total fatty acids present. In contrast to the relative proportion of 18:2 (n-6) in the maternal and fetal plasmas, the proportion of 20:4 (n-6) within the plasma of the fetus was nearly twice that present in the maternal plasma. The fetal plasma contained very much higher proportions of 20:3 (n-9) than the maternal plasma and accordingly displayed a significantly higher 20:3 (n-9)/20:4 (n-6) (triene:tetraene) ratio than the maternal plasma. Fetal plasma also displayed significantly higher proportions of 16:0 and 18:1 but lower proportions of 18:0 than maternal plasma.

Table II shows the absolute concentrations

TABLE I

The Concentration (mg Fatty Acid per 100 ml Plasma) and Fatty Acid Composition (Major Components, Weight Percentages of the Total) of the Total Lipid Present in the Maternal and Fetal Plasma

Animal number	Fatty acid composition (weight %) 16:0	16:1	18:0	18:1	18:2 (n-6)	18:3 (n-3)	20:3 (n-9)	20:4 (n-6)	mg FA/100 ml Plasma
Maternal									
1	17.6 ± 0.13	1.04 ± 0.18	24.3 ± 0.29	22.1 ± 0.02	27.9 ± 0.28	1.08 ± 0.10	tr	6.00 ± 0.13	51.5 ± 1.70
2	16.1 ± 0.21	1.47 ± 0.07	21.6 ± 0.28	30.6 ± 0.13	24.9 ± 0.19	1.68 ± 0.06	tr	3.50 ± 0.13	52.8 ± 1.59
3	16.6 ± 0.16	1.11 ± 0.02	19.9 ± 0.76	31.6 ± 0.46	23.0 ± 0.45	2.38 ± 0.08	tr	5.45 ± 0.32	52.2 ± 0.81
4	17.5 ± 0.22	1.32 ± 0.03	21.7 ± 0.23	28.2 ± 0.13	24.2 ± 0.33	1.38 ± 0.02	tr	5.69 ± 0.10	50.8 ± 3.55
5	19.4 ± 0.13	1.45 ± 0.05	21.0 ± 0.64	29.5 ± 0.13	21.4 ± 0.49	2.10 ± 0.10	tr	5.09 ± 0.18	51.8 ± 2.41
6	17.3 ± 0.26	1.33 ± 0.07	20.3 ± 0.50	29.0 ± 0.25	25.0 ± 0.72	1.65 ± 0.14	tr	5.38 ± 0.23	51.0 ± 1.16
Mean	17.4 ± 0.46	1.29 ± 0.07	21.5 ± 0.63	28.5 ± 1.38	24.4 ± 0.89	1.71 ± 0.19	tr	5.19 ± 0.35	51.7 ± 0.31
Fetal									
1	28.5 ± 0.36	3.59 ± 0.16	13.7 ± 0.25	32.1 ± 0.14	4.90 ± 0.14	1.97 ± 0.13	5.12 ± 0.14	10.1 ± 0.68	13.8 ± 0.88
2	26.8 ± 0.69	3.90 ± 0.23	16.0 ± 0.12	31.1 ± 0.52	4.16 ± 1.15	2.53 ± 0.41	7.18 ± 0.32	8.33 ± 0.15	16.6 ± 0.83
3	26.8 ± 0.29	4.21 ± 0.08	11.9 ± 0.09	43.6 ± 0.28	2.61 ± 0.26	0.88 ± 0.15	3.46 ± 0.08	6.58 ± 0.22	14.6 ± 1.39
4	29.2 ± 0.34	5.03 ± 0.13	13.0 ± 0.06	36.6 ± 0.54	2.07 ± 0.59	1.04 ± 0.15	5.33 ± 0.17	7.31 ± 0.38	13.8 ± 0.17
5	27.5 ± 0.08	4.79 ± 0.09	12.3 ± 0.13	38.4 ± 0.25	2.47 ± 0.15	0.50 ± 0.30	4.46 ± 0.19	9.72 ± 0.22	15.8 ± 0.66
6	32.1 ± 0.28	5.00 ± 0.10	11.7 ± 0.10	34.9 ± 0.31	2.39 ± 0.10	0.45 ± 0.10	4.41 ± 0.08	8.49 ± 0.27	15.6 ± 0.38
Mean	28.5[a] ± 0.81	4.42[b] ± 0.25	13.1[a] ± 0.65	36.1[b] ± 1.85	3.10[a] ± 0.47	1.15[c] ± 0.38	5.02[a] ± 0.51	8.42[a] ± 0.55	15.0[a] ± 0.47

[a]The level of significance of the difference between maternal and fetal values is $P < 0.001$.
[b]The level of significance of the difference between maternal and fetal values is $P < 0.01$.
[c]The level of significance of the difference between maternal and fetal values is $P < 0.05$.

TABLE II

The Concentration (mg Fatty Acid per 100 ml Plasma) and Fatty Acid Composition (Major Components, Weight Percentage of the Total) of the Unesterified Fatty Acids Present in the Maternal and Fetal Plasma.

	Fatty acid composition (weight %)						
	16:0	16:1	18:0	18:1	18:2 (n-6)	18:3 (n-3)	mg/100 ml Plasma
Maternal	19.9	1.43	36.5	34.6	4.66	1.52	14.0
	±0.64	±0.27	±1.36	±0.96	±0.28	±0.18	±1.54
Fetal	36.0[a]	3.53	19.0[a]	27.7[c]	5.71	1.37	7.73[b]
	±2.88	±1.34	±2.73	±1.94	±1.21	±0.25	±0.40

[a]The level of significance of the difference between maternal and fetal values is $P < 0.001$.
[b]The level of significance of the difference between maternal and fetal values is $P < 0.05$.
[c]The level of significance of the difference between maternal and fetal values if $P < 0.01$.

and relative proportions of the major fatty acids present in the unesterified fatty acid fraction of the maternal and fetal plasmas. The concentration of unesterified fatty acids circulating in the fetal plasma was only about one-half that of the mother. In the unesterified fatty acid fraction of the maternal plasma, the proportion of 18:2 (n-6) was extremely low compared to that in the phospholipids and the cholesteryl esters (5) and was not significantly different from the proportion of 18:2 (n-6) found within the unesterified fatty acids of the fetal plasma. However, the unesterified fatty acids of the fetal plasma contained a significantly higher proportion of 16:0 than maternal plasma and there were compensatory decreases in the proportions of 18:0 and 18:1.

The rates of disappearance of ^{3}H-16:0 and ^{14}C-18:2 (n-6) from the maternal circulation and their subsequent accumulation in the fetal circulation following injection into the jugular vein of the mother are shown in Figure 1, a and b, respectively. The changes in the specific activities of the ^{3}H-16:0 and ^{14}C-18:2 (n-6) within the unesterified fatty acids of the maternal and fetal plasmas are shown in Figure 2 a and b, respectively. It is clear from the slopes of the activity-time curves that the rates of disappearance of both ^{3}H-16:0 and ^{14}C-18:2 (n-6) following their injection into the maternal circulation were rapid. Radioactivity was detectable in the fetal plasma within 2.5 min of its infusion into the maternal circulation. However, in contrast to the similar rates of disappearance of the ^{3}H-16:0 and ^{14}C-18:2 (n-6) from the maternal circulation, there was a large difference between the appearance of ^{3}H-16:0 and ^{14}C-18:2 (n-6) within the plasma of the fetus. An appreciable accumulation of ^{3}H-16:0 within the plasma of the fetus was accompanied by a very much lower accumulation of ^{14}C-18:2 (n-6); the amount of maternally administered ^{14}C-18:2 (n-6) subsequently found within the fetal plasma never exceeded one-fifth of that of the ^{3}H-16:0. Clearly the relative efficiency of tranfer of the ^{14}C-18:2 (n-6) from the maternal plasma across the placenta into the fetus was considerably lower than for the ^{3}H-16:0. The specific activity-time curves also illustrate the decrease in the efficiency of transfer of 18:2 (n-6) relative to 16:0 from mother to fetus. Thus, the relationship between the specific activities of the ^{3}H-16:0 in the plasma unesterified fatty acids of the mother and fetus (Fig. 2a) is clearly similar to that of a product-precursor relationship indicating more or less direct transfer of the acid across the placenta. In contrast, the specific activity of the ^{14}C-18:2 (n-6) in the fetal plasma unesterified fatty acids remained lower than that displayed by the maternal plasma (Fig. 2b).

DISCUSSION

As has already been shown for the newborn ruminant animal (6-10), the plasma fatty acid composition of the fetal lamb differed markedly in its polyunsaturated fatty acid composition from that of its mother. Within the fetal plasma, there was an extremely low proportion of 18:2 (n-6) and an appreciable proportion of 20:3 (n-9) thereby contrasting with the high proportion of 18:2 (n-6) and low proportion of 20:3 (n-9) in the maternal plasma. However, in spite of the lack of 18:2 (n-6), the proportion of 20:4 (n-6) in the plasma of the fetal lamb was significantly higher than its proportion within the maternal plasma; in the rabbit, similar differences between the proportions of 20:4 (n-6) in maternal and fetal plasmas have been noted within the unesterified fatty acid fraction (17). Thus, with regard to the distribution of C18 and C20 polyunsaturated fatty

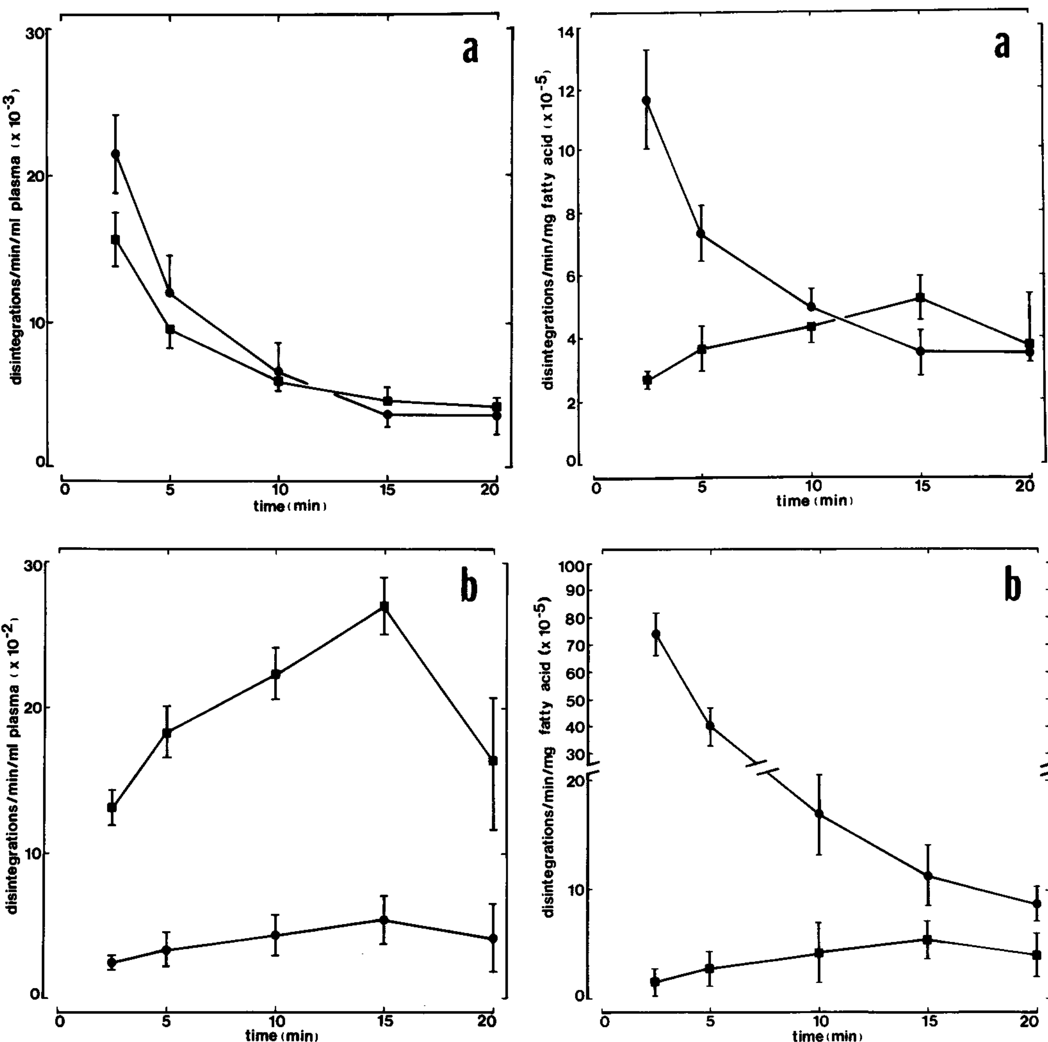

FIG. 1. The rates (mean ± standard error) of (a) disappearance from the maternal plasma and (b) the accumulation in the fetal plasma of the maternally administered ^{3}H-16:0 (■—■) and ^{14}C-18:2 (n-6) (●—●).

FIG. 2. The changes in the specific activities (mean ± standard error) of (a) ^{3}H-16:0 and (b) ^{14}C-18:2 (n-6) within the fetal (■—■) and maternal (●—●) plasmas.

acids of the (n-6) series, there exists a characteristic difference between the plasmas of the maternal ewe and the developing fetus. Whereas, in the plasma of the maternal ewe, the 20:4 (n-6)/18:2 (n-6) ratio during gestation was only 0.21; within the fetus, it was 2.72. The higher proportions of 20:4 (n-6) in the fetal circulation compared with that of the mother may arise either through a specific preferential transfer of the acid from the maternal circulation into that of the fetus or through a secondary source of 20:4 (n-6) via its synthesis from 18:2 (n-6) in the placenta or fetal tissues. In the rabbit, both the preferential transfer of 20:4 (n-6) between the maternal and fetal circulations and any extensive conversion of 18:2 (n-6) to 20:4 (n-6) within the fetal tissues have been eliminated as possibilities (17). A placental source of 20:4 (n-6) through conversion from 18:2 (n-6) has, therefore, been suggested.

The absolute concentrations and relative proportions of unesterified fatty acids in the maternal and fetal plasmas obtained in the present investigations are similar to those which have been obtained previously for sheep. Thus, in contrast to the newborn lamb where the plasma displays high concentrations of unesterified fatty acids (7,9,13,18), the concentration of

unesterified fatty acids in the plasma of the developing sheep is significantly less than that of its mother (9,13,18,19). In the adult sheep, the high concentrations of 18:2 (n-6) within the plasma may be almost exclusively accounted for by the high C18 polyunsaturated fatty acid content of the cholesteryl ester and phospholipid fractions, which together may account for some 70-80% of the total plasma lipids present (2,4). The unesterified fatty acid fraction of the adult plasma contains only very low proportions of 18:2 (n-6) and hardly detectable amounts of 20:4 (n-6). It is clear from comparative fatty acid compositional studies of maternal and fetal plasma lipids (6,7,9,20) and direct transfer experiments involving the use of labeled metabolities (9,13,21) that in the sheep, as in other animal species, little if any esterified fatty acids of the maternal circulation are taken up by the fetus. Maternal contribution to the fetus is almost entirely through the transfer of unesterified fatty acids. In view of the extremely low proportions of 20:4 (n-6) within the maternal plasma unesterified fatty acids, it is unlikely, therefore, that the differences between the proportions of 20:4 (n-6) and the ratios of 20:4 (n-6)/18:2 (n-6) in the plasmas of the mother and fetus can be the result of a preferential transfer of 20:4 (n-6) across the placenta. This suggests, therefore, that during development when the essential fatty acid supply is so extremely limited and in order to satisfy a preferential requirement for 20:4 (n-6), the enzyme system involved in the synthesis of 20:4 (n-6) from 18:2 (n-6) is more active in the tissues of the fetal lamb than in the tissues of the mother. However, although in vitro experiments with tissue homogenates and ^{14}C-labeled 18:2 (n-6) have failed to indicate that the synthesis of 20:4 (n-6) from 18:2 (n-6) within the liver of the fetal lamb is more active than in the liver of the ewe (22), more recent analyses and in vitro incubations (Noble, Shand, and Moore, unpublished observations) have provided strong evidence that the placental tissue is an active site for the synthesis of 20:4 (n-6) from 18:2 (n-6). The significant differences in the percentages of 16:0, 18:0. amd 18:1 obtained between the unesterified fatty acids of the maternal and fetal plasmas in the present experiment with sheep contrast with observations with other animals (17) in which the fatty acid composition of maternal and fetal plasma unesterified fatty acids were very similar.

The transport of unesterified fatty acids from the maternal circulation across the placenta into the fetus has been studied in a number of animal species including the sheep (13-15,17,23-25). As in the present investigations, injection of labeled fatty acids into the maternal circulation leads to a rapid appearance of radioactivity within the fetal plasma; apart from the single observation of James et al. (23), it is also clear from measurements of arterial and venous concentrations of the unesterified fatty acids within both the maternal and fetal circulations that the net flux of unesterified fatty acids into the fetus occurs through passage down a maternal-fetal gradient with a continuous exchange between the fatty acid pools of the maternal plasma, placenta, and fetal plasma. Although the injection of ^{3}H- and ^{14}C-labeled substrates into other animal species has demonstrated similar turnover rates within the maternal plasma unesterified fatty acids for 16:0 and 18:2 (n-6) (14,17), it would appear from both the present and previous observations (13) that in the sheep their rates of turnover are significantly lower than in many other species studied. The relative rates of passage of individual maternal plasma unesterified fatty acids across the placenta into the fetal circulation would also appear to reveal distinct species differences. Thus, in the rabbit, comparative specific activity data of the unesterified fatty acids within the maternal and fetal plasmas have indicated that 16:0 and 18:2 (n-6) have a similar ability to cross the placenta (17); in the guinea pig, in spite of a lower maternal-fetal concentration gradient of 18:2 (n-6) relative to 16:0, there would appear to be a greater ability for 18:2 (n-6) to cross the placenta than 16:0 (14). The present results with sheep clearly contrast with those obtained for the rabbit and guinea pig as, relative to 16:0, there is an obvious discrimination against the passage into the fetal circulation of the 18:2 (n-6) available from the unesterified fatty acid fraction of the maternal plasma. From previous evidence (13), it would appear that the overall transfer and contribution of maternal unesterified fatty acids to the fetus is considerably less in the sheep than in other animal species. Furthermore, in nonruminant animals, 18:2 (n-6) comprises a relatively high proportion of the maternal unesterified fatty acids, and is, therefore, able to ensure an adequate supply of 18:2 (n-6) to the fetus, whereas in the sheep the low proportion of 18:2 (n-6) in the maternal plasma unesterified fatty acids would impose a severe limitation on the supply of this fatty acid to the fetus. The present results clearly show that in the sheep the acquisition of 18:2 (n-6) by the developing fetus is open to further restrictions, in addition to those already imposed by the low proportion of 18:2 (n-6) in the unesterified fatty acids of the maternal plasma,

through the differential permeability of the placenta to the passage of 18:2 (n-6) relative to other long chain fatty acids. However, there is a distinct possibility that the relative inability of 18:2 (n-6) to pass freely into the fetus and the consequential presence in the neonatal lamb at birth of extremely low tissue concentrations of 18:2 (n-6) may be associated with an active synthesis within the placenta of 20:4 (n-6) from the available 18:2 (n-6).

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METHODS

Analysis of Alpha Tocopherol in Blood Plasma and Platelets by Gas Liquid Chromatography

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ABSTRACT

A method is described for the analysis of α-tocopherol by gas liquid chromatography (GLC) with 0.3% Apiezon L as liquid phase. Impurities that interfere with GLC are removed by saponification. Cholesterol and other nonsaponifiables are separated from α-tocopherol by GLC.

INTRODUCTION

A previous publication (1) described a method for the analysis of tocopherols in 0.1 ml of blood plasma by gas liquid chromatography (GLC) that had adequate sensitivity for the low levels of tocopherol expected in blood platelets. Occasionally, however, there were difficulties with low recoveries attributable to thin layer chromatography (TLC). The method was modified to eliminate TLC by substituting saponification to remove interfering substances. Both precision and sensitivity were increased by the modification.

MATERIALS AND METHODS

Reagents

Petroleum ether, nanograde, and carbon disulfide (Fisher Scientific Co., Silver Spring, MD) were used as received. Pyridine (Fisher Scientific Co.), hexamethyldisilazane, trimethylchlorosilane (Analabs Inc., New Haven, CT), and absolute ethanol (U.S. Industrial Chemical Co., Louisville, KY) were all distilled before use. Saturated aqueous KOH was prepared with boiled deionized water. α-Tocopherol was from Distillation Products, Rochester, NY, and 5,7-dimethyltocol was synthesized (2) and donated by Hal T. Slover of the Nutrient Composition Laboratory, Beltsville, MD. Concentration tubes were purchased from Laboratory Research Co., Los Angeles, CA. Trimethylsilylation reagent (TMS) was prepared by mixing pyridine, hexamethyldisilazane, and trimethylchlorosilane in the ratio of 10:9:6.

Internal standard: An ethanolic solution of 5,7-dimethyltocol (5,7,-T), 0.5 μg/ml, was prepared for use as an internal standard with 10% pyrogallol as antioxidant. This solution was standardized against α-tocopherol (1).

Analysis

Sample preparation: Plastic centrifuge tubes and syringes were used in all manipulations involving platelets. Cardiac blood from laboratory animals or venous blood from humans was mixed immediately with EDTA in a conical-tip centrifuge tube and centrifuged at 1000 rpm at 4 C for about 15 min. Platelet-rich plasma was transferred with a siliconized Pasteur pipet to a clean conical-tip centrifuge tube and centrifuged at 3000 rpm at 4 C for 10 min to produce a platelet pellet. Platelet-poor plasma was stored at -40 C until analyzed. Platelets were washed twice with cold physiological saline and resuspended in 2-3 ml of saline (~3 x 10^8 cells/ml). Cell counts were made with a Coulter Counter Model B.

Saponification: Exactly 0.5 ml of platelet suspension or 0.1 ml of plasma was transferred to a 20-ml screw-cap tube. Exactly 2 ml of the 5,7-T standard solution was added, and the tube was flushed with a slow flow of nitrogen for 2 min. Saturated aqueous KOH (0.5 ml) was added. The tube was capped immediately with a Teflon-lined cap, heated for 5 min in a boiling water bath, and cooled rapidly in ice. Ice-cold water (2.5 ml) and petroleum ether (10 ml) were added, and the mixture was shaken well and allowed to separate. The petroleum ether layer was transferred to a clean 40-ml centrifuge tube, and the saponification mixture was

TABLE I

Recoveries of α-Tocopherol Added to Rat Platelets and Plasma

Analysis number	α-Tocopherol Present (μg)	Added (μg)	Found (μg)	Recovery (%)
		Platelets		
E-Deficient				
1	0	1.39	1.24	89.2
2	0	1.39	1.42	102.2
3	0	1.39	1.42	102.2
4	0	1.39	1.34	96.4
E-Adequate				
1	0.32	1.39	1.65	96.5
2	0.32	1.39	1.72	100.6
3	0.32	1.39	1.65	96.5
4	0.32	1.39	1.78	104.1
		Plasma (0.1 ml)		
E-Deficient				
1	0	1.13	1.00	88.5
2	0	1.13	1.02	90.3
3	0	1.39	1.24	89.2
4	0	1.39	1.18	84.9
E-Adequate				
1	0.83	1.13	2.00	102.0
2	0.63	1.13	1.62	92.0
3	0.78	1.13	1.83	95.8
4	1.07	1.13	2.22	100.9

reextracted with an additional 5 ml of petroleum ether. The petroleum ether extracts were combined, washed with 10 ml of ice-cold water, transferred to a concentration tube, and evaporated to dryness. TMS reagent (20 μl) was added, mixed, and evaporated with nitrogen. The silylated residue was dissolved in 10 μl of carbon disulfide, and 2 μl was injected on the gas chromatograph. Nonsilylated samples could be stored overnight in petroleum ether at -40 C. Best recoveries were obtained when extracts were kept ice cold after saponification and still in the presence of base. All tocopherol analyses were made under yellow lights.

Gas liquid chromatography: GLC was essentially as described before (1) except that the liquid phase loading was 0.3% instead of 0.5% Apiezon L. Briefly, a F&M Model 810 gas chromatograph equipped with a 1/8 in. x 15 ft silanized glass coil column packed with 0.3% Apiezon L on 100/120 mesh Gas Chrom Q was operated isothermally at 245 C. Detection was by flame ionization, and the carrier gas was helium at a flow rate of about 25 ml/min. Peak areas were measured either by triangulation or by electronic integration (Hewlett Packard Model 3370B).

RESULTS AND DISCUSSION

Recoveries

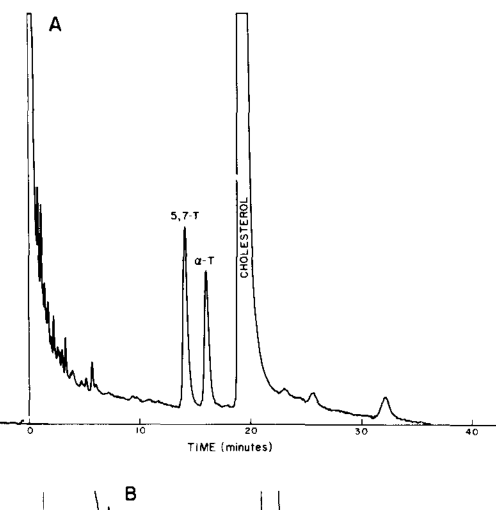
For evaluation of recoveries, an ethanolic solution of 5,7-T internal standard containing a known amount of α-tocopherol and 10% pyrogallol was added either to 0.1 ml of plasma or to 0.5 ml of the platelet suspension. Recoveries of the total tocopherol expected, added plus sample, ranged from 85-104% (Table I). Average recoveries (N=4) from vitamin E-deficient platelets and plasma were 98 and 88%, respectively, and from vitamin E-adequate platelets and plasma were 99 and 98%, respectively. Recoveries of α-tocopherol relative to 5,7-T from pure standard mixtures averaged 95%. This contrasted to 83% recovery from pure standard mixtures when

TABLE II

Replicate Analyses of α-Tocopherol Content of Platelets and Plasma from Four Different Animal Species

Species	Animal number	α-Tocopherol Platelets ($\mu g/10^9$ cells)	Plasma (μg/ml)
Rat	1	3.2, 2.8	4.4, 4.4
	2	4.2, 4.1	7.4, 7.2
Rabbit	1	0.56, 0.60	2.2, 2.0
	2	0.57, 0.50	3.3, 3.6
Human	1	0.62, 0.66	8.6, 9.4
	2	0.69, 0.79	8.7, 9.2
Swine	1	0.14 ± 0.0029[a]	1.0, 1.4

[a]N = 7, mean ± SE.



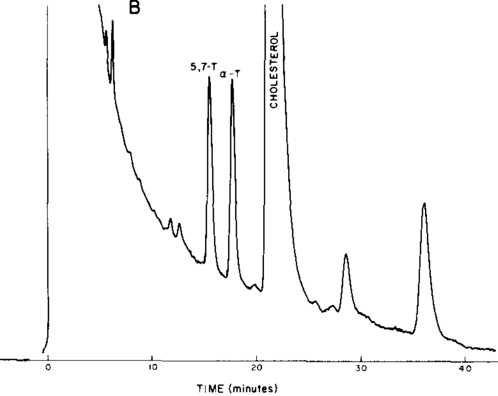


FIG. 1. Gas chromatographic separation of rat (A) platelet and (B) plasma α-tocopherol TMS ether on 0.3% Apiezon L. Chromatographic conditions are described in the text.

TLC was included in the procedure (1).

Reproducibility

Replicate analyses of platelet and plasma samples from four different species are shown in Table II. For a series of seven analyses of the same platelet sample containing 0.14 μg α-tocopherol/10^9 cells, the coefficient of variation was 5.4%. This variation of the method was compared with daily variation of the gas chromatograph. Aliquots of a standard mixture containing α-tocopherol and 5,7-T in isooctane were evaporated, silanized, dissolved in carbon disulfide, and injected on the gas chromatograph on different days. Under these conditions, the coefficient of variation for ten injections was 4.1% for α-tocopherol.

Interferences

Most methods for the GLC analysis of α-tocopherol require prior removal of cholesterol. Under the GLC conditions described for this procedure, however, cholesterol and α-tocopherol were completely separated. Furthermore, chromatograms derived from platelets and plasma from vitamin E-depleted rats showed no impurities at the α-tocopherol retention time.

If cholesterol is a dietary component, 5,7-T is not a suitable internal standard for those animals that are coprophagous. The fecal sterol, coprostanol, was identified by mass spectrometry as a substance that co-chromatographed with 5,7-T during analysis of tissues from rabbits fed cholesterol. This interference was not found with human subjects and was negligible with rats fed diets that did not contain cholesterol. Since TLC is not used with this procedure, possible alternative internal standards include long chain hydrocarbons (C_{28}-C_{30}) or δ-tocopherol.

Screw caps and some lots of pyrogallol were troublesome sources of extraneous peaks. Only solvents of high purity can be used.

Gas Chromatograph

Typical chromatograms for platelets and plasma are shown in Figure 1. Retention times for 5,7-T and α-tocopherol were about 15 and 18 min, respectively, and column efficiencies for α-tocopherol exceeded 5000 theoretical plates. Under the conditions described, cholesterol had a retention time of about 20-23 min and did not interfere.

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COMMUNICATIONS

The Influence of Purines on Plasma Lecithin: Cholesterol Acyltransferase Activity in the Rat

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ABSTRACT

The activity of plasma lecithin:cholesterol acyltransferase (LCAT) in the rat is significantly inhibited in vitro by guanine, xanthine, and hypoxanthine. LCAT activity decreases with increase in xanthine concentration. The other two purines, adenine and uric acid, had no significant effect.

INTRODUCTION

Disturbances of uric acid metabolism are frequent in cases of idiopathic hyperlipidemia (1), and gouty patients have higher blood lipid levels than subjects with normal plasma uric acid concentrations (2). To determine whether there is a direct link between purine and lipid metabolism, we have studied the in vitro effect of uric acid and its direct precursors on the activity of one of two enzymes involved in lipoprotein catabolism, namely lecithin:cholesterol acyltransferase (LCAT), and on the activities of microsomal enzymes involved in lipid metabolism in the rat.

MATERIALS AND METHODS

LCAT Assay

The plasma LCAT activity was determined by measuring [4-^{14}C] cholesterol esterification. Blood from a Wistar rat was collected and treated with EDTA (1 mg/ml), and after separation, the plasma was incubated for 24 hr at 37 C with 0.05 μCi of [4-^{14}C] cholesterol (specific activity, 44 mCi/mmole) and an antibiotic mixture (sodium benzyl penicillinate 400 u., streptomycin sulfate 2 mg) at pH 7.0 (3). Another assay was performed using a substrate composed of heat-inactivated plasma and of labeled unesterified cholesterol added as an albumin-stabilized emulsion (4). After incubation, lipids were extracted according to Folch et al. (5) and fractionated by thin layer chromatography on Silica Gel G (0.25 mm). Spots were located by means of autoradiography and after exposure to iodine vapors. The esterification rate was determined by scraping off each area and measuring its radioactivity in a Packard Tricarb 3390 liquid scintillation spectrometer.

Using this method, LCAT activity was measured after adding a purine to the incubation mixture to give a final concentration of 1.4 μM. By this procedure, the effects of uric acid, adenine, guanine, xanthine, and hypoxanthine

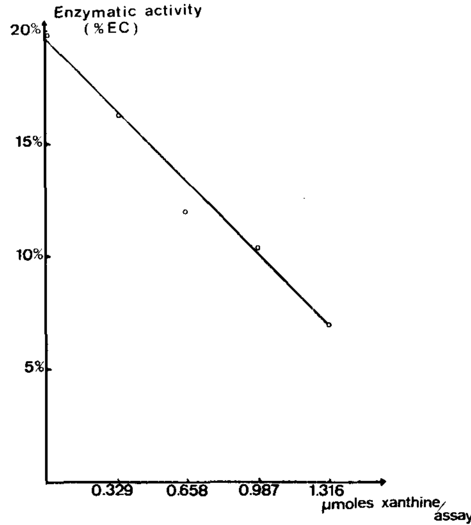

FIG. 1. Decrease of LCAT activity with increase in xanthine concentration. The experimental points in the figure each represent means of three separate experiments. EC: esterified cholesterol.

TABLE I

Influence of Purines on LCAT Activity[a]

	Assay without purine	Assay + uric acid (1.4 μM)	Assay + adenine (1.4 μM)	Assay + guanine (1.4 μM)	Assay + xanthine (1.4 μM)	Assay + hypoxanthine (1.4 μM)
Percentage of radioactivity incorporated into EC[b]						
Method (1) 24 hr	15.93 ± 7.4	12.7 ± 4.4	16.07 ± 5.5	7.2 ± 3.65	7.04 ± 3.98	5.56 ± 2.90
Method (2) 4 hr	18.32 ± 2.19	17.54 ± 2.99	17.75 ± 3.45	11.61 ± 1.43	11.84 ± 4.41	10.62 ± 2.25
p Method (1)		NS[c]	NS	< 0.001	< 0.001	< 0.001
Method (2)		NS	NS	< 0.001	< 0.001	< 0.001
Inhibition percentage						
Method (1)		20		55	56	65
Method (2)				37	36	42

[a]The results represent the average values of 18 experiments with different rats ± their standard errors.
[b]EC: esterified cholesterol.
[c]NS: nonsignificant.

were determined separately. Purines were added in a solution of tris 0.025 M and NaOH 0.5 M. Uric acid was measured at the end of the incubation period to check for absence of uricase activity, and a check was also made that the pH remained at 7.0.

We also studied the effects of purines on the incorporation of [1-^{14}C]palmitic acid into phospholipids and neutral lipids by hepatic microsomal enzymes. The enzyme assay was performed according to Magdalou et al. (6) after isolation of microsomes (7).

RESULTS AND DISCUSSION

Adenine appeared to have no effect on plasma LCAT activity, whereas uric acid had a slight inhibitory effect, and guanine, xanthine, hypoxanthine caused marked inhibition.

The reductions in mean LCAT activity in the presence of guanine, xanthine, and hypoxanthine were very significant ($p < 0.001$). Further assays using different concentrations of xanthine showed that LCAT activity decreases with increase in xanthine concentration (Fig. 1). The inhibitory effects of guanine, xanthine, and hypoxanthine were maintained with increased concentrations of [4-^{14}C]cholesterol in the incubation mixture (up to 0.25 μCi in one assay).

Hepatic microsomal enzyme assays showed that the purines have no significant effect on the incorporation of [1-^{14}C]palmitic acid.

Rat hepatic microsomes contain the lipid synthesis enzymes: an acyl-CoA synthetase (8), three glycerol-3-phosphate acyltransferases (9), a phosphatidate phosphatase (10), and an acyl-CoA cholesterol acyltransferase (11), allowing esterification of cholesterol by acyl-CoA. So palmitic acid is incorporated into triglycerides (4.85% of radioactivity ± 0.78), into cholesteryl esters (5.45% ± 2.11), and into phospholipids (30.65% ± 6). The incorporation of palmitic acid into different lipid fractions is not modified in any statistically significant way by addition of uric acid or its immediate precursors. The incorporation of palmitic acid is included for triglycerides between 3.70% and 5.70%, for cholesteryl esters between 5.33% and 6.83%, and for phospholipids between 28% and 34.3%. Stimulation of the microsomal transacylase activities by purines cannot be accepted as an explanation of the association of hyperlipidemia with hyperuricemia. On the contrary, we find an important decrease of LCAT activity with an increase in guanine, xanthine, or hypoxanthine. It seems likely that this inhibition may happen at any of two stages in the reaction: (a) either

at the moment of the exchange of [4-^{14}C] cholesterol with cholesterol from the high density liprotein (HDL) surfaces; or (b) in the reaction itself by inhibiting the transfer of the acyl group of lecithin to [4-^{14}C] cholesterol fixed to the HDL molecule, HDL carrying at the same time the substrate and the product of the reaction.

In the rat, LCAT only acts on HDL, while in man it also contributes to a small degree to catabolism of very low density lipoproteins with lipoprotein lipase. In hyperuricemia in man, an increase in xanthine and hypoxanthine levels can be observed. Also, increases can be observed during treatment with allopurinol which inhibits the xanthine-oxidase activity. So, there may be a relationship between hyperlipidemia frequently observed in these patients, and inhibition of LCAT activity by the purines, xanthine, and hypoxanthine.

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Stearoyl-CoA Desaturase Activity in Adipose Tissue and Liver of the Cardiomyopathic Hamster

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ABSTRACT

Stearoyl-CoA desaturase activity and the fatty acid compostion of lipids of adipose tissue and liver were determined in 35- and 180-day-old cardiomyopathic hamsters and age-matched normal controls. Enzyme activity was unchanged in the adipose tissue of 35-day-old animals but was significantly depressed in the 180-day-old cardiomyopathic hamsters. In the liver, stearoyl-CoA desaturase activity was significantly lower in the 35-day-old diseased animals but was unchanged in the 180-day-old animals. The analysis of the fatty acid composition of the lipids isolated from adipose tissue showed an increase in the relative percentage of saturated fatty acids accompanied by a decrease in the relative percentage of unsaturated fatty acids in both age groups of the cardiomyopathic hamsters. However, linoleic acid content was increased in the diseased animals. Similar changes in fatty acid composition of lipids from the livers of 35-day-old cardiomyopathic hamsters were observed, but no significant differences in the fatty acid composition between 180-day-old cardiomyopathic hamsters and normal controls were observed. The changes in the fatty acid composition appear to be related to the observed changes in desaturase activity. It is concluded that such changes in desaturase activity and fatty acid composition could affect the normal structure and functions of membranes and membrane-related processes.

INTRODUCTION

The final step in the synthesis of oleic acid is the desaturation of stearoyl-CoA by a microsomal enzyme system in the liver and adipose tissue. The stearoyl-CoA desaturase is a microsomal, multi-component, membrane- bound enzyme system involving lipid and at least three protein components (1). Changes in physiological conditions (2) and diet have been shown to have a considerable effect on the desaturation activity of microsomes. The enzyme system is depressed by fasting (3,4), stimulated by recovery from fasting (3,4), depressed by feeding diets containing the lipid end-product (18:1) (3,5), and stimulated by feeding diets devoid of end-products (4,5).

In a previous study (6), we reported alterations in the activities of fatty acid synthetase, glucose-6-phosphate dehydrogenase, citrate lyase, and malic enzyme in liver and adipose tissue homogenates from cardiomyopathic hamsters of various ages. The results suggested that lipogenesis was depressed in those tissues of the diseased animals as compared to age-matched normal controls. Another study (7) of the fatty acid composition of neutral lipids isolated from heart and skeletal muscle of cardiomyopathic hamsters showed a relative increase in the concentration of saturated fatty acids (mainly 16:0 and 18:0) accompanied by a relative decrease in the concentration of unsaturated fatty acids (mainly 16:1 and 18:1). The concentration of 18:2 was found to be elevated. Such a variation in the concentration of fatty acids could be due to a preferential utilization of unsaturated fatty acids or to a decrease in the desaturating capacity of liver and adipose tissue.

In the present study, the conversion of stearic acid (18:0) to oleic acid (18:1) has been measured in vitro under optimal conditions using microsomes isolated from the liver and adipose tissue of 35- and 180-day-old cardiomyopathic hamsters and age-matched controls. Additionally, the fatty acid composition of lipids isolated from the liver and adipose tissue from the same animals was determined.

MATERIALS AND METHODS

Cardiomyopathic (BIO 82.62) and randomly bred female hamsters were obtained from TELACO (Bar Harbor, ME). All animals were allowed free access to water and food. At an average age of 35 and 180 days, at least six cardiomyopathic and six age-matched hamsters were sacrificed by decapitation, and the liver and subcutaneous adipose tissues were excised and kept in ice-cold isotonic KCl. Because of the small size of the adipose pad in the 35-day-old hamsters, the pads from two animals were pooled and used for subsequent determination of stearoyl-CoA desaturase activity.

The excised tissues were homogenized in 0.25 M sucrose containing 50 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA, and 1 mM dithiothreitol. Microsomes were prepared by differential centrifugation (8) and were suspended in the homogenization medium to yield a concentration of 10 mg of protein/ml for liver

TABLE I

Adipose Tissue Stearoyl-CoA Desaturase Activity, Microsomal Protein Concentration, Lipid Content, Tissue and Body Weights, and Food Consumption of 35- and 180-Day-Old Randomly Bred (RB) and Cardiomyopathic (BIO) Hamsters

	Age 35 days		Age 180 days	
	RB	BIO	RB	BIO
Oleate formed (nmol/min/mg prot)	2.02 ± 0.24[a] (5)	1.82 ± 0.23 (5)	0.82 ± 0.08[c] (9)	0.26 ± 0.04[c] (8)
Microsomal protein concentration (mg/g tissue)	0.28 ± 0.01 (5)	0.29 ± 0.01 (5)	0.21 ± 0.01 (9)	0.19 ± 0.01 (8)
Total lipid Content (mg/g tissue)	948 ± 5.80[d] (9)	889 ± 7.40[d] (9)	947 ± 6.40[e] (9)	796 ± 15.0[e] (9)
Tissue weight (g)	2.10 ± 0.20[bf] (5)	1.30 ± 0.10[f] (5)	3.70 ± 0.20[g] (9)	2.80 ± 0.10[g] (8)
Hamster weight (g)	48.1 ± 1.0 (9)	44.9 ± 2.0 (9)	135 ± 7.6[h] (9)	99.0 ± 1.8[h] (8)
Food consumption (g/day/g body wt)	0.31 ± 0.01 (9)	0.30 ± 0.03 (9)	0.07 ± 0.004 (9)	0.08 ± 0.001 (9)

[a]Values are $\overline{X}$ ± SEM. Number in parentheses indicates number of animals.
[b]Weights of adipose tissue from two animals (4 pads).
[c-h]Differences between values with similar superscripts are statistically significant ($P < .05$).

TABLE II

Liver Stearoyl-CoA Desaturase Activity, Microsomal Protein Concentration, Lipid Content, and Liver Weight of 35- and 180-Day-Old Randomly Bred (RB) and Cardiomyopathic (BIO) Hamsters

	Age 35 days		Age 180 days	
	RB	BIO	RB	BIO
Oleate formed (nmol/min/mg prot)	0.51 ± 0.04[ab] (13)	0.32 ± 0.04[b] (13)	0.22 ± 0.03 (12)	0.18 ± 0.02 (12)
Microsomal protein concentration (mg/g tissue)	11.0 ± 0.5 (13)	12.5 ± 0.5 (13)	12.4 ± 0.8 (12)	12.6 ± 0.4 (12)
Total lipid content (mg/g tissue)	38.9 ± 0.3[c] (13)	43.6 ± 0.2[c] (13)	35.5 ± 0.9 (13)	38.9 ± 1.4 (13)
Liver weight (g)	2.5 ± 0.1 (13)	2.6 ± 0.1 (13)	5.5 ± 0.3[d] (13)	3.9 ± 0.1[d] (13)

[a]Values are $\overline{X}$ ± SEM. Number in parentheses indicates number of animals.
[b-d]Differences between values with similar superscripts are statistically significant ($P < .05$).

TABLE III

Relative Percent Fatty Acid Composition of Lipids Isolated from Adipose Tissue of 35- and 180-Day-Old Randomly Bred (RB) and Cardiomyopathic (BIO) Hamsters

Fatty acid	Age 35 days		Age 180 days	
	RB	BIO	RB	BIO
14:0	1.85 ± 0.06[a]	1.99 ± 0.05	1.44 ± 0.04	1.78 ± 0.10
16:0	22.1 ± 0.16	21.4 ± 0.28	19.9 ± 0.29	18.7 ± 0.87
16:1	6.01 ± 0.17[b]	5.17 ± 0.12[b]	5.60 ± 0.24[c]	3.84 ± 0.08[c]
18:0	5.14 ± 0.09[d]	5.97 ± 0.09[d]	3.90 ± 0.27[e]	5.74 ± 0.09[e]
18:1	40.0 ± 0.39[f]	38.4 ± 0.34[f]	36.3 ± 0.66[g]	33.8 ± 0.33[g]
18:2	20.5 ± 0.29[h]	22.4 ± 0.26[h]	29.3 ± 0.74[i]	33.9 ± 0.28[i]
18:3	2.73 ± 0.03	3.11 ± 0.17	2.68 ± 0.09	2.71 ± 0.07
Others	1.70 ± 0.14	1.50 ± 0.12	1.00 ± 0.12	1.00 ± 0.10
(16:0 + 18:0) / (16:1 + 18:1)	0.597	0.630	0.568	0.648

[a]Values are $\overline{X}$ ± SEM of at least six observations.
[b-i]Differences between values with similar superscripts are statistically significant ($P < .05$).

TABLE IV

Relative Percent Fatty Acid Composition of Lipids Isolated from Liver of 35- and 180-Day-Old Randomly Bred (RB) and Cardiomyopathic (BIO) Hamsters

Fatty acid	Age 35 days		Age 180 days	
	RB	BIO	RB	BIO
10:0	7.43 ± 1.60[a]	8.25 ± 2.10	9.31 ± 1.60	7.22 ± 1.60
14:0	1.33 ± 0.12	1.22 ± 0.15	1.28 ± 0.05	1.60 ± 0.38
16:0	22.3 ± 0.29[b]	24.2 ± 0.58[b]	20.9 ± 0.53	19.2 ± 1.20
18:0	13.1 ± 0.47[c]	14.7 ± 0.79[c]	15.4 ± 1.0	15.8 ± 0.41
18:1	15.9 ± 1.10[d]	12.1 ± 0.88[d]	10.9 ± 0.6	9.2 ± 0.45
18:2	15.9 ± 0.46	15.3 ± 0.51	16.0 ± 1.20[e]	20.3 ± 0.66[e]
18:3	1.37 ± 0.14	1.36 ± 0.27	Trace	Trace
20:0	Trace	Trace	1.95 ± 0.17	2.16 ± 0.14
20:4	8.30 ± 0.10[f]	7.76 ± 0.13[f]	9.94 ± 0.61	10.4 ± 0.27
22:0	1.28 ± 0.05	1.43 ± 0.09	Trace	Trace
Others	9.86 ± 0.25	10.3 ± 0.30	11.6 ± 0.30	11.0 ± 0.32
(16:0 + 18:0) / 18:1	2.232	3.225	3.341	3.318

[a]Values are $\overline{X}$ ± SEM of at least six observations.
[b-f]Differences between values with similar superscripts are statistically significant ($P < .05$).

and 5 mg of protein/ml for adipose tissue. Protein concentration was determined by the method of Lowry et al. (9).

Stearoyl-CoA desaturase activity was determined as described by Holloway (10) except that fatty acid methyl esters were prepared according to the method of Metcalf and Schmitz (11). All assays were performed in triplicate and were linear with time of incubation and amount of enzyme added.

Extraction and quantitation of tissue lipids and determination of their fatty acid composition were performed as described earlier (7).

RESULTS

Table I summarizes the results of adipose tissue stearoyl-CoA desaturase activity, microsomal protein content, total lipid content, tissue weight, animal weights, and food consumption per day per g body weight. No significant differences were found in animal weights between the 35-day-old cardiomyopathic hamsters and their normal counterparts, but a significant decrease in body weight was seen in the 180-day-old diseased animals. Additionally, no significant differences were found in the amount of food consumed per day per g body weight in either age group.

The activity of adipose tissue stearoyl-CoA desaturase of 35-day-old cardiomyopathic hamsters was not different from that of the age-matched normal controls (Table I). In the older group, however, enzyme activity (nmoles/min/mg protein) in the cardiomyopathic hamsters was only 32% of that of the controls. Similar results were obtained when the activity was expressed on the basis of adipose tissue weight, i.e., nmoles/min/g tissue. Adipose tissue weight and the total lipid content of the two age groups of the cardiomyopathic hamsters were significantly lower than those of the normal controls (Table I).

In liver (Table II), stearoyl-CoA desaturase activity was significantly depressed in the 35-day-old diseased animals but was not changed in the 180-day-old group. No differences in the microsomal protein concentration were observed. There was a significant increase in the total lipid content of livers of 35-day-old diseased animals, but no significant differences were observed in the older group. Livers of the 180-day-old cardiomyopathic hamsters were significantly lighter than their normal counterparts.

The fatty acid composition of lipids isolated from adipose tissue and liver is shown in Tables III and Table IV, respectively. In adipose tissue, there was a significant increase in the relative concentration of stearic acid and linoleic acid of the diseased animals of both age groups. Concomitantly, there was a significant decrease in the relative concentration of palmitoleic and oleic acid in the diseased animals. Similar results were obtained from the analysis of the fatty acid composition of liver lipids (Table IV). There was an increase in the concentration of palmitic and stearic acids of the young diseased animals accompanied by a decrease in the concentration of oleic and arachidonic acids. In the 180-day-old group, linoleic acid concentration was significantly elevated in the diseased animals.

DISCUSSION

Stearoyl-CoA desaturase activity has been found to be influenced by various factors including diet (3,4). It is unlikely, however, that the enzyme activity in the liver and adipose tissue of the diseased animals is depressed as a result of feeding diets containing the lipid end-product since food sonsumption by the two age groups of cardiomyopathic hamsters per day per g body weight was similar to that of the age-matched normal controls. The decreased enzyme activity that was observed in the diseased animals may be a result of other changes in the tissues of these animals.

Although the fatty acid composition of adipose tissue is changed in both the 35- and 180-day-old cardiomyopathic hamster (Table III), desaturase activity was depressed only in the 180-day-old diseased animals. Since the fatty acids that are stored in adipose tissue are derived from various sources – such as diet, de novo fatty acid synthesis in liver, etc. – the alterations in the fatty acid compostion of adipose tissue lipids of 35-day-old cardiomyopathic hamsters could reflect the alterations in the desaturase activity in the liver of those animals (Table IV). In the 180-day-old diseased animals, the alterations in the fatty acid composition may be related to the observed changes in the desaturase enzyme. The decrease in the lipid content of adipose tissue could be due to the decrease in glyceride synthesis that we reported earlier (12).

The changes in the relative concentration of some of the fatty acids of the lipids isolated from the livers of the cardiomyopathic hamsters parallel changes in stearoyl-CoA desaturase activity. Thus, when desaturase activity was lowered (35-day-old group), the relative concentration of palmitic and stearic acids was elevated and the concentration of oleic acid was lowered. Where no changes in desaturase activity were observed (180-day-old group), no

changes in the fatty acid composition were observed.

The major fatty acid in the diet is linoleic acid (48.51% of the total fatty acids – unpublished data). At the present time, we have no experimental data to explain the relative increase in its concentration or the observed decrease in arachidonic acid concentration in the 35-day-old cardiomyopathic hamsters.

The changes in the fatty acid compostion of the lipids of adipose tissue and liver, and similar changes in the heart and muscle that we reported earlier (7) in the cardiomyopathic hamsters, would result in alterations in the ratio of saturated to monounsaturated fatty acids. The resultant disturbance in this ratio may, in turn, influence the normal assembly and fluidity of cellular membranes. Evidence exists of alterations in mitochondrial function (13), sarcoplasmic reticulum (14), microsomal function (12), and cell permeability (15) in the diseased animals. It is possible that the alterations in the normal function of these membranes may be related to alterations in their fatty acids. Work is presently underway to resolve this point.

ACKNOWLEDGMENTS

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ERRATUM

In "Δ15,18-Tetracosadienoic Acid Content of Sphingolipids from Platelets and Erythrocytes of Animals Fed Diets High in Saturated or Polyunsaturated Fats" [*Lipids* 13:551 (1978)], "Platelets" was printed incorrectly.

changes in the fatty acid composition were observed.

The major fatty acid in the diet is linoleic acid (48.51% of the total fatty acids – unpublished data). At the present time, we have no experimental data to explain the relative increase in its concentration or the observed decrease in arachidonic acid concentration in the 35-day-old cardiomyopathic hamsters.

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ERRATUM

In "Δ15,18-Tetracosadienoic Acid Content of Sphingolipids from Platelets and Erythrocytes of Animals Fed Diets High in Saturated or Polyunsaturated Fats" [*Lipids* 13:551 (1978)], "Platelets" was printed incorrectly.

Arylsulfonate Esters of Fatty Alcohols: IV. Effects on Cholesterol Catabolism[1]

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ABSTRACT

Hypercholesterolemic rats, fed 1% cholesterol and 0.5% glycocholate, were treated with arylsulfonates in various ways to observe the pattern of cholesterol elimination. Dietary linoleyl p-toluenesulfonate (LTS) hastened return to normocholesterolemia and lowered hepatic cholesterol either with or without continued cholesterol feeding. LTS administered via the portal vein significantly lowered plasma cholesterol in 48 hr; ethyl linoleate and monoolein porduced no lowering. LTS administered via the portal vein to glycocholate-infused rats increased the biliary excretions of label from [4-^{14}C] cholesterol administered intracardially and also increased total bile acid excretion 21% without increased bile volume when compared to similar injection of ethyl linoleate. No change in biliary excretion of cholesterol was seen. Bile acid kinetics were studied by using isotopic dilution techniques. Cholate turnover was enhanced by feeding oleyl p-toluenesulfonate (OTS) and oleyl *p*-(*n*-decyl)-benzenesulfonate (ODS) as suggested by a 16-35% decrease in half-life in both normal and hypercholesterolemic rats. Rats consuming a grain-based colony diet had a 54% increase in cholate synthesis when OTS was included in the diet. The composition of bile was changed when either OTS or ODS was fed; an increase in chenodeoxycholate was noted. This change was gradual with OTS but rapid with ODS and paralleled enhanced decay of chenodeoxycholate specific radioactivity in response to treatment. ODS and OTS also increased $^{14}CO_2$ expiration from oral [26-^{14}C] cholesterol in hypercholesterolemic rats. Dietary OTS and ODS elevated hepatic free cholesterol in hypercholesterolemic rats; ODS also elevated plasma free cholesterol and increased cholesteryl ester hydrolase activity in the liver. The data suggest that arylsulfonates stimulate cholesterol catabolism, in addition to the reported inhibition of cholesterol absorption [Lipids 12:819 (1977)].

INTRODUCTION

Various arylsulfonates of long chained fatty alcohols lower plasma and hepatic cholesterol levels in the cholesterol-fed rat and rabbit (1,2). Oleyl p-toluenesulfonate (OTS) decreases the rate of arterial cholesterol accumulation in cholesterol-fed rabbits (2). Oleyl p-(n-decyl)-benzene-sulfonate (ODS) appears to be the most potent hypocholesterolemic compound among a large number produced by modification of the arylsulfonate portion of the molecule, but there are no apparent differences in hypocholesterolemic activities of oleyl and linoleyl p-toluenesulfonates (2).

The mechanism of cholesterol-lowering by these compounds has not been completely established. Linoleyl p-toluenesulfonate (LTS) does not inhibit cholesterol synthesis in liver in vivo or in vitro nor does it cause a redistribution of cholesterol to other tissues (3). LTS does appear to inhibit cholesterol absorption from the intestine of prophylactically treated rats (3) in a manner similar to the structurally related hypocholesterolemic agent, (-)N-[α-phenyl-β-(p-tolyl)ethyl] linoleamide (4). Although initial studies with normocholesterolemic rats showed no increase in fecal excretion of acidic steroids (3), this report presents evidence that arylsulfonates can effect bile acid metabolism and cholesteryl ester hydrolase activity.

MATERIALS AND METHODS

Ethyl linoleate and arylsulfonates were prepared as reported elsewhere (2,5). Monoolein prepared by the method of Daubert and Baldwin (6) was purified by preparative thin layer chromatography (TLC) prior to use. [4-^{14}C] Cholesterol-labeled serum lipoproteins were prepared (7) by incubating serum at 37 C with [4-^{14}C] cholesterol (Calbiochem, LaJolla, CA; 34.3 mCi/mmole) dispersed on Celite 545. [4-^{14}C] Cholesteryl oleate was prepared from [4-^{14}C] cholesterol (New England Nuclear, Boston, MA; 50mCi/mmole) and oleyl chloride (Hormel Institute, Austin, MN), as described by Pinter et al. (8). The product was purified on alumina columns as described by Sgoutas (9) and diluted to desired specific radioactivity with nonlabeled cholesteryl oleate (Hormel Institute).

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Animals and Diets

Inbred-Wistar male rats were housed at constant temperature (72 F) and humidity (50%) in wire-bottomed cages. Feed and water were available ad libitum unless otherwise specified. Weanling rats were fed a natural grain based colony diet or purified, hypercholesterolemic diet A (5) containing 1% cholesterol and 0.5% sodium glycocholate (Nutritional Biochemicals Corp., Cleveland, OH) for 3 to 4 weeks, except as otherwise indicated, prior to supplementation. Fat contained in diet A was 2% hydrogenated coconut oil (diet AH-2), 5% lard (diet AL-5), or 10% beef tallow plus 10% safflower oil (diet ABS-20). Hydrogenated coconut oil (Procter and Gamble, Cincinnati, OH), lard (Stark and Wetzel, Indianapolis, IN), beef tallow (Swift and Co., Chicago, IL), and safflower oil (Pacific Vegetable Corp., Richmond, CA) were obtained commercially. In the early experiments, the linoleyl arylsulfonate derivatives were used as supplements. Since later work could not demonstrate differences in the hypocholesterolemic activities of oleyl and linoleyl tosylates (2), the less readily oxidizable oleyl derivatives were used in subsequent experiments.

Bile Duct Experiments

In chronic fistula experiments, polyethylene tubing (PE 10) was inserted into the common bile duct, exited through the incision, and taped on the animal's back. For rats receiving infusion fluid, a second piece of PE 10 tubing inserted into a separate incision in the bile duct, distal to the first and secured as above, extended to a graduated cylinder (50 ml) positioned at a height (18 in) to provide a flow rate of 1 ml/hr. Flow of infusate was started before insertion of the tube into the bile duct. Cannulated animals were housed in sliding-bar restraining cages. At the end of cannulation, rats received cardiac injections of [4-^{14}C]cholesterol-labeled serum lipoproteins. Whole bile was fractionated by saponification-extraction into total bile acids and neutral sterols (10). Cholesterol was determined (11) and total bile acids were measured spectrofluorometrically (12). Radioactivity was measured by liquid scintillation counting (Packard, Tri Carb). Whole bile (0.1 ml) was diluted with 15 ml of dioxane scintillation fluid (13) and counted directly. Quench corrections were made by addition of an internal standard to the samples and recounting.

In bile duct experiments with T-cannulated rats, surgery was performed as previously outlined (14). The animals were not restrained and had free access to both water and diet. After a 3 to 4 day recovery period, rats with sufficient bile flow and no abnormalities were injected by cardiac puncture with 0.5 μCi of [24-^{14}C]-chenodeoxycholic acid (35.8 mCi/mmole, International Chemical and Nuclear Corp., Irvine, CA) and 2.0 μCi of [G-^{3}H]cholic acid (2.12 mCi/mmole, New England Nuclear) in 0.25 ml of 40% ethanolic saline. The 15 min bile samples were collected daily under ether anesthesia starting 24 hr after injection of labeled bile acid. Bile samples were saponified, and the free bile acids were separated by continuous development, thin layer chromatography (TLC) (14). The eluates were analyzed for radioactivity by scintillation counting and for mass by ultraviolet spectrophotometry as previously outlined (14). From the results of photometric and scintillation analysis, specific radioactivity (SR) was then calculated and half-life, pool size, and synthesis rate were determined by following the decay of SR with time.

Cholesterol Oxidation Study

[26-^{14}C]Cholesterol (Amersham/Searle Corp., 50 mCi/mmole) was dissolved in olive oil and given to ether-anesthetized animals by stomach tube. To collect expired CO_2, animals were then placed in sealed chambers through which Ascarite-scrubbed air was circulated and then bubbled through 2 N KOH in two consecutive 1500 ml traps at a rate of 1.25 l/min. Preliminary experiments showed that 99.5% of the CO_2 was removed. Carbonate was precipitated from aliquots of the KOH traps with saturated $BaCl_2$. The precipitate was filtered and washed with water in a Buchner funnel, dried at 110C for 1 hr, and weighed. An aliquot of $BaCO_3$ (0.5 g), thus prepared, was pulverized in a mortar, suspended in a toluene scintillation fluid containing 4.5% Thixotropic Gel Powder (Packard Instrument Co., Inc.), and counted. Efficiency (38%) of this method was determined by acidification of aliquots of selected samples, and collection of evolved CO_2 in Hydroxide of Hyamine, which was then dissolved in toluene scintillation fluid and counted. Radioactivity in plasma and liver was extracted as described for lipid analyses. Aliquots of extracts were taken to dryness and counted in toluene scintillation fluid. Counting efficiency was determined by the channels ratio method.

Plasma and Tissue Lipid Analyses

Blood samples were withdrawn by cardiac puncture, and livers were excised and frozen for later analysis. Livers were thawed and extracted with chloroform-methanol for hepatic choles-

TABLE I

Effect of Linoleyl Tosylate on Cholesterol and Hepatic Total Lipid[a]

Dietary addition (%)[b]			Days on experiment			
CH	LTS	GC	5	10	15	20
			Hepatic total cholesterol, mg/liver			
1.0	None	0.5	1161 ± 36[c]	1399 ± 91	1494 ± 33	1708 ± 159
None	None	0.5	1015 ± 113	1063 ± 83	935 ± 198	714 ± 168
1.0	0.15	0.5	1076 ± 88	927 ± 252	678 ± 86	517 ± 55
None	0.15	0.5	1058 ± 125	762 ± 106	616 ± 120	639 ± 103
			Hepatic total lipid, mg/liver			
1.0	None	0.5	2072 ± 89	2566 ± 125	2512 ± 37	2834 ± 219
None	None	0.5	1952 ± 294	1958 ± 140	1910 ± 342	1524 ± 228
1.0	0.15	0.5	1846 ± 212	1707 ± 418	1393 ± 70	1102 ± 116
None	0.15	0.5	1843 ± 138	1516 ± 125	1368 ± 164	1332 ± 233
			Plasma cholesterol, mg/100 ml			
1.0	None	0.5	354 ± 49	332 ± 20	418 ± 45	451 ± 69
None	None	0.5	283 ± 31	227 ± 18	198 ± 33	232 ± 9
1.0	0.15	0.5	300 ± 45	129 ± 6	136 ± 14	114 ± 7
None	0.15	0.5	210 ± 19	145 ± 11	127 ± 5	133 ± 16

[a]After bleeding 6 animals to establish initial plasma cholesterol levels (308 ± 29, mg/100 ml), 48 hypercholesterolemic male rats (212 ± 6 g) were randomly divided into 4 groups of 12 rats and were fed the variations of diet AH-2 during a 20 day period. At 5-day intervals, 3 from each group were selected for analyses of plasma and liver by methods described in the text. All groups of rats maintained or showed small increases in body weight. The liver weights of 3 groups were similar (13.2 g), but cholesterol-fed rats with no LTS had slightly larger livers (15.1 g).

[b]CH, cholesterol; LTS, linoleyl tosylate; GC, glycocholate.

[c]Values are means for 3 rats ± SE.

terol and total lipid analysis (15). Plasma and hepatic cholesterol was determined by the method of Sperry and Webb after digitonin precipitation (11).

Cholesteryl Ester Hydrolase Experiments

Cholesteryl ester hydrolase activity was determined essentially by the method of Deykin and Goodman (16) with modifications described recently (17). The 100,000 x g supernatant fraction was freed of endogenous cholesteryl ester by hexane extraction. Hydrolase activity was assayed in a final volume of 2 ml, to which [4-^{14}C]cholesteryl oleate was added in 100 μl of acetone, and the mixtures were incubated at 37 C for 1 hr. Isolation of radioactive cholesterol and cholesteryl ester for scintillation counting has been described in detail (17). Protein was determined by the method of Lowry et al. (18).

RESULTS AND DISCUSSION

Previous studies (3) have shown that arylsulfonates cause an inhibition of the intestinal absorption of dietary cholesterol. Additional mechanisms by which the arylsulfonates reduce hepatic and plasma cholesterol of hypercholesterolemic rats seemed possible. In a preliminary experiment, hypercholesterolemic rats continued to receive bile salts but no cholesterol in their diet (Table I). In the presence or absence of dietary cholesterol, LTS feeding accelerated the lowering of plasma and hepatic cholesterol. When animals were killed after 10 days, those that were fed LTS without (or with) cholesterol had a 36% (or 43%) lower plasma cholesterol and a 28% (or 13%) lower hepatic cholesterol than those fed neither cholesterol nor LTS. Evidence that LTS enhanced cholesterol lowering persisted in the data from animals killed after 15 days and 20 days.

This enhanced lowering of plasma and hepatic cholesterol could be the result of increased cholesterol oxidation to bile acids, increased biliary cholesterol excretion, or decreased intestinal absorption of endogenous cholesterol. It is unlikely that LTS inhibits cholesterol biosynthesis (3).

The effect of intravenous injection of LTS was observed (Table II). A single portal vein injection of LTS significantly lowered plasma cholesterol within 48 hr, but these levels were not maintained at 72 hr. Ethyl linoleate and monoolein were essentially without effect. Sham operated animals showed no drop in plasma cholesterol levels at 48 and 72 hr. The

TABLE II

Comparative Responses of Hypercholesterolemic Rats to Single Portal Vein Injections[a]

Test substance	No. of rats	Diet ingested (g/72 hr)	Total plasma cholesterol (mg/100 ml)		
			0 hr	48 hr	72 hr
Ethyl linoleate	8	34 ± 2	383 ± 51	364 ± 34	446 ± 52
Monoolein	8	29 ± 4	446 ± 62	383 ± 42	414 ± 25
Linoleyl tosylate	8	27 ± 2	409 ± 39	260 ± 18[b]	387 ± 33
None (control)	8	32 ± 4	391 ± 25	452 ± 38	443 ± 40

[a]Portal veins of hypercholesterolemic rats which had been fed the diet AH-2 for 5 weeks were exposed by surgery. The test substance (60 mg) was suspended by sonication in an equal volume of 1% Tween 20 in 0.9% saline and was injected into the portal vein. Rats were then sutured and returned to their cages with food and water ad libitum. Control animals were sham-operated but received no injections. Blood samples were taken for analysis at time of surgery and 48 and 72 hr later. Values are the means for 8 rats ± SE.

[b]$P<0.005$ when compared to the 48 hr control value.

TABLE III

Biliary Excretion of ^{14}C-Label after Injection of [4-^{14}C]Cholesterol[a]

Portal vein injection	Duodenal infusion of glycocholate (mg/hr)	No.	% of injected radioactivity		Total steroid recovery in bile, 12-24 hr	
			0-12 hr	12-24 hr	Cholesterol (mg)	Bile acids (mg)
None	None	3	12.52 ± 0.92[b]	10.74 ± 0.30	--	--
None	2.5 - 3.5	3	14.45 ± 0.58	7.76 ± 0.67	--	--
EL[c]	2.5	7	9.41 ± 0.44	5.09 ± 0.56	2.1 ± 0.3	44.7 ± 3.5
LTS	2.5	7	10.42 ± 0.51	6.36 ± 0.96	2.0 ± 0.2	54.0 ± 5.1

[a]Hypercholesterolemic male rats (200-225 g) were bile-fistulated and then given an intracardiac injection of rat serum containing [4-^{14}C]cholesterol-labeled lipoprotein (0.20-0.30 μCi). Portal vein injections (60 mg) were made immediately and duodenal infusions then followed as indicated in the text. Bile was collected for 12-hr periods and analyzed for total radioactivity, cholesterol and bile acids. Animals had free access to a 5% dextrose solution.

[b]Values are means for the group ± SE.

[c]EL, ethyl linoleate; LTS, linoleyl tosylate.

reason for the return to initial levels of plasma cholesterol by the tosylate-treated group may be catabolism or cellular binding of the tosylate. Although 97% of the label remained in the liver 72 hr after a single portal vein injection of linoleyl [1-^{14}C]tosylate, no studies were undertaken to determine the chemical form or bound state of the compound. It is evident that LTS can exert a hypocholesterolemic effect when given intravenously to the rat, and that effect is not a general response to injection of a lipid material since ethyl linoleate and monoolein do not produce it. Ethyl linoleate, which has been shown to exert a hypocholesterolemic effect when fed in the diet to hypercholesterolemic rats (5,19,20), appears to exert its hypocholesterolemic effect by different mechanisms than LTS.

To test whether arylsulfonates increase secretion of biliary cholesterol or increase degradation of cholesterol to bile acids, the biliary excretion of labeled cholesterol and its metabolites was measured after injection of [4-^{14}C]cholesterol-labeled serum lipoproteins into rats fed hypercholesterolemic diet AH-2 from weaning (Table III). Since bile acid excretion is greatly increased (21) 12-18 hr after fistulation, rats were infused with ca. 3 mg/hr of bile acids to partially offset this response (22). Bile samples were collected at 0-12 and 12-24 hr after bile duct cannulation and LTS injection. The fraction of injected radioactive cholesterol excreted in the bile was similar for all groups during the 0-12 hr period, although rats which received portal injections tended to be lower. In all groups infused with glycocholate, biliary excretion of radioactivity was depressed during the 12-24 hr period to about 60% of the 0-12 hr excretion, while the 12-24 hr excretion of radioactivity by noninfused rats declined only 14%. In rats that received LTS injections, biliary excretion of radioactivity tended to be higher than in those that received ethyl linoleate (Table III), although the difference was not statistically significant. Since ethyl linoleate did not lower plasma cholesterol in response to portal vein injection (Table II), this treatment may represent a better control

for comparison to LTS injection. Comparison of the two groups that received portal vein injections further showed no difference in volume of bile excreted nor amount of biliary cholesterol excreted over the 12-24 hr period, but the LTS treated group excreted more total bile acids than did the ethyl linoleate treated rats. The lack of any effect of LTS injection on biliary cholesterol excretion and the small amount of cholesterol eliminated by this route (4 mg/day) suggest that arylsulfonates do not exert hypocholesterolemic activity by this mechanism.

Since the previous experiments suggested that arylsulfonates may have an effect on bile acid metabolism, bile acid turnover was studied by the isotope dilution method, using techniques recently described (14). In spite of a decrease in plasma cholesterol with OTS or ODS treatment, a linear relationship of log specific radioactivities (SR) of cholate to time was obtained (Fig. 1), and, therefore, the kinetics of cholate metabolism could be defined (Table IV). It appeared that OTS and ODS caused a slight decrease in cholate pool size. The half-life of cholate decreased with treatment in all cases. In rats fed the hypercholesterolemia-producing diet AL-5, the half-life decreased 16% on supplementation with OTS ($P < 0.02$) and 21% with ODS ($P < 0.01$). In rats fed the colony diet, OTS supplementation decreased the cholate half-life by 35%. Calculation of synthesis rates was limited to colony diets since commercial sodium glycocholate was added to the other diets and dietary intake was not monitored. OTS caused a 54% increase in cholate synthesis. Although a small number of the untreated colony rats was used, the value for cholate synthesis agreed with the previously obtained value (14). These experiments suggest that arylsulfonate feeding can exert hypocholesterolemic activity by increasing catabolism of cholesterol to cholate. Although the SR of chenodeoxycholate declined faster with arylsulfonate treatment (Fig. 2), these same parameters could not be calculated since a nonlinear

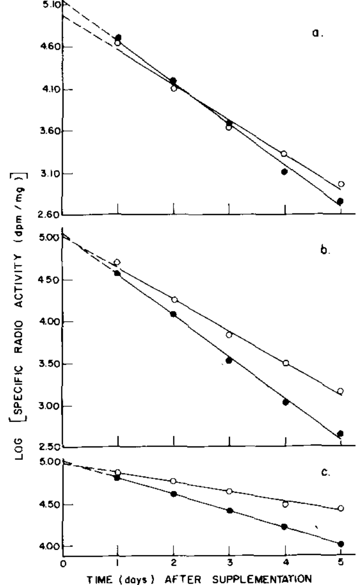

FIG. 1. Decline in cholate specific radioactivity as affected by arylsulfonates. Hypercholesterolemic diet AL-5 (a,b) or our colony diet (c) were fed to male rats from weaning. After bile duct T-cannulation and recovery period, [^{3}H]cholate was injected by cardiac puncture, and the same diet was fed with (•) or without (○) supplements of 0.5% OTS (a,c) or 0.1% ODS (b). The log of the mean specific radioactivity for each diet was plotted. See Table IV for more details.

TABLE IV

Effect of Arylsulfonates on Cholate Metabolism[a]

Basal diet	Supplements[b]	No. of rats	Plasma cholesterol (mg/100 ml)	Half-life (day)	Pool size (mg)	Synthesis rate (mg/day)
AL-5	None	14	211 ± 9	0.67 ± 0.04	53.5 ± 8.4	---
AL-5	0.5% OTS	12	147 ± 9	0.56 ± 0.02	36.6 ± 6.6	---
AL-5	None	13	281 ± 22	0.76 ± 0.05	40.1 ± 4.7	---
AL-5	0.1% ODS	14	177 ± 14	0.60 ± 0.03	49.2 ± 7.6	---
Colony	None	2	---	2.36 ± 0.64	48.5 ± 6.2	14.9 ± 2.3
Colony	0.5% OTS	3	---	1.54 ± 0.15	50.2 ± 0.9	23.0 ± 2.6

[a]Rats were fed a basic diet from weaning through the bile duct operation and recovery period. After intracardiac injection of [^{3}H]cholate, each group was fed the same basal diet plus indicated dietary supplements. Bile samples were collected daily and cholate specific radioactivities were determined. A plot of log specific radioactivity vs time was a straignt line for each rat (the average coefficient of determination for all rats was 0.979 ± 0.003). Kinetic values were calculated from these data by using least squares equations to obtain slope and ordinate intercept for each rat. Plasma cholesterol was determined one week after supplementation. Values are expressed as means ± standard errors of the mean.

[b]OTS, oleyl tosylate; ODS, oleyl *p*-(*n*-decyl) benzenesulfonate.

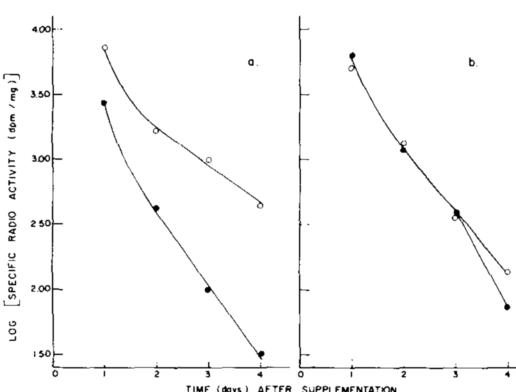

FIG. 2. Decline in chenodeoxycholate specific radioactivity as affected by arylsulfonates. The same rats that were fed hypercholesterolemic diet AL-5 in Figure 1 were also injected with [24-^{14}C]chenodeoxycholate. Nonsupplemented diet (o) or diets supplemented (•) with 0.1% ODS (a) or 0.5% OTS (b) were then fed after the cardiac injection. Each point represents the mean specific radioacitivty of 12-14 animals.

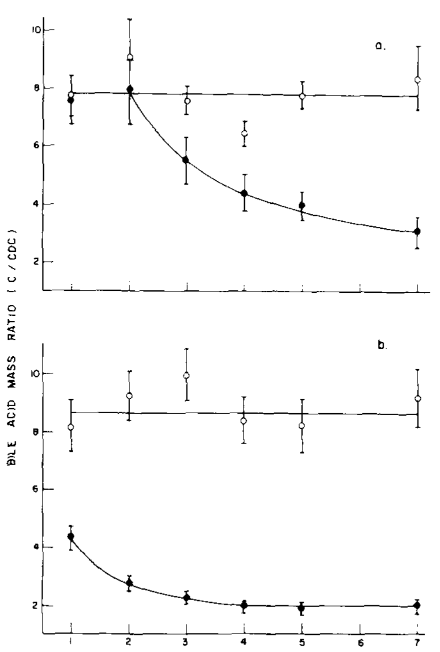

FIG. 3. Change in the ratio of cholate to chenodeoxycholate in rat bile in response to arylsulfonate feeding. Rats were fed hypercholesterolemic diet AL-5 from weaning through the bile duct T-cannulation and recovery period. Nonsupplemented (o) or diets supplemented (•) with 0.5% OTS (a) or 0.1% ODS (b) were then fed and 15 min bile samples were taken daily. Cholate and chenodeoxycholate mass values were determined spectrophotometrically after separation by continuous development TLC. The mass ratios were calculated for individual animals, and then the mean and standard error of the mean (bars) were plotted.

TABLE V

Effect of OTS and ODS on Cholesterol Concentration and Metabolism in Hypercholesterolemic Rats Fed [26-^{14}C]Cholesterol Prior to Supplementation[a]

Dietary supplement	No. of rats	$^{14}CO_2$ Expired during days of supplementation (dpm/hr)				Cholesterol[b] Specific radioactivity (dpm/mg) liver		plasma	Concentration liver (mg/liver)		plasma (mg/100μl)	Metabolism[c] (mg/hr)
		0	1	2	3	TC	FC	TC	TC	FC	TC	
None	6	8151	2200	1220	792	320	322	256	716	35	431	2.5 ± 0.1
0.5% OTS	6	7796	1816	1112	1060	308	308	276	644	50	252	3.4 ± 0.7
0.1% ODS	5	8334	2454	1878	1800	300	315	308	563	76	386	5.7 ± 0.8

[a]Weanling rats fed Diet ABS-20 for 18 days were given by stomach tube 0.5 μCi [26-^{14}C]cholesterol in 0.3 ml olive oil, and fasted 24 hr while expired CO_2 was collected (Day 0). They were then fed Diet ABS-20 with supplement for 3 days (Days 1-3). CO_2 was collected continuously from administration of radioactivity, and liver and plasma analyses were performed after 3 days of supplementation.

[b]TC, total cholesterol; FC, free cholesterol.

[c]Cholesterol metabolism was calculated by dividing the rate of $^{14}CO_2$ expiration on day 3 by the specific radioactivity of liver free cholesterol. Values are averages ± standard error of the mean and were calculated from pooled $^{14}CO_2$ collections (2 pools/group).

TABLE VI

Effect of OTS and ODS on Cholesterol Concentration and Metabolism in Hypercholesterolemic Rats Fed [26-^{14}C]Cholesterol during Supplementation[a]

Dietary supplement	No. of rats	$^{14}CO_2$ Expired (dpm/hr)	Liver cholesterol[b] Specific radioactivity TC (dpm/mg)	FC (dpm/mg)	Concentration TC (mg/liver)	FC (mg/liver)	Metabolism[c] (mg/hr)
None	6	2436	614	798	1468	44	3.0 ± 0.1
0.5% OTS	6	4118	322	672	1044	69	6.1 ± 0.7
0.1% ODS	5	3398	211	527	887	112	6.4 ± 1.3

[a]Weanling rats were fed Diet ABS-20 for 7 weeks, and Diet ABS-20 with supplement for 4 days. After 2 days of supplementation, they were given by stomach tube 1.1 μCi [26^{14}C]cholesterol in 0.5 ml olive oil, and expired CO_2 was collected for 2 days, after which liver analyses were performed.

[b]TC, total cholesterol; FC, free cholesterol.

[c]Cholesterol metabolism was calculated by dividing the rate of $^{14}CO_2$ expiration by the specific radioactivity of liver free cholesterol. Values are averages ± standard error of the mean and were calculated from pooled $^{14}CO_2$ collections (2 pools/group).

plot was again (14) obtained with chenodeoxycholate.

Analysis of the bile collected daily throughout the isotope dilution experiments for the amounts of cholate and chenodeoxycholate secreted suggested an increase in chenodeoxycholate secretion after arylsulfonate treatment. Since total biliary diversion may not have been established during the collection of bile using our T-cannulated rat model (14), a measure of the relative amounts of primary bile acid secretion was obtained by comparing the ratio of cholate to chenodeoxycholate (Fig. 3). While control diets maintained constant relative amounts of cholate and chenodeoxycholate throughout the experiment, addition of OTS to the diet produced a gradual decrease starting on the third day of treatment. ODS which was fed at a fifth of the amount of OTS caused a much more rapid decrease, and a constant ratio was obtained by the third day. This increase in chenodeoxycholate secretion probably reflects an increase in chenodeoxycholate synthesis, since a faster decline of the SR of chenodeoxycholate was seen when these changes in bile acid secretion were taking place (Fig. 2).

The decreasing ratio of cholate to chenodeoxycholate in relation to hypocholesterolemic activity may prove to be important. Thyroxine or other thyroid hormones which can act as hypocholesterolemic agents have produced a similar change in relative amounts of cholate and chenodeoxycholate (23-25). This change was accompanied by an increased excretion of chenodeoxycholate and its metabolites (24) and possibly a decrease in cholesterol absorption (23,26). If there is any correlation between these effects of thyroxine and arylsulfonates, it would most likely be at

TABLE VII

Effect of ODS on Plasma Free Cholesterol during Initial Period of Supplement[a]

Dietary supplement	No. of animals	Period of intake	Plasma free cholesterol[b]
		(days)	(mg/100 ml)
None	6	5	58 ± 13
0.1% ODS	6	5	125 ± 35
None	6	3	44
0.1% ODS	3	3	136

[a]Weanling rats were fed Diet ABS-20 for 7 weeks before supplement.

[b]Mean values with standard error when analyzed individually, and without standard error when pooled for analysis.

the cellular level, for after 5 or 8 weeks the levels of plasma protein-bound iodine were not different in rats fed diets with or without 0.15% LTS (27). It has also been shown that decreasing cholate while increasing chenodeoxycholate in diets which also contain cholesterol reduced the hypercholesterolemia-producing effects of such diets (28). Thus, changes in bile acid composition can have a hypocholesterolemic effect. It is possible that the hypocholesterolemic action of arylsulfonates is also due to the change in bile composition.

The metabolic fate of cholesterol in hypercholesterolemic rats treated with arylsulfonates was investigated further. [26-^{14}C]Cholesterol was administered to hypercholesterolemic rats by stomach tube either prior to or after dietary supplementation with OTS or ODS. By 3 days of supplementation, a greater hourly output of radioactivity in CO_2 was detected in arylsulfonate treated animals irrespective of when the

TABLE VIII

Effect of Dietary ODS on Cholesteryl Ester Hydrolase Activity in the Liver[a]

Experiments	Dietary supplement	No. of animals	Age from weaning (wks)	Protein (mg/assay)	[4-^{14}C]Cholesteryl oleate hydrolyzed 120 mμmoles substrate (μmoles/mg protein/hr)	200 mμmoles substrate
I	None	2	4	24	164 ± 21	204 ± 17
	0.1% ODS	3	4	24	316 ± 15	362 ± 4
II	None	3	5	20	172 ± 18	---
	None	3	5	10	171 ± 14	---
	0.1% ODS	3	5	10	273 ± 55	---
III	None	3	6	18	176 ± 6	---
	0.1% ODS	3	6	20	271 ± 4	---

[a]Weanling rats were fed Diet ABS-20 for the number of weeks indicated, and Diet ABS-20 with supplement for 3 days. All values for ester hydrolysis are corrected for boiled controls.

radioactive cholesterol was given (Tables V, VI). During the baseline Day 0 period, when animals had not yet received supplements, $^{14}CO_2$ output was essentially the same for all 3 groups (Table V). After treatment was begun, $^{14}CO_2$ output declined more rapidly in nonsupplemented animals than in supplemented. Until Day 3, average output was higher in both OTS and ODS groups than in nonsupplemented groups. Similar results were seen during the third and fourth day of supplementation when radioactive cholesterol was given after 2 days of supplementation (Table VI). The OTS group was slower to respond to treatment than the ODS group which was similar to the changes seen in bile composition (Fig. 3). Since the hepatic cholesterol SR of treated animals did not exceed the nonsupplemented (Tables V, VI), the increase in $^{14}CO_2$ output probably represented an increase in cholesterol catabolism in response to arylsulfonate treatment.

Along with expected decreases in total hepatic and plasma cholesterol, supplementation produced marked elevations in hepatic and plasma FC during the initial period of supplementation (Tables V, VI, VII), although the elevations have not been observed after total cholesterol levels decline (2). This FC increase is apparently due to an increase in liver cholesteryl ester hydrolase (CEH) activity (Table VIII). In a preliminary experiment, ODS did not simulate CEH when added to an incubation from nonsupplemented animals. This probably indicates that ODS does not directly activate CEH. The accumulation of FC, the probable substrate for oxidation to bile acids (17), suggests the stimulation of CEH as an initial event in the mode of action of arylsulfonates in hypercholesterolemic rats. Although other studies from this laboratory suggest that CEH action is not a rate-limiting step in cholesterol oxidation (17), increases in hepatic FC during the early phase of cholesterol feeding (29) have also been followed by an increase in bile acid synthesis (30,31) and a decrease in the ratio of cholate to chenodeoxycholate (31).

From the accumulated evidence, it now appears that the mode of action of cholesterol-lowering by arylsulfonates involves at least two mechanisms. One of these is stimulation of cholesterol catabolism, in which increased CEH activity may be an initial event, and the other (3) is a decrease in the intestinal absorption of cholesterol possibly due to increases in the relative amount of CDC in the bile. The inhibition of cholesterol absorption is apparent in both normocholesterolemic and hypercholesterolemic rats. The catabolic effect is more prominent in hypercholesterolemic than normocholesterolemic rats.

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Indomethacin Stimulation of Lipid Peroxidation and Chemiluminescense in Rat Liver Microsomes

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ABSTRACT

Peroxidation of endogenous lipid by rat liver microsomes, coupled with oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and measured as thiobarbituric acid reactive materials, is markedly stimulated in the presence of indomethacin [1-(p-chlorobenzyl)-5-methoxy-2-methyl-3-indole acetic acid] (0.1-1.0 mM). Concurrently, indomethacin enhances the lipolysis of membrane phospholipid containing arachidonic acid but has no effect on the rate of O_2 uptake in these samples. The system generates a rapidly developed chemiluminescense (CL), the intensity and rate of development of which are related to indomethacin concentration. The microsomal CL generated in the presence of indomethacin is distinct from the previously reported CL in that the time required for maximum intensity development is a matter of seconds (20-180) rather than hours. The enhanced CL is believed to be due to an energy transfer reaction whereby a high energy species transfers energy to the indomethacin molecule, which, in turn, decays via chemiluminescense. An enhanced chemiluminescense was also observed when indomethacin was added to a lipoxidase system and superoxide generating system (xanthine oxidase). Based on inhibitor studies, the rapidly developed chemiluminescense of the microsomal system requires cytochrome P-450 in addition to NADPH and coordinated iron ions. The results indicate that the CL is related to neither hydroxyl free radical nor superoxide anion formation.

Peroxidation of phospholipid-bound arachidonic acid in rat liver microsomes, coupled to reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidation, generates a chemiluminescense that reaches a maximum over several hours (1-3). It has been reported that this chemiluminescense is a property of intermediates common to drug metabolism, prostaglandin synthesis, and lipid peroxidation (4), possibly including superoxide, hydroxyl free radical, or hydroperoxide free radical. Addition of various compounds including benzopyrene and rose bengal to the peroxidizing system alters the time course of the chemiluminescense maximum development. In the case of polynuclear hydrocarbons and luminol, the changes in chemiluminescense appear to result from the generation of unstable intermediates that can decay via chemiluminescense (5-7); in other cases, the mechanism of the addition effect is not clear (8). Several other enzyme-catalyzed systems generate ultra weak light, including lipozygenase, xanthine oxidase, and the fatty acid cyclooxygenase of prostaglandin synthesis (9).

During experiments in which the peroxidation of endogenous arachidonic acid by liver microsomes was being compared to the oxidation of arachidonate by fatty acid cyclooxygenase, an unusual effect of indomethacin [1-(p-chlorobenzyl)-5-methoxy-2-methyl-3-indole acetic acid] on lipid peroxidation and chemiluminescense was observed. Indomethacin is a potent nonsteroid inhibitor of fatty acid cyclooxygenase and is prescribed clinically for its anti-inflammatory, antipyretic, and analgesic properties. When added to peroxiding microsomes, it was observed that indomethacin rapidly induces an intense, short-lived chemiluminescense and stimulates lipid peroxidation in these samples. We have investigated the source of this chemiluminescense and its possible relationship to lipid peroxidation.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents

Xanthine oxidase, catalase, lipoxidase, superoxide dismutase, NADH, NADPH, $NADP^+$, and β-carotene were purchased from Sigma Chemical Company, St. Louis, MO. Various fatty acids were purchased from Nu-Chek-Prep Inc., Elysian, MN. Radioactive $1\text{-}^{14}C$ fatty acids were obtained from New England Nuclear, Boston, MA. 2-Thiobarbituric acid and acetaldehyde were "white label" grade purchased from Eastman Chemicals, Rochester, NY. The indomethacin was a gift of Merck, Sharp and Dohm, Rahway, NJ. All inorganic chemicals were obtained from Fisher Chemical Company, Fair Lawn, NJ. 2,5 Dimethylfuran was purchased from Aldrich Chemical Company, Milwaukee, WI. 1,2-3,3-Tetraethoxypropane (malondialdehyde tetraethylacetal) was purchased from Fluka Chemical Company,

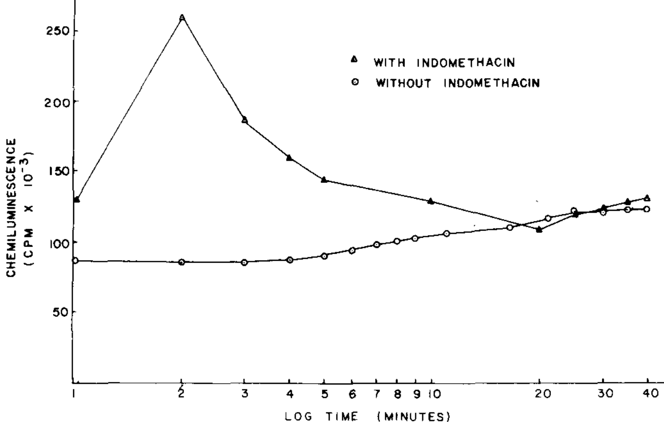

FIG. 1. Relationship between time and chemiluminescense for rat liver microsomes oxidizing NADPH in the presence or obsence of indomethacin. The reaction mixture contained 1.0 mM indomethacin, 200 μg NADPH, 1 mg microsomal protein and phosphate buffer 0.1 M, pH 7.0 to a total volume of 1.0 ml. Both curves are corrected for a blank reaction with NADPH omitted. ○—○, reaction in the absence of indomethacin; △—△, reaction in the presence of indomethacin.

Switzerland. Silica gel was purchased from Applied Science Labs, State College, PA.

Microsomal Preparation

Liver microsomes were prepared from 300-500 g male Holtzman rats by rapidly excising the liver following sacrifice of the animal by decapitation. The livers were washed in 1.15% KCl at 0-4 C and homogenized in 4 vol of the same solution. The microsomal fraction was isolated by differential centrifugation and the microsomal pellet obtained at 105,000 x g was resuspended in 1.15% KCl solution such that microsomes from 2.0 g of liver (wet weight) were suspended in 1.0 ml of solution. This suspension was used immediately, or, as noted in certain experiments, the resuspended microsomes were frozen over night at -10 C. The protein content of the suspensions was determined by the biuret method.

Chemiluminescense System

The chemiluminescense (CL) of all reactions was measured in a Beckman model LS-233 liquid scintillation counter with the coincidence circuit disconnected. Commercial polyethylene counting vials were used as reaction vessels. To measure the CL in the microsomal samples, 0.7 ml of 0.1 M phosphate buffer, pH 7.0, was placed in the vial, and 0.1 ml of indomethacin solution (0.84 mM) was added. The reaction was initiated by addition of NADPH solution (0.1 ml containing 0.25 micromoles). The vial was capped, mixed by agitating, and immediately placed in the counting chamber. All reactions were run at room temperature (25±2 C). Counts (CPM) were recorded at 6 sec intervals for varying time periods (generally 5 min). CPMs per 6 sec interval were plotted vs. time and the maximum (peak) CL during the reaction period determined.

The lipoxidase mediated CL reaction was run in 0.047 M borate-HCl buffer, pH 7.5. In these reactions, the substrate fatty acid (50 μl in 3.0 ml of borate buffer) was sonicated, diluted to 50 ml, and 2.0 ml was used for the reaction. Three μmol of indomethacin were added, and the reaction was initiated with 500 units of lipoxidase (total reaction volume was 3.0 ml).

The superoxide-mediated CL reaction was run in 0.1 M phosphate buffer, pH 7.4. To 0.7 ml of the phosphate buffer were added in order, 3.0 μmol indomethacin in 0.1 ml of buffer, 0.1 ml of 10 μM acetaldehyde in water, 20 λ xanthine oxidase suspension (0.25U). When superoxide dismutase was used, 0.2 mg was dissolved in 10 ml buffer to give an activity of 50 U/ml, and varying amounts of this solution were added.

Thiobarbituric Acid (TBA) Reagent

A stock solution of 2-thiobarbituric acid reagent was prepared by dissolving 4.0 gm TBA in 250 ml of 1.5% NaOH. Immediately before use, an aliquot of the stock solution was adjusted to pH 7.0 by adding 7% $HClO_4$. Two volumes of the pH 7.0 solution were mixed with one volume of 7% $HClO_4$ and 1.5 ml of this solution were added to 1.5 ml of a sample which consisted of the 1 ml chemiluminescense reaction mixture and 0.5 ml of 10% TCA used

TABLE I

Influence of Various Parameters on Maximum Chemiluminescense and Lipid Peroxidation as TBA Reactive Material

Sample	Relative maximum chemiluminescense	TBA reactive material (OD)
Complete[a]	100	0.597
- Indomethacin	16[b]	0.531
- NADPH	23[b]	0.113
- Microsomes	5[b]	0.066
- NADPH + NADP+	14[b]	0.107
+ Fe++ (1.0 mM)	117	0.728
+ ADP (1.0 mM)	125	0.645
+ Fe++ + ADP	140	0.722
+ Frozen microsomes	125[c,d]	0.740
In Tris buffer	9[b]	0.143
In Krebs buffer	36[b]	0.143
+ ADP (1.0 mM)	358[e] (124)	0.513
+ Fe++ (1.0 mM)	124[e] (50)	0.269
+ Fe++ + ADP	426[e] (154)	0.567
+ Indomethacin after 5 min	43[b]	---
+ Indomethacin after 15 min	15[b]	---

[a]Complete reaction contained 1.0 mM indomethacin, 200 μg NADPH, ca. 1 mg protein and phosphate buffer 0.1 M, pH 7.0, in a total volume of 1.0 ml.
[b]No peak in CL observed within 10 min.
[c]Time of maximum CL shorter than "complete."
[d]Frozen microsomes were assayed at pH 7.4.
[e]Value relative to "complete" reaction in Krebs bicarbonate buffer, value in parenthesis is relative to "complete" in phosphate buffer.

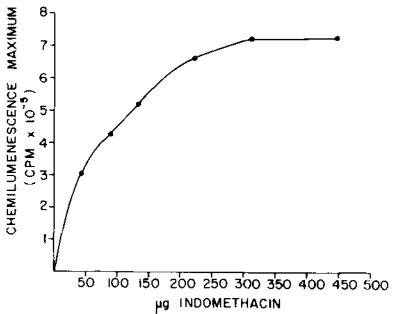

FIG. 2: The influence of indomethacin concentration on maximum chemiluminescense (CL) of rat liver microsomes oxidizing NADPH. The conditions are as described in Figure 1 with the absolute amount of indomethacin varying as indicated. In addition, the time necessary for the reaction to reach a maximum CL was decreased by increasing indomethacin concentration.

to terminate the chemiluminescense reaction. The sample-TBA mixture was heated in a covered boiling water bath for 15 min cooled in tap water, centrifuged at 500 x g for 5 min, and the OD at 535 nm was recorded. 1,1-3,3-Tetraethoxy propane was used to generate malondialdehyde as a standard.

Thin layer Chromatography (TLC) of Fatty Acids

Lipoxidase reaction mixtures were separated by TLC on activated Silica Gel G plates following extraction (X2) of the reaction mixture with 10 ml of redistilled diethyl ether. The ether was evaporated under nitrogen and the residue was redissolved in 1.0 ml of absolute ethanol. The redissolved sample (10 μl) was spotted and the plate developed in isopropanol-hexanes-acetic acid (10:130:10). The radioactive spots were located by scanning on a Berthold thin layer scanner. The spots were scraped from the plate and counted in a Beckman 3100 liquid scintillation counter.

Determination of Microsomal Arachidonate

Following various treatments, microsomal arachidonic acid content was determined by extracting the lipids by the method of Folch et al. (10) and converting the fatty acids to their methyl esters via the method of Metcalf et al. (11). C_{17} acid was used as an external standard. The samples were separated on a Hewlett-Packard Model 5730A gas chromatograph equipped with dual six foot EGGS-X (10%) on Gas Chrom Z columns operated isothermally at 195 C. The detector was flame ionization.

Inhibitor Additions

The addition of various inhibitors and radical scavengers was accomplished in phosphate buffer whenever possible. In the case of SKF-5285 and 2,5 dimethylfuran, methyl cellosolve was used to dissolve these compounds. All

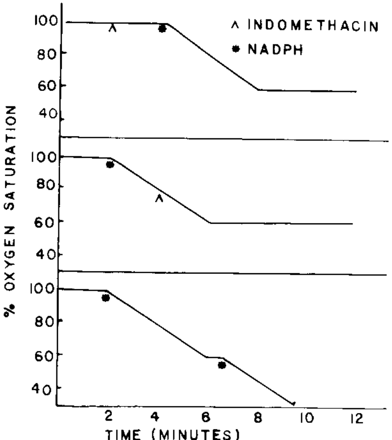

FIG. 3: The effect of indomethacin on oxygen uptake by rat liver microsomes. Upper panel – microsomes preincubated 2 min with indomethacin before NADPH added. Middle panel – indomethacin added 2 min after reaction initiated with NADPH. Lower panel – reaction reinitiated with NADPH after exhaustion of original substrate. Reaction run in air saturated phosphate buffer. Protein, indomethacin and NADPH concentrations as given Figure 1 and Table I. A Yellow Spring polarographic 0_2 electrode equipped with Fisher strip chart recorder was used to obtain the readings.

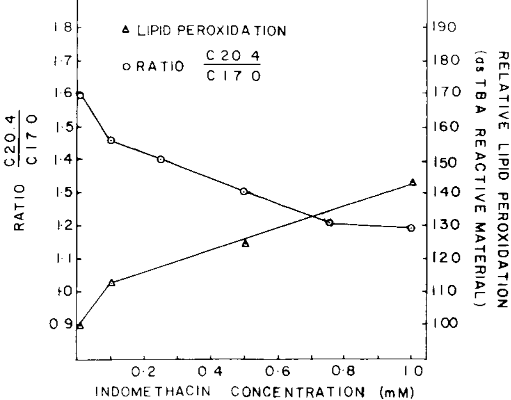

FIG. 4. Effect of indomethacin on the loss of microsomal membrane arachidonic acid and lipid peroxidation. Rat liver microsomes were incubated with NADPH and varying amounts of indomethacin as indicated. After 5 min, the amount of arachidonate relative to a C_{17} fatty acid external standard as well as the amount of lipid peroxidation in the same reaction vessel was determined. Values represent the average of duplicate determinations for three animals. ○—○ $\frac{20:4}{17:0}$ ratio; △—△ lipid peroxidation.

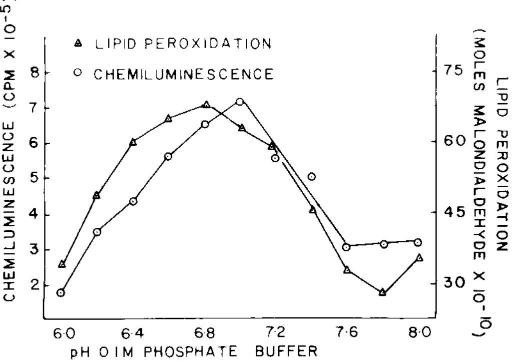

FIG. 5: Relationship of pH optimum for maximum chemiluminescense (CL) and lipid peroxidation. Reactions were run for 5 min in phosphate buffer as described in Figure 1. Maximum CL and the lipid peroixdation was assayed in the same vial. ○—○ lipid peroxidation; △—△ maximum chemiluminescense. Similar results were obtained for frozen microsomes except the maximum was shifted ca. 0.4 pH units higher.

reactions in which methyl cellosolve was used were blank corrected for the presence of this solvent.

RESULTS AND DISCUSSION

In the presence of NADPH, rat liver microsomes generate a slowly developing chemiluminescense (CL) which reaches a maximum value in 2-5 hr. Addition of certain oxidizable and nonoxidizable substrates leads to enhanced CL. The mechanism of the CL and the associated process had been investigated and appears to be rather complex, possibly involving radical initiated chain reactions. Indomethacin also elicits such an effect as illustrated in Figure 1. In the presence of 1.0 mM indomethacin, a rapidly developed CL appeared with a maximum being reached within 120 sec. Following maximal development, the CL curve decayed back to the curve generated in the absence of indomethacin and the two became indistinguishable.

The intensity and rate of development of the CL curve in the presence of indomethacin was directly related to its concentration and showed saturation kinetics with respect to maximal CL intensity (Fig. 2). The apparent Km for indomethacin was 2.0 x 10^{-4} mM. Time of maximal intensity was inversely related to indomethacin concentration with the apparent minimum time necessary to reach maximum CL intensity being shorter than could be measured by this technique. An even more rapid and intense CL in the presence of indomethacin was generated with microsomes frozen and thawed (see Table I).

These data suggested that the event or process that causes indomethacin to stimulate

TABLE II

Effect of Various Additions on Maximum Chemiluminescense and Lipid Peroxidation as TBA Reactive Material

Sample	Relative maximum chemiluminescense	TBA reactive material (OD)
Complete[a]	100	0.597
+ H_2O_2	116[b]	0.877
+ Catalase	93	0.609
+ Ascorbate	79	0.478
+ Heme	132[c]	0.639
+ Aspirin minus indomethacin	22[d]	0.585
+ PCMB (0.1 mM)	62	---
+ PCMB (0.5 mM)	8[d]	---
+ N_3- (0.1 mM)	80	---
+ N_3- (1.0 mM)	65	---
+ GSH + epinephrine	20[d]	---
+ 70 Units SOD	95	---
+ Ethanol (0.07 M)	98	---

[a]Complete reaction contained 1.0 mM indomethacin, 200 μg NADPH, ca. 1 mg protein and phosphate buffer 0.1 M, pH 7.0, in a total volume of 1.0 ml.
[b]Time of maximum CL longer than "complete."
[c]Time of maximum CL shorter than "complete."
[d]No peak observed within 10 min.

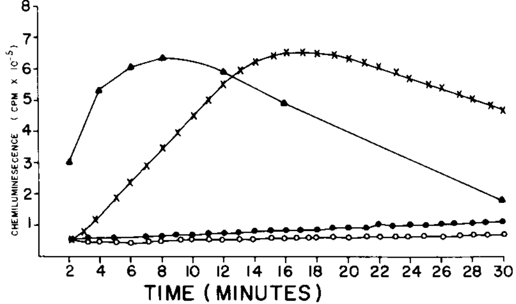

FIG. 6. Relationship between maximum chemiluminescense (CL) in the presence and absence of indomethacin in the lipoxygenase system and the superoxide generating (xanthine oxidase and acetaldehyde) system. Reaction conditions are given in the text. All curves are blank corrected. ○—○ lipoxygenase CL in the absence of indomethacin; X—X lipoxygenase CL in the presence of indomethacin; ●—● superoxide CL in the absence of indomethacin; ▲—▲ superoxide CL in the presence of indomethacin.

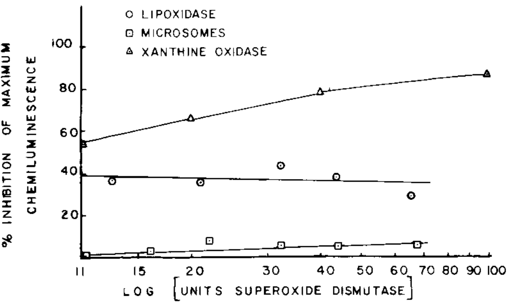

FIG. 7. Effect of superoxide dismutase on the chemiluminescense of rat liver microsomes (□—□), lipoxygenase reaction CL (○—○) and xanthine oxidase-acetaldehydr reaction CL (△—△). Reaction conditions are described in Figure 1. Values are maximum CL in the absence of superoxide dismutase, minus the CL in the presence of the given concentration of superoxide dismutase, divided by the CL in the absence of superoxide dismutase times 100.

CL in this system occurred very early in the reaction sequence and was apparently dependent on membrane organization. More probably, the access of the exogenous indomethacin to the reaction site may be related to the membrane integrity.

Because the indomethacin appeared to be interacting with an early intermediate in the reaction process to generate a CL, the influence of indomethacin on the various reaction parameters and particularly on substrate utilization and product formation was examined. It was theorized that the CL might serve to alter the normal reaction pathway; however, as opposed to its effect on prostaglandin synthetase, Figure 3 reveals that indomethacin had no effect on O_2 uptake initiated by the addition of NADPH to liver microsomes. Neither preincubation of the microsomes with indomethacin nor its addition during the period of oxidation showed any effect on the rate of O_2 uptake.

Liver microsomes oxidizing NADPH are known to peroxidize large amounts of membrane-bound polyunsaturated fatty acids (PUFA), particularly arachidonate (12,13). The determination of the amount of arachidonate remaining in the membrane after incubation for 5 min with NADPH and varying amounts of indomethacin is shown in Figure 4. These data

TABLE III

Effect of Radical Scavengers, Oxidants, Reductants and a P-450 Blocking Agent on Maximum Chemiluminescense and Lipid Peroxidation

Sample	Relative maximum chemiluminescense	TBA reactive material (OD)
Complete[a]	100	0.597
+ Mannitol (5 mM)	107[b]	0.579
+ KCN (5 mM)	67	0.490
+ DTT (5mM)	51[c]	0.364
+ SKF-5285 (5 mM)	8[d]	0.000
+ 2,5 Dimethylfuran (11 mM)	36[c]	0.573
+ β-Carotene	105	0.585

[a]Complete reaction contained 1.0 mM indomethacin, 200 μg NADPH, ca. 1 mg protein and phosphate buffer 0.1 M, pH 7.0, in a total volume of 1.0 ml.
[b]Time of maximum CL shorter than "complete."
[c]Time of maximum CL longer than "complete."
[d]No peak observed within 10 min.

gave the first indication of a relationship between the observed CL and lipid peroxidation. As is shown, addition of increasing amounts of indomethacin led to enhanced loss of arachidonate during the incubation and to increased amounts of thiobarbituric acid (TBA) reactive materials. It must be noted, however, that on a molecular basis, the amount of TBA reactive material (as malondialdehyde) represents only a small portion of the arachidonate lost. Any change in the composition of peroxidation products arising from the addition of indomethacin may not be reflected by the TBA reaction because of its rather indiscriminate reaction with peroxidized materials (14).

The simultaneous determination of pH optima values for CL and the amount of lipid peroxidation using freshly prepared microsomes is shown in Figure 5. The close relationship of the pH optima of the two processes is apparent.

Having determined that the stimulation of lipid peroxidation by indomethacin was linked to a microsomal CL generating system, a study of several parameters influencing both CL and lipid peroxidation was carried out as described in Table I. Deletion of any of the components of the reaction mixture eliminated the early CL associated with the reaction, and peroxidation was also reduced. NADPH could be replaced by neither $NADP^+$ nor NADH. The stimulatory effects of ferrous iron and ADP on peroxidation are well known (15), and their stimulation of the enhanced CL appeared to be additive. The relatively small response of the system employed in the present study to added iron probably reflected the fact that the phosphate buffer contained significant ferrous iron (16). In Tris-HCl or Krebs-bicarbonate buffer prepared from iron free salts, both CL and peroxidation were drastically reduced (Table I). In these buffers, the addition of ferrous iron and ADP further enhanced the CL, and their effects were additive.

As has been reported, freezing and thawing of the microsomes stimulated the lipid peroxidation and CL as well. The addition of indomethacin at time periods after the addition of NADPH showed little enhanced CL, further suggesting that the reactive species occur early in the reaction sequence relative to the CL that develops in the absence of indomethacin. Overall, the correlation between effects on indomethacin stimulated CL and peroxidation gave a calculated r value of 0.98.

In order to investigate the possible mechanisms of the CL and associated lipid peroxidation, several oxidants, reductants, and sulfhydryl reagents were added to the reaction mixture as described in Table II. Again, the striking factor was the extremely high correlation between the effect on indomethacin-mediated CL and lipid peroxidation. The possible role of catalase and H_2O_2 on the CL and peroxidation are consistent with the inhibition observed with N_3^-. These effects were more pronounced in frozen microsomes (Pennington, unpublished observation).

The effect of p-chloromercuribenzoate (PCMB) on peroxidation has been reported, and a similar effect on CL was observed in the present studies. Also, the influence of several reducing agents on the CL generation was the same as had been reported for their effect on peroxidation (17).

The stimulation of CL by the addition of indomethacin was also investigated in two other systems known to generate CL. One of these, lipoxidase acting on PUFA, has been shown to emit ultra weak light (Pennington, unpublished observation). Upon the addition of indomethacin to this reaction, an enhanced CL was observed (Fig. 6). The maximal CL peak

generated by lipoxidase in the presence of indomethacin was much earlier than the CL generated in the absence of indomethacin but not as rapid as in the liver microsomal system. Studies with radiolabeled substrate ([1-^{14}C]-linolenic and arachidonic acids) indicate no qualitative change in the reaction products in the presence of indomethacin. Indomethacin was also observed to accelerate and enhance the CL (Fig. 6) when added to a superoxide generating system described by Fridovich and Kellogg (18). They had shown that xanthine oxidase utilizing acetaldehyde as a substrate generated superoxide and a CL which may be inhibited by superoxide dismutase (SOD). Because superoxide has been proposed as a possible reactant in the peroxidation reactions of liver microsomes (19), the effect of superoxide dismutase on the various CLs generated in the presence of indomethacin was determined. As shown in Figure 7, SOD markedly suppressed the CL intensity generated by xanthine oxidase in the presence of indomethacin. Effects of addition of SOD to the lipoxidase CL system, on the other hand, were equivocal. For example, addition of as little as 15 log units of SOD to the reaction mixture caused a 40% to 50% reduction in the CL maximum, but futher additions were essentially without effect. In the microsomal system, SOD was without effect on the CL generated in the presence of indomethacin. This result indicated a lack of contribution of superoxide to the enhanced microsomal CL process.

In the microsomal system, the early appearance of the intense CL maximum indicated that the metabolism of indomethacin itself did not contribute to the CL generation. In several animals, including man, indomethacin is metabolized by the microsomal mixed function oxidase system, and a large portion of the drug and several products are excreted as conjugates (20). Within the time period of the CL maxima, no detectable amount of indomethacin was metabolized in our in vitro system, indicating that an intermediate of indomethacin metabolism was not responsible for the CL generated.

Several classical radical scavengers and inhibitors were also examined as to their effect on the CL generated in the presence of indomethacin. As shown in Tables II and III, mannitol and high levels of ethanol were without effect on either CL or lipid peroxidation, indicating a lack of contribution by hydroxyl free radicals to either process. The addition of 2,5 dimethylfuran to the reaction mixture in the presence of indomethacin made it possible to differentiate effects on CL and lipid peroxidation, with the CL being markedly depressed by dimethylfuran and the peroxidation only slightly inhibited. Attempts to determine the influence of β-carotene on these two processes proved difficult, owing to the extremely poor solubility of β-carotene even with the addition of miscible solvents. At the low concentration finally achieved, β-carotene was without effect in either process.

The role of microsomal cytochrome P-450 in both the CL and lipid peroxidation is shown by the effect of the potent inhibitor SKF-5285. A low concentration of this compound totally eliminated the CL associated with indomethacin addition (Table III) and reduced peroxidation levels essentially to blank values.

Initial attempts to determine microsomal NADPH oxidase spectrophotometrically and fluorometrically were unsuccessful in the presence of indomethacin because of an apparent inhibition of the reaction. Systematic studies, however, revealed that indomethacin absorbed so intensely in the 340 nm range as to make it impossible to measure NADPH oxidation spectrophotometrically at the concentration of indomethacin used in these experiments. Moreover, these experiments revealed that indomethacin was an extremely potent quencher of NADPH fluorescence, making it impossible to use fluorometry to follow the oxidative reaction. Using lower concentrations, but maintaining the same overall reactant ratios, revealed that NADPH oxidase was enhanced (80-110% relative increase) in the presence of indomethacin. This result was true whether the reaction was measured at 340 nm in the absence of cytochrome *c* or at 550 nm in the presence of cytochrome *c*.

From the foregoing results, we conclude that the addition of indomethacin to several systems capable of generating CL causes enhanced, short-lived CL maxima to develop. The mechanism of this enhanced CL appears to vary from system to system. The unique property of indomethacin is its ability to couple and to convert into light the energy obtained.

In the liver microsomal system, indomethacin also stimulates peroxidation of endogenous membrane arachidonate as well as stimulating NADPH oxidase. A high correlation exists between those factors that affect the enhanced CL in the presence of indomethacin and those which affect lipid peroxidation, thus indicating a coupling between the two processes.

Based on the studies cited, indomethacin appears to interact early in the sequence to generate a CL, compared to the CL developed by microsomes in the absence of indomethacin. The CL reaction requires NADPH and cyto-

chrome P-450, but does not appear to involve either hydroxyl free radical or superoxide. The possible role of P-450 as a peroxidase, as previously reported (21,22), may suggest a mechanism by which the binding of P-450 blocks the CL; however, P-450 may also be involved in the peroxidation and CL reactions via activation of oxygen. No data are available to distinguish these two possibilities.

It is not yet possible to conclude that singlet oxygen contributes to the CL formed in the presence of indomethacin. While a singlet oxygen scavenger such as 2,5 dimethylfuran did influence CL and lipid peroxidation differentially, its poor solubility and its tendency to influence membrane stability make interpretation of the results difficult. Attempts to use β-carotene as a scavenger showed no effect on the enhanced CL or lipid peroxidation at the low concentration achieved.

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The Effect of Arylsulfonate Esters on Cholesterol—Aggravated Atherosclerosis in White Carneau Pigeons

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ABSTRACT

The arylsulfonate esters of linoleyl, stearyl, and decyl alcohols were found to reduce significantly the accumulation of cholesterol in the plasma and livers of White Carneau pigeons subjected to a diet of Purina pigeon pellets coated with 0.25% cholesterol and 10% lard when fed for periods ranging from 9-12 months; no effects were observed in normocholesterolemic pigeons. These compounds produced no toxic side effects and were found to significantly attenuate the development of aortic atherosclerosis. The effect on aortic atherosclerosis was most likely the result of the lowering of plasma cholesterol concentrations. Linoleyl p-toluenesulfonate appeared to be the most effective of the three arylsulfonates tested, both with respect to the reduction of plasma and liver cholesterol accumulation and attenuation of the atherosclerotic process.

INTRODUCTION

It has been shown by Quackenbush et al. (1) and MacNintch et al. (2) that arylsulfonate esters of various log chain fatty alcohols are potent cholesterol-lowering agents when administered orally or parenterally to the cholesterol-glycocholate-fed rat. Results indicate that these compounds lower body cholesterol levels by inhibiting cholesterol absorption from the gastrointestinal tract.

In the present study, three of these arylsulfonate esters, linoleyl p-toluenesulfonate, stearyl p-toluenesulfonate, and decyl p-toluenesulfonate, were studied for their long term effects on the development of cholesterol-aggravated aortic atherosclerosis.

The species chosen for these studies was the White Carneau pigeon. Clarkson et al. (3), Lofland (4), and Prichard et al. (5) have found the White Carneau pigeon to be useful for the study of both aortic and coronary artery atherosclerosis. The White Carneau pigeon spontaneously develops atherosclerotic lesions that resemble morphologically those observed in human beings. In addition, small amounts of exogenous cholesterol significantly aggravate the atherosclerotic process in this species.

MATERIALS AND METHODS

For these studies, random mixtures of both male and feamale atherosclerosis susceptible White Carneau pigeons were obtained from our stock colony when the birds were ca. 6 weeks of age. These birds were group housed (5-6 per group) in growing batteries indoors in the vivarium in light-controlled (7 a.m.-5 p.m.) rooms with an ambient temperature of 72 C ± 2 C. Blood samples were drawn without anaesthesia from the alar vein at 2 week intervals. At the termination of the experiments, the birds were subjected to complete autopsies. It has previously been demonstrated that there is no male-female difference in susceptibility to aortic atherosclerosis in pigeons, (6) and consequently any transient hypertriglyceridemia produced as a result of egg laying would not be expected to alter materially the conclusions of the study.

In the first study, birds were maintained for 1 month on a diet of Purina pigeon pellets coated with 10% lard and 1% cholesterol and then divided into three groups. Each group received a basal diet of Purina pigeon pellets coated with 10% lard and 0.25% cholesterol. Groups 2 and 3 received, in addition, 0.25% linoleyl p-toluenesulfonate and 0.25% stearyl p-toluenesulfonate, respectively, coated on the pellets. These diets were fed continuously for 9 months.

In the second study, birds were divided into four groups. Groups 1 and 2 received Purina pigeon pellets coated with 10% lard and no cholesterol. Groups 3 and 4 received pellets coated with 10% lard and 0.25% cholesterol. Groups 2 and 4 received, in addition, 0.25% decyl p-toluenesulfonate coated on the pellets. These diets were fed continuously for 1 yr.

Serum and liver cholesterol content was determined in each case using the autoanalyzer (7) Pigeon liver cholesterol content was determined on each homogenate of whole liver after digestion for 1 hr in hot 15% alcoholic potassium hydroxide (10 mg alcholic KOH per gram of tissue), extracted three times with hexane, filtered, and made to volume (suitable aliquots

[1]Deceased.

were evaporated to dryness and taken up in isopropanol for total cholesterol determination). The entire aorta was removed from the base of the heart to the bifurcation of the ischiadic arteries (comparable anatomically to the iliac bifurcation in primates). The aorta was divided into a thoracic and abdominal segment immediately inferior to the bifurcation of the celiac artery. Thus, the area of the aorta most susceptible to atherosclerosis (immediately proximal to the celiac bifurcation) was included in the thoracic segment. The extent of grossly visible atherosclerosis was evaluated as an atherosclerosis index (percent of the aortic surface involved) based on visual inspection of the unstained aortas. The aortic segments were then split longitudinally into halves by dividing the aorta so that approximately one-half of the grossly visible atherosclerosis was contained in each half. One-half of the aorta was analyzed for cholesterol content (8) while the other half was stained with Sudan IV. The results from the Sudan IV stained arteries were not used for the purposes of this study. The sulfonate esters were prepared as outlined previously (9).

RESULTS

Study I

Treatment of White Carneau pigeons with a diet containing 0.25% cholesterol and 10% lard for a 9 month period resulted in a mean plasma cholesterol concentration of 1109 ± 103 mg/dl throughout the study with a mean of 1276 ± 133 mg/dl for the final 2 weeks of the study and a mean of 876 ± 87 mg/dl for the first 11 weeks (Table I). Treatment with 0.25% dietary linoleyl p-toluenesulfonate resulted in a striking reduction of plasma cholesterol accumulation at all time periods examined. Linoleyl p-toluenesulfonate-treated birds exhibited mean plasma cholesterol concentrations that were 51.1% ($p < .001$) lower than nontreatment control mean plasma cholesterol levels throughout the duration of the study. A 35.7% ($p < .001$) lower mean was observed with linoleyl p-toluenesulfonate treatment during the initial 11 weeks and a 58.6% ($p < .001$) lower mean was observed during the final 2 weeks of the study.

Linoleyl p-toluenesulfonate also prevented the accumulation of hepatic cholesterol to a marked degree resulting in a mean level which was 66.3% ($p < .01$) lower than that of the nontreatment control group.

Stearyl p-toluenesulfonate, incorporated as 0.25% of the hypercholesterolemic diet, was also effective in preventing the accumulation of plasma cholesterol although less effective than

TABLE I

Serum Cholesterol Concentrations of White Carneau Pigeons with and without Addition of 0.25% Linoleyl or Stearyl Sulfonate Ester in the Diet after 9 Months on Experiment

Treatment	Serum cholesterol (mg/dl)			Liver cholesterol mg/g of wet tissue	Body weight change (g)
	Mean level for initial 11 weeks	Mean level throughout study	Mean level for final 2 weeks		
No treatment control	876 ± 87	1109 ± 103[a]	1276 ± 133	19.6 ± 4.0	-22
Stearyl p-toluene sulfonate	698 ± 70 (-20.3%)	716 ± 66 (-35.4%, $p < .01$)[b]	729 ± 208 (-42.8%, $p < .01$)	12.8 ± 7.0 (-34.7%)	-8
Linoleyl p-toluene sulfonate	563 ± 37 (-35.7%, $p < .001$)	542 ± 32 (-51.1%, $p < .001$)	527 ± 36 (-58.6% $p < .001$)	6.6 ± 0.5 (-66.3%, $p < .01$)	-8

[a]Mean ± standard error of the mean, 10 birds per group except for the linoleyl p-toluenesulfonate group which contained 9 birds.
[b]Figures in parentheses indicate percent change from control mean and level of statistical significance.

TABLE II

Parameters of Aortic Atherosclerosis in White Carneau Pigeons with and without Addition of 0.25% Linoleyl or Stearyl Sulfonate Ester in the Diet after 9 Months on Experiment

Treatment	Number of birds per group	Thoracic aorta		Abdominal aorta	
		A.I.[a]	Cholesterol (mg/g wet tissue)	A.I.	Cholesterol (mg/g wet tissue)
No treatment control	10	34 ± 7[b]	34.9 ± 6	8 ± 4	22.7 ± 5.4
Stearyl p-toluene-sulfonate	10	14 ± 4	10.1 ± 2 (-71.1%, $p < .001$)[c]	6 ± 7	9.8 ± 1.5 (-57.0%, $p < .05$)
Linoleyl p-toluene-sulfonate	9	19 ± 8	11.8 ± 3 (-66.2%, $p < .01$)	11 ± 7	7.8 ± 1.5 (-65.6%, $p < .05$)

[a]A.I. = Atherosclerosis Index (% of surface involved in plaques).
[b]Mean ± standard error of the mean.
[c]Figures in parentheses represent percent change from no treatment control group and level of statistical significance.

TABLE III

Plasma and Liver Cholesterol Concentrations of White Carneau Pigeons with and without Addition of 0.25% Decyl p-Toluenesulfonate in the Diet after 12 Months on Experiment

Treatment	Number of birds per group	Serum cholesterol (mg/dl)			Liver cholesterol (mg/g wet tissue)	Weight change (g)
		Mean level for initial 9 weeks	Mean level through-out study	Mean level for final 43 weeks		
			0.25% Dietary cholesterol			
No treatment control	16	868 + 73	1171 ± 110[a]	1267 ± 139	14.9 ± 2.2	+95
Decyl p-toluene-sulfonate	22	756 ± 52 (-12.4%)	804 ± 163 (-22.8%)	820 ± 57 (-34.4%, $p < .01$)[b]	9.3 ± 10 (-37.5%, $p < .001$)	+73
			No dietary cholesterol			
No treatment	17	- - -	- - -	304 ± 30	2.9 ± 0.18	+96
Decyl p-toluene-sulfonate	23	- - -	- - -	303 ± 30	3.5 ± 0.20 (+20.7%, $p < .05$	+75

[a]Mean ± standard error of the mean.
[b]Figures in parentheses represent percent difference from no treatment control and level of statistical significance.

TABLE IV

Parameters of Aortic Atherosclerosis in White Carneau Pigeons with and without Addition of 0.25% Decyl p-toluenesulfonate in the Diet after 12 Months on Experiment

Treatment	Number of birds per group	Thoracic aorta	
		A.I.[a]	Cholesterol (mg/g of wet tissue
		0.25% Dietary cholesterol	
No treatment control	16	37 ± 8[b]	40.0 ±12.0
Decyl p-toluene-sulfonate	22	17 ± 4 (-54%, p < .05)[c]	24.0 ± 4.0 (-40%, p < .01)
		No dietary cholesterol	
No treatment control	17	2 ± 0.7	3.7 ± 0.4
Decyl p-toluene-sulfonate	23	2 ± 0.5	3.0 ± 0.8

[a]A.I. = Atherosclerosis Index (percent of surface involved in plaques).
[b]Mean ± standard error of the mean.
[c]Figures in parentheses represent percent difference from control mean with level of statistical significance.

linoleyl p-toluenesulfonate in this regard. The mean plasma cholesterol level throughout the study of birds treated with stearyl p-toluenesulfonate was 35.4% ($p < .01$) lower than that of nontreated control birds with a greater difference in means (-42.8%, p .01) during the final 2 weeks than during the initial 11 weeks (-20%, N.S.). Hepatic cholesterol levels resulting from stearyl p-toluenesulfonate treatment averaged 34.7% lower than nontreatment control levels, but this difference was not statistically significant at the 5% confidence level. No appreciable body weight losses were observed in any of the groups during the study.

Examination of the aortae of those birds revealed significantly less aortic atherosclerosis as indicated by the cholesterol content of aortic tissue (Table II). Linoleyl p-toluenesulfonate resulted in 66.2% ($p < .01$) less cholesterol in the thoracic aorta and 65.6% ($p < .05$) less cholesterol in the abdominal aorta on a mg per gram of wet tissue basis than nontreated controls. Stearyl p-toluenesulfonate resulted in 71.1% ($p < .001$) less cholesterol in the thoracic aorta and 57.0% ($p < .05$) less cholesterol in the abdominal aorta than nontreated controls. A comparison of the less objective mean atherosclerosis index in each group, defined as the percent of aortic surface involved in atherosclerotic plaque formation, parelleled the tissue cholesterol concentration in the thoracic but not in the abdominal aorta. Consequently, both arylsulfonate esters produced significant attenuation of the atherosclerotic process in cholesterol-fed White Carneau pigeons, presumably by limiting the amount of cholesterol in the blood-liver pool.

An examination of the organ weights of the birds in this study revealed no significant differences in kidney, heart, liver, or thyroid weight in reference to total body weight, and no other evidence of toxicity was observed.

Study II

Treatment of cholesterol-fed White Carneau pigeons for a 1 yr period with 0.8% dietary decyl p-toluenesulfonate resulted in significantly lowered plasma cholesterol levels (34.4%, $p < .01$) during the final 43 weeks of the study (Table III).

Hepatic cholesterol levels were 37.5% ($p < .001$) lower in decyl p-toluenesufonate-treated birds than in nontreated control birds, and body weight gain was slightly less compared with controls. In general, decyl p-toluenesulfonate was less effective than either linoleyl p-toluenesulfonate or stearyl p-toluenesulfonate in preventing the accumulation of cholesterol in blood and liver.

Decyl p-toluenesulfonate was ineffective in lowering plasma cholesterol levels in birds receiving no exogenous cholesterol in the diet. This response is consistent with the cholesterol absorption inhibition properties of the arylsulfonates (2) and is in agreement with results using normocholesterolemic rats. Hepatic cholesterol levels were slightly but significantly elevated in these birds.

Decyl p-toluenesulfonate treatment resulted in significantly less thoracic aorta atherosclerosis in cholesterol-fed birds, in comparison with controls as manifested by a 40% ($p < .01$) lower thoracic aorta cholesterol content and a 54% lower atherosclerosis index (Table IV). No differences in thoracic aorta atherosclerosis parameters were observed as a result of decyl

p-toluenesulfonate treatment of birds receiving no exogenous cholesterol, as would be expected from the lack of atherosclerotic lesions in the control group. No adverse side effects were observed in decyl p-toluenesulfonate-treated birds.

DISCUSSION

These studies demonstrate that arylsulfonate esters of fatty alcohols when incorporated into the diet at a level of 0.25% effectively reduce the accumulation of both plasma and liver cholesterol in White Carneau pigeons receiving a hypercholesterolemic regimen of 0.25% cholesterol and 10% lard coated on Purina pigeon pellets. These results are comparable to those observed in rats subjected to diets containing 1% cholesterol and 0.5% sodium glycocholate and dietary levels of arylsulfonate esters of fatty alcohols of 0.15% (2). These compounds were found to be ineffective in lowering plasma and liver cholesterol concentrations in normocholesterolemic pigeons, an observation consistent with their lack of effect in normocholesterolemic rats (2) and consistent with inhibition of cholesterol absorption as a mode of action. Linoleyl p-toluenesulfonate was found to be the most effective of the three arylsulfonates tested.

These studies also demonstrate that arylsulfonate esters of fatty alcohols effectively attenuated the development of both thoracic and abdominal aorta atherosclerosis in White Carneau pigeons based on aortic cholesterol content. The percent of aortic surface involved in atherosclerotic plaques (the atherosclerosis index) followed the same pattern in the thoracic aorta but not in the abdominal aorta. Linoleyl and stearyl sulfonate esters were comparable in their attenuation of the atherosclerotic process and more effective than the decyl sulfonate ester.

Linoleyl p-toluenesulfonate exhibited a trend toward more effective prevention of cholesterol accumulation in plasma and liver than did stearyl p-toluenesulfonate, but the difference was not significant at the 5% confidence level. The comparable effects of the two compounds in attenuating the atherosclerotic process correlates with their ability to lower plasma cholesterol concentrations, although linoleyl p-toluenesulfonate was more effective in reducing liver cholesterol accumulation.

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Effect of Catecholamines and β-Blockers on Linoleic Acid Desaturation Activity

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ABSTRACT

The effect of catecholamines and adrenergic blocking agents on the oxidative desaturation of linoleic acid in rat liver microsomes was studied. Epinephrine (1 mg/kg/body weight) produced a significant decrease on the conversion of $[1\text{-}^{14}C]$linoleic acid to γ-linolenic acid. The effect of epinephrine was blocked by single injections of the β blockers propranolol (10 mg/kg body weight) or dichloroisoproterenol 30 min before the hormone treatment. Isoproterenol (100 μg/kg body weight) produced a significant decrease on the activity of the linoleyl-CoA desaturase. The effect of the catecholamines was postulated to be mediated through β receptors by an enhancement of the intracellular levels of cyclic AMP.

INTRODUCTION

Since the work of Ahlquist (1), the mechanism of action of catecholamines has been explained through the presence of α and β-receptors in the membrane of the cell at the so-called adrenergic effector site. The description by Powell and Slatter (2) of the β-receptor blocking properties of dichloroisoproterenol did much to confirm the adrenergic receptor classification proposed by Ahlquist. Since then, numerous compounds classified as β-receptor antagonists have been described. One of them that gained general approval was propranolol. There is general agreement to consider that glycogenolytic effects of the catecholamines are related to their ability to activate adenyl cyclase and thus increase tissue levels of cyclic AMP. β-Adrenergic blocking agents, such as propranolol, competitively inhibit the effect of catecholamines on the activation of adenyl cyclase.

Δ 6 Desaturase is a regulatory enzyme that begins the synthesis of polyunsaturated fatty acids of the essential series (3). In a previous work, we have demonstrated that epinephrine produced a decrease of Δ 6 desaturation activity in rat liver microsomes (4). This effect was postulated to be mediated through an enhancement of the intracellular cyclic AMP levels.

The purpose of the experiment reported in this paper was to study the effect of epinephrine and some pharmacological drugs that stimulate or antagonize the β-receptors on the desaturation of linoleic acid in rat liver microsomes.

[1]The authors are members of the Carrera del Investigador Científico of the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina.

MATERIALS AND METHODS

Chemicals

$[1\text{-}^{14}C]$linoleic acid (58 mCi/mmole, 99% radiochemical purity) was purchased from the Radiochemical Centre, Amersham, England. NADH, ATP, CoA, and other cofactors were provided by Boehringer Argentina. The following drugs were used: L-epinephrine (Biol. Argentina), isoproterenol sulfate (Boehringer, Germany), dichloroisoproterenol hydrochloride (Aldrich Chem. Co. Inc., Milwaukee, WI) and propranolol HCl (Beta Lab. Argentina).

Animals and Treatment of Animals

Adult female Wistar rats, weighing 180-220 g and maintained on standard Purina chow, were used. All rats were fasted for 24 hr and then re-fed with Purina chow for 1 hr. Water was given ad libitum. Three hr later, the rats were separated into several groups. One group was injected with epinephrine at a dose of 1 mg/kg body weight. Another group was treated with isoproterenol (100 μg/kg body weight) and a third group was injected with the same dose of epinephrine and propranolol (10 mg/kg body weight). Propranolol was administered 30 min before epinephrine. A fourth group that received saline solution in place of the drugs was used as control. All compounds were injected intraperitoneally. The animals were killed 12 hr after the injection. In the second experiment, the rats received the same treatment as in experiment one, but an additional group was injected with dichloroisoproterenol (50 mg/kg body weight) and epinephrine. Dichloroisoproterenol was injected 30 min before the epinephrine. All the groups were killed 3 hr after the injections.

TABLE I

Effect of 12 hr Adminstration of Catecholamines and Propranolol on the Hepatic Microsomal Desaturation of Linoleic Acid, Glycemia[a] and Liver Glycogen[a]

Treatment	Conversion 18:2 → 18:3 (%)	Glycemia (mg %)	Liver glycogen (mg/mg prot.)
Control	[b]33.5 ± 1.8	97 ± 4	0.112 ± 0.007
Epinephrine	17.7 ± 2.4	74 ± 3	0.108 ± 0.005
Propranolol + epinephrine	11.5 ± 1.4	91 ± 5	0.114 ± 0.010
Isoproterenol	15.6 ± 3.3	105 ± 2	0.112 ± 0.008

[a]For experimental conditions, see materials and methods.
[b]Averages of the analysis of four rats ± one standard error of the mean (SEM).

Isolation of Microsomes

The rats were killed by decapitation without anesthesia. The blood was allowed to drain and was collected for glucose determination. Livers were rapidly excised and immediately placed in ice cold homogenizing medium (5). After homogenization, samples were taken to measure protein and glycogen content. Microsomes were separated by differential centrifugation at 100,000 x g as described previously (5).

Experimental Procedures

Desaturation of the fatty acids by liver microsomes was measured by estimation of the percentage conversion of [1-^{14}C]linoleic acid to γ-linolenic acid. Three nmoles of the labeled acid and 97 nmoles of unlabeled acid were incubated with 5 mg of microsomal protein in a Dubnoff shaker at 35 C for 20 min in a total volume of 1.5 ml of 0.15 M KCl-0.25 M sucrose solution. The medium contained 4 μmoles of ATP, 0.1 μmole of CoA, 1.25 μmoles of NADH, 5 μmoles of $MgCl_2$, 2.25 μmoles of glutathione, 62.5 μmoles of NaF, 0.5 μmoles of nicotinamide, and 62.5 μmoles of phosphate buffer (pH 7). The reaction was stopped by addition of 2 ml of 10% KOH in methanol. The fatty acids were recovered by saponification of the incubation mixture (45 min at 85 C), acidification and extraction with petroleum ether (bp 30-40 C). The fatty acids were esterified with methanolic 3 M HCl (3 hr at 68 C), and the distribution of the radioactivity between substrate and product was measured by gas liquid radiochromatography in an apparatus equipped with a Packard Proportional counter. Percentage conversion was calculated from the distribution of radioactivity between substrate and product measured directly on the radiochromatogram (6).

Protein content in the homogenate and in the microsomal fraction was determined by the biuret method of Gornall et al. (7) using cystalline bovine serum albumin as a standard. Blood glucose was measured by the o-toluidine method (8) and liver glycogen by the method of Van Handel (9).

RESULTS AND DISCUSSION

The effect of the administration of epinephrine and the blocking agent propranolol on linoleic acid desaturation activity of rat liver microsomes in shown in Table I. Epinephrine markedly depressed Δ 6 desaturation of linoleic acid 12 hr after the injection. Neither glycemia nor liver glycogen showed significant differences in the epinephrine group compared to the controls. These results are consistent with experiments published earlier (4). They showed that epinephrine evokes an ordered sequence of events in liver. It begins with cAMP increase, followed by a glycogen breakdown that leads to a blood glucose increase, and then a decrease of Δ 6 fatty acid desaturation occurs. This decrease of the microsomal Δ 6 desaturase activity is maintained for a long time and is still shown 12 hr after epinephrine injection when the glycogenolytic effect has already faded.

Table I also shows that pretreatment of the rats with propranolol did not inhibit the epinephrine effect in Δ 6 desaturation activity in contrast to what was expected. However, this result can be attributed to the fact that the β-blockade weakens with time. In this connection, it has been published that propranolol is extensively metabolized during the first passage through the liver (10). On the other hand, Evans et al. found that, in the rat, the mean half-life of propranolol was about 40 min (11). Epinephrine has a prolonged duration of action (4) while propranolol's effect is relatively short (12). Therefore, the results shown in Table I may be explained by fast fading of propranolol blocking effect that happens before epinephrine declines. Consequently, Δ 6 desaturase was inhibited by the remaining effect of epinephrine.

TABLE II

Effect of 3 hr Adminstration of Catecholamines and β-Blockers on the Hepatic Microsomal Desaturation of Linoleic Acid, Glycemia, and Liver Glycogen[a]

Treatment	Conversion 18:2 → 18:3 (%)	Glycemia (mg %)	Liver glycogen (mg/mg prot.)
Control	34.2[b] ± 2.7	90 ± 4	0.103 ± 0.006
Epinephrine	19.4 ± 0.8	324 ± 6	0.038 ± 0.004
Propranolol + epinephrine	30.3 ± 1.1	165 ± 8	0.091 ± 0.010
Propranolol	27.3 ± 0.8	87 ± 6	0.077 ± 0.012
Dichloroisoproterenol + epinephrine	28.8 ± 1.5	170 ± 5	0.087 ± 0.006
Isoproterenol	22.0 ± 1.9	83 ± 1	0.082 ± 0.006

[a]For experimental conditions, see materials and methods.
[b]Averages of the analysis of four rats ± one standard error of the mean (SEM).

Table I also shows the effect of an adrenergic agonist, isoproterenol, in rat liver microsomal desaturation of linoleic acid. Isoproterenol produced a 50% decrease in Δ 6 desaturation activity, an effect similar to that obtained with epinephrine. Isoproterenol produced no significant variations in the glycemia and in liver glycogen levels.

Table II shows the results obtained 3 hr after the injection of the adrenergic agonists or antagonists in microsomal Δ 6 desaturation activity, glycemia and liver glycogen. Epinephrine produces a significant decrease of the microsomal conversion of linoleic acid to γ-linolenic acid in liver. This effect is completely blocked if 30 min before the injection of epinephrine the rats received either propranolol or dichloroisoproterenol. Propranolol alone did not modify significantly the results obtained in the control group. Besides, this experiment shows that propranolol and dichloroisoproterenol are also able to inhibit epinephrine induced hyperglycemia and glycogenolysis as was previously reported (13-14).

Therefore, we conclude that the β-adrenergic blocking agents, propranolol and dichloroisoproterenol, are also antagonists of epinephrine effect in the activity of linoleyl CoA desaturase in the conditions of this experiment. The effect obtained with epinephrine can be mimicked with isoproterenol which also produces a significant decrease in Δ 6 desaturation activity (Table II).

It has been suggested that epinephrine initiated its biological effects in several tissues by increasing the intracellular concentration of cyclic AMP (15-16). β-Adrenergic blocking agents competitively inhibit the ability of catecholamines to activate adenyl cyclase activity (17) and the formation of cyclic AMP (18). From the results obtained in the literature, it is evident that cyclic AMP mediates the activity of the β-receptor. Epinephrine produces a decrease in the linoleyl CoA desaturase activity, an effect that is antagonistic with propranolol or dichloroisoproterenol. Dibutyryl cyclic AMP also decreases fatty acid Δ 6 desaturation (19). Thus, it is reasonable to assume that the depressing effect in Δ 6 desaturase activity produced by epinephrine is mediated by a β-receptor through an increase of the intracellular levels of cyclic AMP. It is also substantiated by the observation that isoproterenol, a β-adrenergic agonist, produces both an increase in the intracellular cyclic AMP concentration (20-21) and a significant decrease in Δ 6 desaturation activity. However, isoproterenol produces no significant differences in the glycemia in spite of the slight decrease in liver glycogen levels (Table II). This apparent inconsistency would be particularly important since it has been shown by Newton and Hornbrook (22) that a high dose of isoproterenol does not stimulate glycogen phosphorylase in rat liver. In contrast, it increases the intracellular levels of cyclic AMP (20-21) and the activity of rat liver adenyl cyclase in vitro (22).

However, isoproterenol produces an elevation of the nonesterified fatty acids in blood through an activation of adipose tissue lipase (23-24). Therefore, it is possible to speculate that the in vivo effect of isoproterenol and even at least partially of epinephrine in the activity of rat liver Δ 6 desaturase is evoked through an activation of adipose tissue adenyl cyclase followed by an increased lipase activity. The free fatty acids released to the blood and transported to the liver would inhibit the enzyme.

Therefore, all our experiments show that cAMP is involved in the control of fatty acid Δ 6 desaturase activity but we have not determined yet the mechanism of action.

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Differences in the Phospholipid, Cholesterol, and Fatty Acyl Composition of 3T3 and SV3T3 Plasma Membranes

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ABSTRACT

An analysis of the phospholipid, cholesterol, and phospholipid fatty acyl composition of isolated plasma membranes of 3T3 and SV3T3 mouse embryo cells has been performed. The results show that the plasma membrane of SV3T3 cells contain relatively less phosphatidylethanolamine and sphingomyelin and more cholesterol than 3T3 plasma membranes. The fatty acyl composition of individual phospholipid classes as determined by gas liquid chromatography also showed differences between 3T3 and SV3T3 plasma membranes. The plasma membranes of SV40 transformed 3T3 cells contain: (a) a higher percentage of 18:1 and less 20:3 and 20:4 in phosphatidylethanolamine; (b) a higher percentage of 18:1 in phosphatidylserine; and (c) a higher percentage of 18:2 and 20:4 in phosphatidylinositol.

INTRODUCTION

The plasma membranes of transformed cells have been shown to differ from the plasma membranes of normal cells (1,2) in glycoprotein and glycolipid composition, (2-4), in ultrastructural organization (5,6), and in function (7,8). Many of those studies have employed the 3T3 and SV-40 transformed 3T3 mourse embryo fibroblast cell system (3-8). This cell system has been employed, in particular, to study the role of plasma membrane lipid organization in the control of receptor mobility and membrane function (9,10). Despite data which suggest that transformed SV3T3 cells show differences in the physical state of plasma membrane lipids compared to normal 3T3 cells (11,12), no data characterizing the lipid composition of purified 3T3 and SV3T3 plasma membranes have been published.

We have, therefore, isolated purified plasma membranes from 3T3 and SV3T3 cells grown in culture and have characterized plasma membrane lipid composition. In particular, we have quantitated cholesterol and phospholipid composition, determined the fatty acyl profiles of individual phospholipid classes in 3T3 and SV3T3 plasma membranes, and found significant differences.

EXPERIMENTAL

BALB/c 3T3 (clone A31) and SV3T3 mouse embryo cells (obtained from Dr. George Todaro, National Institutes of Health, Bethesda, MD) were grown in antibiotic-free Dulbecco's modified essential medium containing 10% calf serum. Both 3T3 and SV3T3 cell lines were shown to be free of mycoplasma contamination by repeated cultural analysis (Flow Laboratories, Rockville, MO.) and by transmission and scanning electron microscopy.

Rapidly growing (log phase) cells at approximately 75% confluence (in roller bottles with a 490 cm^2 surface area) were used for plasma membrane isolation. The tissue culture medium was decanted, and the cells were washed three times in 30 ml of isotonic phosphate-buffered saline (pH 7.4) containing 0.75 mM calcium and 0.5 mM magnesium (CMPBS). Cells were then induced to shed plasma membrane vesicles by exposure of cell monolayers to 35 ml of the vesiculant solution, 25 mM formaldehyde, and 2 mM dithiothreitol in CMPBS as recently described (13). After incubation for 2 hr at 37 C, the vesiculant solution containing free floating plasma membrane vesicles was decanted, and the plasma membranes were sedimented by centrifugation at 30,000 x g for 30 min at 4 C. The resulting translucent pellet was washed three times in 20 ml of CMPBS. After each washing, the membranes were gently resuspended leaving any whole cell contamination as a dense white aggregate at the bottom of the tube. The final translucent plasma membrane pellet was then suspended in a known quantity of water.

Aliquots of the plasma membrane suspension were taken for protein determination by the Lowry procedure (14) or fluorescamine method (15), using bovine serum albumin as a standard. All protein determinations used sodium dodecyl sulfate solubilization (16). The remainder of the suspension was used for extraction of lipids.

Extraction of lipids was done with chloroform-methanol (2:1, v/v) from lyophilized aliquots of the aqueous plasma membrane sus-

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TABLE I

Major Lipid Classes in Plasma Membranes

	3T3	SV3T3
Phospholipid (μmol/mg protein)	0.229 ± 0.017	0.243 ± 0.031
Phosphatidylethanolamine	0.039 ± 0.003[a]	0.033 ± 0.007[b]
Phosphatidylcholine	0.121 ± 0.016	0.143 ± 0.006
Phosphatidylserine	0.016 ± 0.003	0.016 ± 0.002
Phosphatidylinositiol	0.014 ± 0.007	0.024 ± 0.007
Sphingomyelin	0.037 ± 0.084	0.026 ± 0.002[c]
Cholesterol (free) (μmol/mg protein)	0.17 ± 0.01	0.20 ± 0.01[d]
Cholesterol/phospholipid molar ratio	0.76 ± 0.02	0.84 ± 0.07

[a]The results are expressed as μmol/mg protein of total recovered lipid Pi. Mean of data of four membrane preparations for each cell type ± SD.
[b]Significantly different from 3T3 data ($P < 0.025$).
[c]Significantly different from 3T3 data ($P < 0.005$).
[d]Significantly different from 3T3 data ($P < 0.05$).

pension (17). The chloroform and methanol were distilled in glass grade (Burdick and Jackson Laboratories, Inc., Muskegon, MI). The chloroform-methanol extracted material was assayed for total cholesterol (18) and total phosphorous (19). Phospholipids were separated on silica get 60 plates (E. Merck, Darmstadt, Germany) by two-dimensional thin layer chromatography using a solution of 130 ml chloroform, 70 ml methanol, and 10 ml 28% aqueous ammonia for the first dimensional elution, and a solution of 100 ml chloroform, 35 ml acetone, 25 ml methanol, 20 ml glacial acetic acid, and 10 ml distilled water for second dimensional elution. When phospholipid classes were to be quantitated, the lipids were visualized by placing the plate in I_2 vapor; however, when fatty acid methyl esters were to be prepared, the phospholipid classes were visualized under ultraviolet light after the plates were sprayed with 2′7′dichlorofluorescein in methanol (20). The fatty acid methyl esters were prepared, as previously described (17), by transmethylation of individual phospholipid classes using 4% sulfuric acid (fluorometric grade) in methanol, and were analyzed by gas liquid chromatography on a six foot by two mm id glass column packed with 10% SP2330 on 100/120 chromosorb WAW (Supelco, Inc., Bellefonte, PA). Fatty acids were identified by comparison of retention times with those of commercially available fatty acid methyl ester standard mixtures (Nu-Chek-Prep, Elysian, MN) by using a Hewlett Packard Digital Integrator Model 3380A interfaced to the electrometer of a Beckman gas chromatograph Model GC-65. Each fatty acyl analysis was performed on plasma membrane lipid preparations isolated from at least three different groups of 3T3 and SV3T3 cells.

These studies were performed on extracts of intact plasma membrane vesicles because preliminary studies established that essentially all vesicle lipid was of plasma membrane origin. More specifically, a comparison was made between the lipid content of intact plasma membrane vesicles and that of purified plasma membrane fragments isolated from vesicles. Plasma membrane fragments were obtained by lysis of a 30,000 x g pellet of plasma membrane vesicles by nitrogen decompression at 250 psi for 5 min and resedimentation at 250,000 x g for 1 hr at 4 C. The 250,000 x g purified plasma membrane preparation contained greater than 97% of the vesicle lipid phosphorus and greater than 91% of the vesicle total cholesterol.

RESULTS

Plasma membranes of 3T3 mouse embryo cells and Simian virus 40 transformed mouse embryo cells have been isolated by the method of Scott (13). Such preparations have been shown to represent highly purified plasma membrane preparations. Both 3T3 and SV3T3 plasma membrane vesicles show a tenfold enrichment in 5′ nucleotidase activity compared to whole cell homogenates and contain no detectable NADH-Cytochrome C reductase activity, no detectable succinic dehydrogenase activity, and no DNA (Scott, In press, J. Cell. Sci.).

Plasma membrane vesicles isolated from 3T3 and SV3T3 share many other common properties. They have an identical phase and ultrastructural morphology, and they contain the identical density of intramembranous particles demonstrated by the freeze fracture technique. They are both agglutinated by Concanavalin A and wheat germ agglutinin, and have an essentially identical polypeptide composition determined by polyacrylamide gel electro-

TABLE II

Fatty Acyl Profiles of Phospholipids from Plasma Membranes

Fatty acyl group	Phosphatidylethanolamine		Phosphatidylcholine		Phosphatidylserine		Phosphatidylinositol		Sphingomyelin	
	3T3	SV3T3	3T3	SV3T3	3T3	SV3T3	3T3	SV3T3	3T3	SV3T3
14:0	---	---	3.4 ± 2.8	2.6 ± 0.8	---	---	---	---	---	---
16:0	8.4 ± 1.7[a]	9.2 ± 0.8	33.7 ± 7.2	29.3 ± 4.0	10.4 ± 8.1	4.0 ± 1.9	5.5 ± 1.9	5.9 ± 0.9	59.8 ± 0.6	67.2 ± 17.5
16:1	---	0.8 ± .1	7.3 ± 3.2	9.0 ± 1.3	1.2 ± 0.5	1.1 ± 0.2	---	---	1.7 ± 0.6	0.8 ± 0.7
18:0	13.6 ± 3.0	14.7 ± 3.1	11.6 ± 1.3	11.8 ± 2.2	40.1 ± 3.7	41.5 ± 3.9	51.0 ± 15.8	39.0 ± 2.4	9.5 ± 4.1	7.2 ± 2.2
18:1	10.1 ± 0.1	23.4 ± 0.7[b]	25.8 ± 5.7	30.0 ± 4.0	14.1 ± 6.9	28.0 ± 2.7[c]	20.8 ± 9.8	16.8 ± 5.6	0.6 ± 0.6	0.8 ± 0.1
18:2	4.2 ± 1.2	6.0 ± 0.9	9.2 ± 5.0	9.4 ± 2.1	16.3 ± 9.7	9.7 ± 4.5	1.4 ± 0.2	3.2 ± 0.2[d]	---	---
20:0	---	---	---	---	---	---	---	---	1.3 ± 0.5	1.1 ± 0.5
20:3	6.1 ± 0.4	2.8 ± 0.6[b]	2.1 ± 0.8	1.9 ± 0.5	6.7 ± 4.6	5.6 ± 1.6	4.8 ± 3.1	6.1 ± 0.6	---	---
20:4	35.5 ± 3.8	24.5 ± 6.3[e]	4.9 ± 2.6	3.8 ± 0.6	5.5 ± 2.9	5.3 ± 3.7	14.2 ± 8.7	32.0 ± 3.3[e]	---	---
20:5	4.8 ± 2.0	3.9 ± 0.5	0.7 ± 0.3	0.9 ± 0.4	---	0.5 ± 0.2	2.4 ± 1.9	---	---	---
22:0	---	---	---	---	---	---	---	---	6.6 ± 1.6	6.1 ± 3.4
22:4	5.9 ± 0.3	4.5 ± 0.5[c]	0.6 ± 0.4	0.4 ± 0.1	2.7 ± 4.0	1.8 ± 0.4	---	---	---	---
22:5	11.1 ± 0.7	9.9 ± 1.8	0.8 ± 0.4	0.8 ± 0.2	3.1 ± 3.0	2.8 ± 0.3	---	---	---	---
23:0	---	---	---	---	---	---	---	---	1.0 ± 0.2	1.1 ± 0.7
24:0	---	---	---	---	---	---	---	---	8.0 ± 1.2	7.8 ± 5.4
24:1	---	---	---	---	---	---	---	---	11.5 ± 3.8	7.7 ± 5.4

[a] Calculation based on area percent of total area present.
[b] Significantly different from 3T3 membranes ($P < 0.005$).
[c] Significantly different from 3T3 membranes ($P < 0.05$).
[d] Significantly different from 3T3 membranes ($P < 0.025$).
[e] Significantly different from 3T3 membranes ($P < 0.1$). All data are: expressed as the mean ± SE where N = 3 or 4.

phoresis. Our most recent studies have also shown that 3T3 and SV3T3 plasma membranes have identical cyclic AMP-dependent protein kinase activites and contain a comparable composition of phosphoproteins (Scott, manuscripts submitted or in preparation). These data support our view that the 3T3 and SV3T3 plasma membranes used in this study have comparable general characteristics and do not represent different regions or domains of the cell surface. They should, therefore, serve as a valuable source of material to study differences in plasma membrane lipid composition.

Using these preparations, we have found that the plasma membranes of 3T3 and SV3T3 cells differ in the amount of phosphatidylethanolamine and sphingomyelin/mg protein relative to other phospholipid classes. SV3T3 plasma membranes contained 0.033 ug of the phospholipid/mg protein as phosphatidylethanolamine and 0.026 ug/mg protein as sphingomyelin, whereas 3T3 plasma membranes contained 0.039 ug and 0.037 ug/mg of protein, respectively, for phosphatidylethanolamine and sphingomyelin. These differences were found to be statistically significant (Table I). The remainder of the plasma membrane phospholipid classes, however, were present in the same relative proportions in both cell types.

The results of an analysis of the cholesterol composition in isolated plasma membrane preparations (Table I) show that SV3T3 plasma membranes contained 0.205 umol of cholesterol/mg protein, whereas the cholesterol content in 3T3 plasma membranes was 0.175. Thus, the plasmamembranes of SV3T3 cells contained ca. 1.2 times more cholesterol than 3T3 plasma membranes. The molar ratios of cholesterol to phospholipid for the 3T3 and SV3T3 plasma membranes were, however, not significantly different.

The fatty acyl composition of individual classes of phospholipids was then examined. Each class of plasma membrane phospholipid appeared to have a unique fatty acyl profile. Table II illustrates these compositional peculiarities: (a) phosphatidylethanolamine was characterized by nearly half the fatty acyl moieties as 18:1 and 20:4 combined; (b) one-third of the fatty acyl moieties in phosphatidylcholine were 16:0; (c) phosphatidylserine had about 40% 18:0 in its fatty acyl profile; (d) sphingomyelin, with only one fatty acyl moiety, had a distinctive profile in terms of the predominance of saturated fatty acyl groups, some monoenes, and the presence of 20:0, 22:0, 23:0, 24:0, and 24:1, which were not identified in other membrane phospholipid classes.

Besides the identification of unique fatty acyl characteristics of individual phospholipid classes, specific differences were identified between 3T3 and SV3T3 plasma membranes. In particular, compared to 3T3 plasma membranes, SV3T3 plasma membranes showed enrichment of 18:1 in phosphatidylethanolamine and phosphatidylserine. SV3T3 membranes also contained a lower percentage of 20:4 in phosphatidylethanolamine. The relative amounts of 20:3 and 22:4 were also lower in phosphatidylethanolamine from SV3T3 plasma membranes. By contrast, phosphatidylinositol from SV3T3 plasma membrane had higher relative amounts of 18:2 and 20:4. The fatty acyl profiles of phosphatidylcholine and sphingomyelin from 3T3 and SV3T3 plasma membranes did not reflect any significant differences (Table II).

DISCUSSION

SV3T3 plasma membranes varied in lipid composition from the 3T3 plasma membranes in three general characteristics which have been reported to influence the physical state of lipids in membranes (21). These included increased relative amounts of cholesterol, decreased relative amounts of sphingomyelin, and altered fatty acyl composition. More specifically, SV3T3 plasma membranes contain about 0.8 times the relative amount of phosphatidylethanolamine, 0.7 times the relative amount of phosphatidylethanolamine, 0.7 times the relative amount of sphingomyelin, and 1.2 times the amount of cholesterol relative to 3T3 plasma membranes. Compared to 3T3 plasma membranes, SV3T3 plasma membranes also contain: (a) 2.2 times the amount of 18:1 and about 0.5 and 0.7 times the amount of 20:3 and 20:4, respectively, in phosphatidylethanolamine; (b) nearly 2 times the amount of 18:1 in phosphatidylserine; and (c) more than 2 times the amount of 18:2 and 20:4 in phosphatidylinositol.

These data represent the only published detailed analyses of plasma membrane lipid composition of cultured normal and transformed cells with one exception. The plasma membrane lipid composition of BHK cells and polyoma transformed BHK cells was recently published (22). The authors of that study concluded that their data showed no significant difference between phospholipid composition, cholesterol/phospholipid ratio, or in the fatty acyl profiles of normal and transformed cells. These negative results may be related to the different cell system used, but most likely reflected the marked variation in results obtained in the two separate experiments which

were reported.

Data similar to that reported in our paper have been previously published on studies of plasma membrane isolated from normal liver and hepatomas (23,24). In particular, numerous studies on the plasma membranes isolated from normal and malignant liver cells have shown a general tendency for transformed cells to contain a relatively higher amount of 18:1 in phospholipids (24-27), as have our data on SV3T3 cells. The plasma membranes of normal hepatocytes following a short term exposure to a chemical carcinogen have also been shown to increase relative amounts of 18:1 in their phospholipids (17).

Elevations of 18:1 have also been found in the plasma membrane phospholipids of essential fatty acid deficient animals (28). The elevation of 18:1 in this situation was coupled with a simultaneous decrease in 20:4 and the presence of significantly elevated levels of 20:3. Since no elevation in SV3T3 plasma membrane 20:3 content was observed, it appears likely that fatty acid deficiency of these cultured cells does not account for increased levels of 18:1. It is possible, however, that the decreased level of 20:4 in SV3T3 plasma membrane might correlate with the increased synthesis of prostaglandins (29) in SV3T3 cells, which are thought to be derived from plasma membrane arachidonic acid. It is apparent, however, that the specific cause(s) for the alteration in fatty acyl profiles of phospholipids of plasma membrane from malignant cells has not been explained.

Studies of Morris hepatoma 7777 cells suggested that there were alterations in the deacylation-reacylation cycles of the phospholipids (30) and that this could result in elevations in 18:1 and decreases in 20:4 in microsomal phospholipids. One would expect, therefore, that alterations in plasma membrane lipids in transformed cells might have origin in aberrant microsomal synthesis.

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The Content and Composition of Sterols and Sterol Esters in Low Erucic Acid Rapeseed (*Brassica napus*)

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ABSTRACT

The low temperature crystallization technique for the enrichment of "minor" components, such as sterols and sterol esters, from vegetable oils was applied to low erucic acid rapeseed oils. The recovery of free sterols and sterol esters was estimated by use of ^{14}C-cholesterol and ^{14}C-cholesterol oleate. 80% of the free sterols and 45% of the sterol esters were recovered in the liquid fraction, while in two studies total recoveries were 95% and 99%, respectively. This technique showed some selectivity toward the sterol bound fatty acids when compared to direct preparative thin layer chromatography (TLC) of the crude oil. Gas liquid chromatography (GLC) analysis of the free and esterified sterols as TMS-derivatives showed very little selectivity in the enrichment procedure. The fatty acid patterns of the sterol esters demonstrated, however, a preference in the liquid fraction for those sterol esters which have a high linoleic and linolenic acid content. The content of free sterols was 0.3-0.4% and that of sterol esters 0.7-1.2% of the rapeseed oils in both winter and summer types of low erucic acid rapeseed (*Brassica napus*) when the lipid classes were isolated by direct preparative TLC of the oils. The free sterols in the seven cultivars or breeding lines analyzed were composed of 44-55% sitosterol, 27-36% campesterol, 17-21% brassicasterol, and a trace of cholesterol. The esterified sterols were 47-57% sitosterol, 36-44% campesterol, 6-9% brassicasterol, and traces of cholesterol and Δ5-avenasterol. The fatty acid patterns of these esters were characterized by ca. 30% oleic acid and ca. 50% linoleic acid, whereas these acids constitute 60% and 20%, respectively, of the total fatty acids in the oil. Little or no variation in sterol and sterol ester patterns with locality within Sweden was observed for the one cultivar of summer rapeseed investigated by the low temperature crystallization technique.

INTRODUCTION

Sterols and sterol esters of fatty acids predominate among the nonglyceride lipids of most vegetable oils. Many reports in the literature present figures for the percentage distribution of sterols, analyzed after saponification of the total lipids (1-12). The sterol patterns after saponification have been used to identify vegetable oils in the trade and to detect adulterations, e.g., by animal fats in vegetable oils (13).

During the technological processing of the oil, there are changes in the composition of the sterol mixture (14). The free and esterified sterols behave differently, and their qualitative and quantitative composition in the crude oil is, therefore, of interest. Certain plant sterols have been reported to protect vegetable oils from oxidative polymerization during heating (15-17). Furthermore, the oils from different types of low erucic acid rapeseed varied in their oxidative stability, although their fatty acid patterns and tocopherol contents were similar (U. Holm, personal communication; R. Ohlson, personal communication). The relatively high linoleic acid content of low erucic acid rapeseed (8-12%) makes any variation in content of antioxidative components of interest.

These facts stimulated a program on qualitative and quantitative analyses of the minor components of the oils of different types of low erucic acid rapeseed, a crop of great importance in the Swedish agriculture.

The present paper presents data on sterols and sterol esters, using the low temperature crystallization technique of Jacini et al. (18) for enrichment of minor components. A preliminary report from our studies has previously been presented (19).

MATERIALS AND METHODS

Seed material

High quality seeds of *Brassica napus* from isolated multiplications of cultivars and breeding lines were supplied by the Swedish Seed Association, Svalöv (marked Sv in tables), and the Weibullsholm Plant Breeding Institute, Landskrona (marked WW in tables). Two summer and two winter cultivars of low erucic acid rapeseed (LEAR) and three lines with low content of erucic acid and of glucosinolates (double-zero types) were investigated. Five samples of one summer rapeseed cultivar harvested in different areas in Sweden were also analyzed.

Reagents

Solvents were of "pro analysi" grade from Merck. Cholesterol, thin layer chromatography (TLC) reference mixture No. 1 and AOCS No. 11, reference mixture for gas liquid chromatog-

raphy (GLC), were purchased from Nu-Chek-Prep., Inc., Elysian, MN. Pure samples of brassicasterol, campesterol, stigmasterol, and sitosterol were gifts from Dr. Henry Kirscher, University of Arizona, Tucson, AZ. [4-^{14}C]Cholesterol and [oleate-1-^{14}C]-cholesteryl oleate were purchased from NEN Chemicals GmbH, Dreieichenham, West Germany, and Tri-Sil from Pierce, Rockford, IL. Other chromatographic materials were obtained from Analabs, Inc., North Haven, CT.

Extraction

The extraction was performed by vigorous shaking of 5 g of seeds and four steel balls in stainless steel tubes (20,21) with 30 ml hexane-ethanol (3:1) for 1 hr. The extract was filtered through fat-free filters. The solvent was removed by vacuum evaporation and the oil was stored at -20 C.

Enrichment of Minor Components

Nontriacylglycerols were enriched in a noncrystallizable fraction obtained by low temperature crystallization of the oil. Ten grams of oil were dissolved in 195 ml of ethyl acetate and the solution was left to crystallize overnight at -80 C (So-Low Environmental Equipment Co., Inc., Cincinnati, OH). The mother liquor was separated from the precipitate, mainly triacylglycerols, through reversed filtration by applying a slight vacuum. The precipitate was recrystallized twice with 150 ml ethyl acetate at -80 C. The washings were added to the mother liquor. After removing the crystallization solvent, the fractions were dissolved in hexane and stored at -20 C.

^{14}C-cholesterol and ^{14}C-cholesteryl oleate were added to the crude rapeseed oil in two studies, and their recoveries in the fractions after crystallization were measured in a Beckman LS-230 Liquid Scintillation system, using a liquid scintillation counting solution containing 4 g PPO (2,5-Diphenyloxazole) and 50 mg POPOP, 1,5-bis[-2-(5-Phenyloxazolyl)]-benzene per liter of toluene.

Thin Layer Chromatography

The lipids of the concentrated fraction or, in special cases, of the crude oils, were separated on silica gel plates (Anasil H, 0.5 mm, from Analabs, Inc.) with hexane-diethyl ether-acetic acid (70:30:1) as developing solvent. In this system, the sterols (Rf ~0.17) were well separated from the triterpene alcohols (Rf ~0.30). A reference mixture was applied at one side of the plate. After development, this reference lane was sprayed with dichlorofluoroscein (0.025% in ethanol), and the sterols and sterol esters were located under UV-light (365 nm). The corresponding areas from the actual sample were quickly removed from the plate, and the sterols and the sterol esters were eluted from the silica gel with diethyl ether. These lipids were isolated in duplicate and to one of the two samples, ca. 10% cholesterol was added as an internal standard.

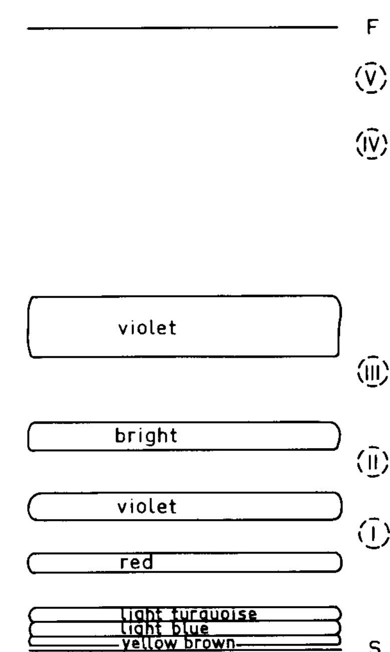

FIG. 1. Thin layer chromatogram of a precipitate from the crystallization of crude, laboratory extracted rapeseed oil (Oro). Absorbent: Silica gel H. Solvent: hexane-diethyl ether-acetic acid (70:30:1, by vol). The colors noted are those observed for nonsterolic components fluorescent under UV-light (365 nm). The location of the known standards, visible under UV-light after spraying with dichlorofluorescein, are denoted by Roman numerals: I = sterols (R_f = 0.17-0.21); II = triterpene alcohols (R_f = 0.27-0.30); III = free fatty acids (R_f = 0.37-0.41); IV = triacylglycerols (R_f = 0.73-0.78), V = sterol esters (R_f = 0.87-0.90).

Hydrolysis of Sterol Esters Followed by Isolation of Sterols and Fatty Acids

Sterols esters (~1 mg) were refluxed for 2-3 hr in 5 ml of 1 M methanolic NaOH. The solution was then acidified with conc. HCl, the sterols and the free fatty acids were extracted with hexane (2 x 2 ml) and separated in the same TLC system as previously described.

Gas Chromatography

The sterols were silylated by heating at 60-65 C for 45 min using 200 μl Tri-Sil to 1000 μg of sterols. The silyl ethers were analyzed in a

TABLE I

Recovery of ^{14}C-Cholesterol and ^{14}C-Cholesteryl Oleate in the Two Fractions Obtained by Crystallization of Rapeseed Oil (WW Oro) at -80 C

Added compound	Recovery in the mother liquor		Recovery in the precipitate		Total recovery	
^{14}C-cholesterol	80 77	78	13 20	17	93 97	95
^{14}C-cholesteryl oleate	49 41	45	50 62	56	99 100	99

TABLE II

Pattern of Free and Esterified Sterols Isolated by Low Temperature Crystallization of Rapeseed Oil Followed by Preparative TLC (A) and by Direct, Preparative TLC (B) of the Oil

Lipid classes	Technique	Percentage distribution of sterols[a]		
		Brassicasterol	Campesterol	Sitosterol
Free sterols	A	16.6	29.8	53.6
	B	16.0	28.9	55.0
Esterified sterols	A	7.0	35.7	57.3
	B	4.7	37.4	57.9

[a]Traces of other sterols were also registered. See Table IV.

Varian Aerograph Model 2100 equipped with two 180 cm x 2 mm glass columns kept at 270 C. The columns were packed with 3% OV-1 on Anachrom ABS 90/110 mesh, and 1% OV-17 on HP Chromosorb G 80/100 mesh, respectively. Nitrogen was used as carrier gas at 20 ml/min.

The TMS-sterols were identified by comparison of the retention times of actual TMS-sterols with those of known standards. For one sample, WW Oro, mass spectra of TMS-sterols from the free and esterified fractions were compared with those reported in the literature. Possible differences in GLC response for different sterols have been ignored in the calculation by direct normalization.

Fatty acid methyl esters were prepared according to Appelqvist (22), but with 0.01 M $NaOCH_3$ as catalyst in the methanolysis of crude oils and analyzed in the Varian instrument using a 180 cm x 2 mm glass column containing 6% EGA on Anachrom ABS 90/105 mesh at a column temperature of 175 C. Nitrogen was used as carrier gas at 20 ml/min. The fatty acids were identified by comparison of the retention times of actual fatty acids with those of reference mixtures. The percentage of each fatty acid was calculated by direct normalization since the reference mixture AOCS No. 11 analyzed under the same conditions yielded area percent close to known weight percent. A Varian 480 digital integrator has been used for calculation of peak areas.

Combined GC-Mass Spectrometry

Analyses were performed on a LKB-9000 gas chromatograph-mass spectrometer. In the chromatograph, a glass column 150 cm x 2 mm packed with 1% OV-17 on HP Chromosorb G 80/100 mesh was operated at 240 C with helium at 20 ml/min as carrier gas. Other conditions were: molecular separator temperature 240 C, ion source 240 C, ionizing voltage 70 eV, trap current 50 μA, and acc. voltage 3500 V.

RESULTS

Isolation Techniques

Low temperature crystallization of crude rapeseed oil yielded a liquid fraction, which amounted to 6-7%, with very small differences between the various cultivars. The liquid fraction contained about 50% triacylglycerols and 50% other "minor" substances, whereas the precipitate was composed of almost 100% triacylglycerols with trace amounts of other components.

Some minor components in the liquid fraction, separated by TLC, displayed fluorescent colors under UV-light (365 nm). These fluorescent bands were slightly different for the oils investigated. Samples from WW Oro from different areas in Sweden showed, however, identical patterns. A schematic drawing of the colors observed under UV-light of the concen-

TABLE III

Content of Free and Esterified Sterols Isolated in Crude, Laboratory Extracted Rapeseed Oil by Low Temperature Crystallization of the Oil Followed by Preparative TLC (A) and by Direct, Preparative TLC (B) of the Oil

			% of oil[a]		
	Cultivar or line	Technique	Free sterols	Esterified sterols	Calculated[b] sterol esters
Summer rapeseed LEAR type	WW Oro	A	0,35	0,65	1,1
	WW Olga	B	0,36	0,71	1,2
Summer rapeseed Double-zero type	WW 1568	A	0,25	0,56	0,9
	WW 1568	B	0,34	0,43	0,7
Winter rapeseed LEAR type	Sv Brink	A	0,24	0,56	0,9
	Sv Brink	B	0,31	0,51	0,9

[a]The figures obtained by technique A have been corrected according to the recoveries shown in Table I.

[b]Calculations have been done with the assumption that the sterol esters are composed entirely of sitosteryl linoleate.

trated fraction of WW Oro oil is given in Figure 1. The sterols were well separated from the triterpene alcohols, and the methyl sterols were located between these two lipid classes. Although the triacylglycerols were well separated from the sterol esters, the latter might not be separated from some other esters such as the triterpene alcohol esters. The contribution of these other esterified fatty acids to the fatty acid patterns reported for sterol esters is probably negligible in rapeseed oils, since Fedeli et al. (23) demonstrated that the total triterpene alcohol content of this oil was only 0.03%.

A recovery of 78% of ^{14}C-cholesterol and 45% of ^{14}C-cholesteryl oleate was obtained in the mother liquor (Table I). Rather great diversity was found, however, between duplicate recovery studies. The partitioning of sterol esters between solid and liquid phase at -80 C is obviously dependent on experimental conditions, such as the practical performance of the reversed filtration, which might differ from time to time. Quantitative estimations of the various lipid classes after low temperature enrichment are, therefore, of limited value.

Although the low temperature crystallization technique showed selectivity in recovery of the total amount of free and esterified sterols, the sterol patterns displayed almost identical proportions in the crude oil and in the mother liquor fraction (Table II). The greatest deviation was observed for brassicasterol in the sterol ester fraction (7.0% vs. 4.7%). This is, however, regarded as insignificant and no correction for any selectivity has been undertaken in the data presentation.

The sterol patterns of the free and the esterified sterol fractions obtained from duplicate crystallizations of one oil differed by 0.2-0.8 percentage units which indicated good reproducibility.

Content of Sterols and Sterol Esters

The content of free sterols in three different cultivars of rapeseeds was found to be 0.31-0.36% of the oil when analyzed by direct preparative TLC of the oil and 0.24-0.35% when analyzed by preparative TLC of the liquid fraction from the crystallization and application of the recovery factor (Tables I and III). The amounts of esterified sterols were 0.43-0.71% and 0.56-0.65%, respectively, when determined with the two techniques.

It is obvious that the quantities calculated from analyses of the liquid fraction differ somewhat from those arrived at by the straight forward preparative TLC. Since no overall recovery studies were made for preparative TLC, the figures for content of free sterols and sterol esters presented are subject to considerable uncertainties in the second decimal figure.

Calculations of the amount of sterol esters were done with the assumption that they are composed entirely of sitosteryl linoleate. They accounted for 0.7-1.2% of the crude oil.

Sterol Patterns

The major sterols in both the free and esterified sterol fractions of rapeseed oils are in descending order sitosterol, campesterol, and brassicasterol, though their percentage distribution varies in the two fractions (Table IV). Brassicasterol, typical for Brassica and other Cruciferae (24), showed the greatest difference, accounting for almost 20% of the free sterols

TABLE IV

Sterol Composition of Free and Esterified Sterols in Crude, Laboratory Extracted Rapeseed Oils

	Cultivar or line	Free Sterols, %			Esterified sterols, %		
		Brassicasterol	Campesterol	Sitosterol	Brassicasterol	Campesterol	Sitosterol
Summer rapeseed LEAR type	WW Oro[a]	16.6	29.8	53.6	7.0	35.7	57.3
		±0.7	±0.5	±0.5	±0.6	±1.0	±1.5
	WW Olga	17.5	27.4	55.1	7.3	36.5	56.2
Summer rapeseed Double-zero type	WW 1568	21.0	30.6	48.4	8.5	37.8	53.7
	Sv 1535	20.1	36.3	43.6	6.4	41.3	52.4
	Sv 1536	19.2	30.3	50.5	7.0	42.0	51.0
Winter rapeseed LEAR type	Sv Status	20.1	31.2	48.7	8.3	44.0	47.7
	Sv Brink	20.4	34.5	45.1	7.0	42.9	50.1

[a]The figures for WW Oro represent means ± standard deviation of sterol compositon in oils from rapeseed grown in five different localities in Sweden. Sterols from WW Oro subjected to mass fragmentography demonstrated traces of cholesterol in the free and esterified fraction and traces of Δ5-avenasterol in the esterified fraction.

but only ca. 7% of the esterified sterols. This was balanced by both campesterol and sitosterol, which showed slightly higher relative concentrations in the esterified compared to the free fraction. Two or more peaks with retention times longer than that for sitosterol were registered on the chromatograms from the esterified sterol fraction. The larger of the two peaks was identified as Δ5-avenasterol from the following criteria: The mass spectrum of the trimethylsilyl ether derivative showed M^+ at m/e 484 (14%) and other strong ions at m/e 469 (11%), 386 (100%), 296 (87%), 281 (36%), and 129 (98%). The base peak at m/e 386 arises from loss of part of the side chain by a McLafferty rearrangement, and is typical for sterols containing a Δ24(28)-bond. The strong m/e 129 ion is specific for TMS-derivatives of a Δ5-sterol and serves to distinguish monounsaturated Δ5-sterols from corresponding diunsaturated sterols and Δ7-sterols. This fragmentation pattern agrees with that reported for Δ5-avenasterol by Knights (25).

Small amounts of cholesterol were obtained in both the free and esterified fractions. A component with the same retention time as, or slightly longer than, that of stigmasterol was irregularly observed on the chromatograms. The identity of this peak was not established. The sterols of one sample of WW Oro were analyzed by GC-mass spectrometry. In this case, however, stigmasterol could not be detected by mass-fragmentography. In mass spectra of the TMS-derivatives of cholesterol, brassicasterol, campesterol, and sitosterol agreed with those reported by Knights (25).

The standard deviation for percentage content of individual sterols among five samples of the Oro cultivars were 0.5-0.7 for the free sterols (Table IV). The analyses of the esterified sterols involve two additional analytical steps, and this is probably the reason for a somewhat higher variation (SD=0.6-1.5).

Only small differences in the sterol patterns between the investigated cultivars and lines of rapeseed were observed. There is no evidence for any type specificity (summer vs. winter types, LEAR vs double-zero) in sterol patterns.

The distribution of individual sterols as percentage of total sterols (free and esterified) in our samples was calculated in order to permit comparison to literature data. Figures thus obtained were 11.5-14.2% of brassicasterol, 32.2-39.2% of campesterol, and 47.9-55.7% of sitosterol.

Sterol-bound Fatty Acids

The fatty acids bound to sterols accounted for about 0.4% of the total content of fatty

TABLE V

Pattern of Total Fatty Acids, Mainly Triacylglycerols in Rapeseed Oils and of Sterol Bound Fatty Acids (ca. 0.4% of the Total Fatty Acids)

	Cultivar or line	Composition (%) of total fatty acids[a]					Composition (%) of sterol bound fatty acids[b]				
		16:0	18:0	18:1	18:2	18:3	16:0	18:0	18:1	18:2	18:3
Summer rapeseed LEAR type	WW Oro[c]	4.4 ±0.1	1.8 ±0.1	62.6 ±1.3	20.7 ±0.1	8.4 ±0.8	5.6 ±1.6	1.4 ±0.3	24.1 ±2.8	60.3 ±2.5	8.6 ±2.7
	WW Olga	4.6	1.5	61.3	20.8	9.3	9.2	1.0	18.5	58.8	12.5
Summer rapeseed Double-zero type	WW 1568	5.1	1.5	58.0	23.5	10.2	6.2	3.0	19.2	56.4	15.2
	Sv 1535	4.3	1.4	63.0	18.6	11.4	7.3	1.2	24.1	55.5	12.0
	Sv 1536	4.2	1.4	62.0	20.0	11.1	4.9	0.9	21.5	58.9	13.8
Winter rapeseed LEAR type	Sv Status	5.8	1.5	57.5	22.0	11.4	12.4	2.0	18.0	53.6	14.1
	Sv Brink	5.2	1.5	58.4	22.6	10.3	7.7	2.4	19.9	55.8	14.2

[a]0.2-0.5% of 16:1, 1-2% of 20:0 and traces up to 0.7% of 22:1 appears on the chromatograms.
[b]About 1% of 16:1 and traces of 20:0 were also registered.
[c]The values (mean ± SD) of WW Oro are based upon samples harvested from five different areas in Sweden.

acids in the oil. The qualitative composition of these fatty acids was found to be remarkably different from that of total fatty acids (Table V). Although no noticeable crystallization selectivity was found to exist for the sterol pattern of the esterified sterols (Table II), some selectivity according to degree of unsaturation of the fatty acids bound to the sterols was observed. However in the direct TLC technique, one is handling a much smaller amount of esterified sterols and the risk of accumulation of contaminants through the various analytical steps is much greater. Therefore, a "blank" sample, was run through all the steps and was found to contain mainly 16:0, 18:0, 18:1, and 18:2 (cf. ref. 22). Analyses of the sterol-bound fatty acids from WW Olga, isolated by the direct preparative technique, yielded the following percentages after correction for contaminants: 7.3% of 16:0, 1.4% of 16:1, 2.3% of 18:0, 32.1% of 18:1, 51.2% of 18:2, and 5.6% of 18:3.

The sterol esters of the mother liquor are thus enriched with respect to esters containing linoleic (59% in the liquid vs. 51% in the total oil) and linolenic acids (13% vs. 6%).

Regardless of minor errors in the techniques used for the isolation of the sterol esters, it is obvious that the sterol esters are much higher in linoleic acid and lower in oleic acid than the glycerol esters (Table V). Only very small differences in the patterns of sterol bound fatty acids were registered between the investigated cultivars and lines of rapeseeds, as can be seen in Table V.

DISCUSSION

The isolation of the various "minor" components of vegetable oils can be accomplished by different techniques. Classical silicic acid column chromatography of the crude lipids has the advantage of low exposure of the lipids to oxidation during the separation if carefully operated (26). The predominance of triacylglycerols in the crude lipids and the large number of other components present in minor or trace amounts make such separations tedious and requires use of excessive amounts of pure organic solvents besides yielding fractions of doubtful purity. Isolation of sterols and other lipid classes from the nonsaponifiable of vegetable oils has also been done by lipophilic gel chromatography (27). High performance (high pressure) liquid chromatographic (HPLC) separations of lipids using semipreparative columns would provide a very rapid method. Great variation in polarity of the actual lipid classes demands solvent gradient elution and choice of detector might cause problems. Successful HPLC separations have been carried out for lipids in soybeans (28). Preparative TLC can be utilized but requires the use of many plates, risk of oxidation, and the accumulation of contaminants (cf. 22 and loc. cit.). The low temperature crystallization technique of Jacini et al. (18) has been reported to yield a liquid fraction very much enriched in "minor" components. These authors state that although the method is not quantitative (some "minor" components remaining in the solid phase at -70 C), it is not selective toward the standard unsaponifiables. However, no data have been found in the literature to support such statements which appear to refer only to olive oil (29). Our results demonstrate little or no selectivity as regards the free sterols of which ca. 80% are found in the liquid fraction. For the sterol

esters, of which only ca. 50% are recovered in the liquid fraction, there is little if any selectivity as regards the sterol moiety of the sterol esters. However, the sterol esters of the liquid fraction are richer in linoleic and linolenic acids than those of the total sterol esters. Hence, in any application of the low temperature crystallization technique to minor components of oils, selectivity must be considered. Nevertheless, the technique offers many advantages, especially for the trace components which might be present at very low concentrations in the oil (cf. Fig. 1).

To the best of our knowledge, this is the first report on the content and composition of free sterols and sterol esters of oils from rapeseeds of known origin. In previous Canadian studies of high and low erucic acid rapeseed (*B. napus*) (30), the content of sterols was reported to be 0.5% and 1.1% of the oil and that of sterol esters 3.4% and 1.0%, respectively. However, these figures were based on gravimetric analysis of silicic acid fractions.

Our data on total sterols, 0.7-1.2% of the oil, agree with literature data (0.6-1.2%) based on the standard saponification method (2,8,9,23). Since some losses of sterols occur during refining (14,31), any comparison of our data with data from the literature on refined rapeseed oils must be done with caution.

In the Swedish samples of low erucic acid rapeseed, we have identified sitosterol, campesterol, and brassicasterol as the major sterols both in free and esterified form and traces of cholesterol, which is in accordance with literature data (24). Further, we have found Δ5-avenasterol among the esterified sterols. This sterol has an ethylidene group in the side chain. Sterols with such structure are believed to improve the resistance of oils to darkening and polymerization (15-17). Amounts from zero to 5.7% of Δ5-avenasterol are reported to occur in rapeseed (2,6,7,9,10,12). The presence of Δ7-sterols and dienols in rapeseed oils have been stated by others (8) but were not found in our samples. Although the GLC analyses were performed on two columns with different stationary phases, peak-overlapping may occur, and other stationary phases or capillary columns seem to be necessary to obtain a complete separation (12,32).

Stigmasterol has been reported to occur in *B. napus* but not in some other Brassicas studied (1), and this difference was utilized in taxonomic consideration. We did not find any stigmasterol, which is in accordance with detailed German studies (8).

The loss of Δ7-sterols during storage of rapeseed has been observed (4). The extraction of the seeds reported in this paper was performed ca. 6 months after harvest. A further loss of Δ5-avenasterol and Δ7-sterols during refining is likely, and studies on this aspect are currently underway.

German and French studies (3,4) on seeds of known origin performed with the classical saponification technique, which only yields data on total sterol content and composition, were performed with both high erucic and low erucic acid cultivars. Although the German data on winter types of rapeseed *(B. napus)* indicate higher campesterol and slightly lower brassicasterol content in low erucic acid cultivars, their data on summer cultivars of both *Brassica campestris* (turnip rape) and *B. napus* (rape) hardly support such correlations (4). However, the French data tend to indicate higher campesterol and lower brassicasterol content of low erucic acid compared to high erucic acid rapeseed (3). Our data on total sterol patterns, calculated from the analyses of sterols and sterol esters, indicate rather high brassicasterol contents (12-14%) in low erucic acid varieties compared to German (7-12%) or French figures (7-8%). Recent Japanese analyses (11) showed no differences in the content of brassicasterol (7-9%) between rapeseeds with a low and a high content of erucic acid.

The small differences in sterol patterns noted in our studies on low erucic and double-zero types of summer rape and low erucic winter rape are probably within experimental error and are of no practical significance. Similarly, no differences with cultivation conditions of one cultivar was observed. This is interesting for the evaluation of different new cultivars of rapeseed.

The differences in sterol patterns for the free sterols compared to the esterified sterols is indeed noteworthy. If use was made of the sterol patterns of the free sterols instead of the total sterol patterns, as it is now done in the Food Standards for rapeseed oil by the Codex Alimentarius Commission (13) (claiming 8-12% brassicasterol), adulteration appears to be detectable at lower levels of adulterations. Further, no time consuming saponification step would be necessary in this food control analysis.

Also, the great differences in fatty acid patterns of the sterol esters compared to that of triacylglycerols (essentially the total fatty acids) deserves attention.

Since it has been reported that some losses in total sterols occur during industrial processing (14,31), studies are underway on the effect of refining and deodorization on the qualitative and quantitative composition of sterols and

sterol esters of low erucic acid rapeseed oil.

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Fatty Acid Metabolism in L1210 Murine Leukemia Cells: Differences in Modification of Fatty Acids Incorporated into Various Lipids

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ABSTRACT

L1210 leukemia cells can utilize all of the main fatty acids that normally are present in the ascites fluid in which they grow. This finding is consistent with the view that L1210 cells derive most of their fatty acids from the ascites fluid. From 80-90% of each fatty acid was incorporated into cell lipids without structural modification, suggesting that the lipid composition of these cells can be altered by changing the type of fatty acids to which they are exposed. Most importantly, the palmitate that was subsequently incorporated into total cell phospholipids was elongated and desaturated somewhat more than that incorporated into triglycerides. This difference was due primarily to more extensive modification of the palmitate incorporated into the ethanolamine phospholgycerides fraction. Although there was no difference between total phospholipids and triglycerides with linoleate, more of the linoleate incorporated into ethanolamine phosphoglycerides was elongated and further desaturated than that incorporated into choline phosphoglycerides and triglycerides. These findings indicate that fatty acids incorporated into various cell lipid fractions are not structurally modified to the same extent. There appears to be greater modification of fatty acid used for ethanolamine phosphoglyceride synthesis as compared with triglyceride and choline phosphoglyceride synthesis.

INTRODUCTION

L1210 leukemia cells can take up large quantities of palmitic acid (1). Calculations based on the cell lipid content and growth rate suggest that most of the cell lipid requirement could be supplied by uptake from the extracellular fluid. Analysis of the fluid in which the cells grow, however, revealed that it contains a mixture of fatty acids (1). Therefore, in order to examine this question more critically, it was necessary to determine whether the L1210 cell can utilize other fatty acids as readily as palmitate. Another previous observation was that almost 90% of the palmitic acid that was taken up was incorporated into cell lipids without structural modification (1). Furthermore, about 25% of the palmitate uptake was recovered in phospholipids. This suggested that the fatty acyl composition of the L1210 cell membranes might be modified if the fatty acids to which the cells were exposed could be varied. Two additional pieces of data were required in relation to membrane modification. One was whether other fatty acids, like palmitate, were incorporated into phospholipids. The other was whether they also underwent little structural modification, especially fatty acids that were used for phospholipid synthesis. This communication provides information concerning these points. Of particular importance was the new observation that the fatty acids incorporated into the ethanolamine phospholglycerides were modified to a greater extent than those incorporated into other complex lipids.

MATERIALS AND METHODS

L1210 cells were harvested from male DBA/2j mice 7 days after i.p. injection of 1 x 10^5 cells in saline (1). Cells obtained from 6-8 mice were pooled and separated from the ascites plasma by centrifugation at 480 x g for 5 min at 0 C. Contaminating erythrocytes were removed by hypotonic hemolysis and the washed cell pellet was resuspended in Eagle's minimum essential medium with glutamine (Grand Island Biological Company, Grand Island, NY). The concentration of the suspended cells was determined by duplicate cell counts using a clinical hemocytometer after Turk's staining. Viability counts were performed after staining with Erythrosin B, and viability just prior to incubation averaged 91.1% ± 0.6. Fatty acid was combined with albumin by incubating solutions of 0.6 mM fatty acid-poor bovine serum albumin (Pentex, Miles Laboratory, Elkhart, IN) in phosphate-buffered saline with Celite that was coated with fatty acid (2). The fatty acid concentrations in these solutions were determined by titration (3). The specific activity and purity of the [1-^{14}C]fatty acid substrates (Amersham/Searle Co., Arlington Heights, IL) as determined by gas liquid chromatography (GLC) was as follows: myristate 0.382 mCi/mmol, 95.3%; palmitate 0.222 mCi/mmol, 94.7%; stearate 0.084 mCi/mmol, 93.2%; oleate 0.660 mCi/mmol, 93.8%; linoleate 0.3 mCi/mmol, 96.0%; and arachidonate 0.261 mCi/mmol, 94.1%. The major labeled impurities contained in the fatty acid substrates

were myristate (>20:4[1], 0.9%; 18:0, 0.8%; 18:2, 0.6%; 16:0, 0.5%), palmitate (>20:1, 2.1%; 16:1, 0.9%; 18:0, 0.8%; 18:2, 0.6%; 18:1, 0.4%), stearate (>20:4, 2.1%, 18:1, 1.3%; 18:3+20:0+20:1, 1.1%; 18:2, 0.7%, 16:0, 0.5%), oleate (>20:4, 1.5%;18:2, 1.4%; 20:2+20:3+22:1, 1.1%; 18:3+20:0+20:1, 1.0%; 20:4, 0.5%), linoleate (22:4, 1.3%; 20:2+20:3+22:1, 0.9%; 18:3+20:0+20:1, 0.8%) and arachidonate (22:4+22:5+22:6, 5.2%; 20:2+20:3+22:1, 0.2%). Metabolic incubations were carried out in rubber-stoppered 50 ml glass flasks with a gas phase of 95% air-5% CO_2. Each flask contained 5 x 10^7 L1210 cells, 3.6 μmol of a single fatty acid, and 1.2 μmol of albumin in a total volume of 5.0 ml. Previous studies with palmitate had failed to detect any appreciable differences in fatty acid modification at various incubation times and substrate concentrations (1). Therefore, for the present study of multiple fatty acids, we chose one appropriate time point and a substrate concentration which resembles that of L1210 ascites fluid (1). After incubation at 37 C, the cells were washed three times and extracted with 20 ml $CHCl_3$:CH_3OH (2:1, v/v) (4). The $CHCl_3$ phase was evaporated to dryness under N_2 and the residue redissolved in 5 ml $CHCl_3$. One ml of the $CHCl_3$ solution was dried and analyzed for total lipid radioactivity using a dioxane-water (5:1, v/v) scintillation solution containing 5.83 g PPO, 0.25 g POPOP, and 83 g napthalene per liter. The lipids contained in the remainder of the chloroform extract were analyzed by thin layer chromatography (TLC) (5) and GLC. Two-dimensional TLC was used to separate individual phospholipids (6) and then the segments of silica gel containing lipids were scraped directly into dioxane scintillation solution for measurement of radioactivity.

For studies of the esterification of [1-^{14}C]palmitate and [1-^{14}C]linoleate into choline phosphoglycerides and ethanolamine phosphoglycerides, one-dimensional TLC on Silica Gel G plates developed in $CHCl_3$:CH_3OH:CH_3COOH:H_2O (100:50:14:6) was used to separate these phospholipid components. In these studies, the separation was markedly facilitated by prewashing the plates with 100% ethyl acetate. These phospholipids were identified by ninhydrin, Dragendorff, and 40% H_2SO_4 aerosol sprays and extracted immediately by the modified procedure of Raheja et al. (7) with a solution of $CHCl_3$:CH_3OH (1:1). After washing with 9 ml 0.04N HCl, the isolated choline or ethanolamine phosphoglycerides were saponified, the fatty acids were methylated (8) and analyzed by GLC. The fatty acid methyl esters were separated on a 6 ft. x 0.25 in. metal column containing 10% SP-2330 on Chromosorb W (AW) using a Hewlett-Packard 5710 gas liquid chromatograph equipped with a 1:10 stream splitter and a heated collection vent. Individual methyl esterpeaks were identified by comparison with known standards (Supelco Co., Bellefonte, PA). Corresponding effluent fractions were collected by attaching Teflon tubing to the heated collection vent and allowing the effluent gas to bubble into 15 ml of a Toluene-Triton X (8:2, v/v) solution containing 5.5 g PPO and 0.1 g POPOP per l. The radioactivity was measured after the Teflon tubing was cut into small pieces and added to the counting vials (9). In all cases where radioactivity was measured, the degree of quenching and counting efficiency were determined using an external standard, and quench correction curves were constructed using internal standards.

[1]The fatty acids are abbreviated as number of carbon atoms: number of double bonds. > 20:4 refers to fatty acids with more than 20 carbon atoms or 20 carbon atoms and more than 4 double bonds.

RESULTS

Table I shows distribution of the radioactivity incorporated into L1210 cell lipids after 1 hr of incubation with physiologically important long chain fatty acids. Although the bulk of the radioactivity was recovered in neutral lipids, especially triglycerides, there was still an appreciable amount incorporated into phospholipids in every case. This amounted to 22-26% for the main 16-20-carbon atom fatty acids tested. These long chain acids account for 88% of the fatty acid present in the ascites fluid in which L1210 cells grow (1). The differences in incorporation of the several fatty acids could not be accounted for by differences in the inherent fatty acid composition of the various cell lipid fractions. For example, stearate, the most abundant fatty acid in L1210 phospholipids (10), was incorporated into phospholipids at the lowest rate. In all cases, only small amounts of fatty acid radioactivity were incorporated into mono- and diglycerides, cholesteryl esters, and cellular free fatty acids.

Although fatty acids were incorporated into several different phospholipids, 76-91% of the radioactivity recovered in phospholipids was in choline and ethanolamine phosphoglycerides (Table II). These are the phospholipids found in highest amounts in the L1210 cell (1).

The extent to which these fatty acids are modified structurally when they are incorporated into the cell lipids is shown in Table III. In

TABLE I

Incorporation of Labeled Fatty Acids into Cellular Lipid Fractions[a]

Fatty acid	Distribution				
	Phospholipids	Mono- and diglycerides	Free fatty acids	Triglycerides	Cholesteryl esters
Myristate	79.3 ± 1.3 (11.9)[b]	21.7 ± 0.7 (3.3)	3.6 ± 0.4 (0.5)	554.2 ± 10.4 (83.3)	6.0 ± 0.6 (0.9)
Palmitate	104.5 ± 3.5 (26.4)	20.4 ± 0.7 (5.1)	7.2 ± 0.7 (1.8)	256.9 ± 10.2 (64.8)	7.2 ± 0.3 (1.8)
Stearate	75.2 ± 2.2 (21.7)	23.4 ± 0.7 (6.8)	29.9 ± 1.1 (8.7)	201.7 ± 4.0 (58.3)	15.9 ± 2.7 (4.6)
Oleate	101.6 ± 3.8 (22.4)	19.9 ± 0.7 (4.4)	4.4 ± 0.2 (0.9)	314.0 ± 11.3 (68.5)	14.7 ± 1.2 (3.2)
Linoleate	96.1 ± 6.7 (26.6)	14.2 ± 0.7 (4.0)	7.0 ± 1.0 (1.9)	234.1 ± 8.8 (65.4)	7.9 ± 2.0 (2.2)
Arachidonate	97.5 ± 1.5 (25.9)	6.3 ± 0.6 (1.7)	3.9 ± 0.4 (1.0)	264.9 ± 5.1 (70.4)	4.0 ± 0.6 (1.1)

[a]L1210 leukemia cells obtained 7 days after transplantation were harvested, washed, and incubated at 37 C with [1-^{14}C] fatty acids for 60 min at a molar ratio of fatty acid to albumin of 3. The incubations were terminated by transferring the contents of each flask into ice cold phosphate-buffered saline followed by centrifugation. Lipids were extracted with chloroform-methanol (2:1), and the lipid fractions were separated by thin layer chromatography. Radioactivity was determined with a liquid scintillation spectrometer. Values are in nmol/hr per 10^8 cells and are expressed as mean ± standard error of determinations on five to six separate incubations.

[b]Parentheses enclose the percentage of radioactivity in each fraction.

every case, greater than 79% of the radioactivity was incorporated without structural modification. On the other hand, every fatty acid was elongated and, with the exception of oleate, desaturated to some extent. The main elongations were the conversion of 14:0 to 16:0, 16:0 to 18:0, and 20:4 to 22:4. The main desaturation was the conversion of 18:0 to 18:1. There also were appreciable amounts of 22:4 radioactivity recovered when the cells were incubated with linoleate. The mechanism of this is unclear because very little radioactivity occurs in 20:4 under these conditions. In studies of other cells which contain Δ 6-desaturase activity, the main radioactive product of linoleate is 20:4 (11). However, in additional experiments (Table IV), analysis of the individual lipid subfractions following incubation of the cells with labeled linoleate revealed the presence of small amounts of radioactivity in 20:3 as well as 20:4.

As shown in Table IV, the fatty acid incorporated into the main lipid components was not structurally modified to the same extent. For example, palmitate incorporated into total phospholipids was modified more than that incorporated into triglycerides. Furthermore, the palmitate incorporated into choline phosphoglycerides was modified less than that incorporated into ethanolamine phosphoglycerides. Similar results regarding choline and ethanolamine phosphoglycerides were obtained with radioactive linoleate. As opposed to palmitate, however, the linoleate incorporated into the total phospholipid fraction of the cell was not modified to any greater extent than that incorporated into triglycerides. In spite of these somewhat larger effects, from 80-90% of the total amount of these fatty acids incorporated into total phospholipids still occurred without structural modification.

DISCUSSION

These studies demonstrate that the L1210 cell can readily utilize all of the major fatty acids available in the ascites fluid in which it grows. In each case, incorporation into phospholipids accounts for almost 25% of the total radioactivity recovered in cell lipids. At most, only 20% of the total incorporated fatty acid underwent structural modification. This suggests that exposure of the L1210 cell to different fatty acids could produce changes in membrane fatty acyl composition and, therefore, cell properties and function (1).

TABLE II

Distribution of Radioactivity in Phospholipids[a]

Fatty acid	Distribution					
	Ethanolamine phosphoglyceride	Choline phosphoglyceride	Serine and inositol phosphoglyceride	Sphingomyelin	Choline lyso-phosphoglyceride	Other phospholipids
Myristate	6.3 ± 0.5 (7.9)[b]	60.8 ± 1.0 (76.7)	3.6 ± 0.4 (4.4)	1.9 ±<0.1 (2.4)	3.9 ± 0.3 (4.9)	2.8 ± 0.5 (3.6)
Palmitate	20.7 ± 0.7 (19.8)	63.7 ± 2.1 (60.9)	8.6 ± 0.3 (8.2)	1.2 ±<0.1 (1.2)	10.4 ± 0.3 (10.0)	0.0 ± 0.0 (0.0)
Stearate	27.7 ± 3.5 (35.7)	30.9 ± 1.0 (40.1)	8.2 ± 0.6 (10.7)	0.8 ± 0.4 (1.1)	7.0 ± 0.5 (9.1)	2.4 ± 0.6 (3.2)
Oleate	17.7 ± 1.0 (16.9)	72.5 ± 3.8 (69.0	11.5 ± 1.1 (10.9)	0.9 ± 0.3 (0.8)	1.5 ± 0.5 (1.5)	0.9 ± 0.3 (0.8)
Linoleate	19.5 ± 3.0 (20.5)	63.8 ± 6.0 (69.6)	6.5 ± 1.0 (6.9)	1.4 ± 0.3 (1.4)	0.7 ± 0.2 (0.8)	0.8 ± 0.2 (0.8)
Arachidonate	21.0 ± 1.8 (21.2)	64.6 ± 1.8 (65.5)	9.4 ± 0.3 (9.6)	0.4 ± 0.1 (0.5)	1.7 ± 0.2 (1.7)	1.6 ± 0.8 (1.6)

[a]L1210 leukemia cells were incubated with [1-^{14}C]fatty acids in minimal essential medium for 60 min at a molar ratio of fatty acid to albumin of 3. The phospholipids in the cell lipid extract were separated using two-dimensional thin layer chromatography. The regions of the gel that contained lipid material were scraped, and the radioactivity was determined by liquid scintillation spectrometry. Values are the absolute rate of incorporation into each phospholipid expressed as nmol/hr per 10^8 cells and are the mean and SE of determinations on four separate incubations.

[b]Parentheses enclose the percentage incorporation of each phospholipid.

TABLE III

Distribution of Radioactivity in Cell Fatty Acids after Incubation with [1-^{14}C]Fatty Acids[a]

Labeled fatty acid in incubation medium	Distribution (%)												
	<14:0[b]	14:0	16:0	16:1	18:0	18:1	18:2	18:3 + 20:0 + 20:1	20:2 + 20:3 + 22:1	20:4	22:4	22:5	22:6
Myristate	0.2	80.1	14.9	0.9	3.0	0.6	n.d.	n.d.	n.d.	n.d.	--------	0.4	------
Palmitate	n.d.[c]	n.d.	86.8	1.3	10.4	1.5	n.d.	n.d.	n.d.	n.d.	--------	n.d.	------
Stearate	0.4	0.4	1.7	1.8	88.4	4.4	0.3	1.0	0.5	0.5	--------	0.8	------
Oleate	n.d.	0.1	1.1	0.1	4.5	91.1	n.d.	2.5	0.4	0.4	--------	0.1	------
Linoleate	0.3	0.1	1.8	0.1	1.6	2.5	80.4	n.d.	5.7	0.6	3.4	2.1	2.4
Arachidonate	0.3	0.2	0.4	0.1	0.5	0.1	0.3	0.6	1.6	79.3	14.5	0.2	0.8

[a]L1210 leukemia cells were incubated in minimum essential medium with various labeled fatty acids for 60 min at a molar ratio of fatty acid to albumin of 3. The saponifiable lipids of the cell lipid extract were methylated using boron trifluoride, and the fatty acids were separated using gas liquid chromatography. Radioactivity was determined by liquid scintillation spectrometry. Shown are the mean of two determinations (mean variation 0.84%).

[b]Refers to those fatty acids containing less than 14 carbon atoms.

[c]Not detected.

TABLE IV

Distribution of Radioactivity in Fatty Acids of Major Intracellular Lipids after Incubation of L1210 Cells with [1-^{14}C]Palmitate or [1-^{14}C]Linoleate[a]

	Percentage distribution of radioactivity													
	<14:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3 + 20:0 + 20:1	20:2	20:3	20:4	Unidentified[b]	22:4	22:5	22:6
[1-^{14}C]Palmitate															
Total phosphoglycerides	n.d.	n.d.	78.4	0.4	17.9	3.0	0.2	0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Choline phosphoglycerides	n.d.	n.d.	84.6	n.d.	13.2	2.2	n.d.	0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ethanolamine phosphoglycerides	n.d.	0.3	59.1	0.1	31.3	5.2	1.1	0.7	0.1	0.4	0.8	0.2	0.3	0.2	0.3
Triglycerides	n.d.	n.d.	89.0	n.d.	9.1	1.8	n.d.	0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
[1-^{14}C]Linoleate															
Total phosphoglycerides	n.d.	n.d.	0.5	n.d.	1.2	n.d.	91.4	n.d.	3.5	1.4	1.1	0.3	0.2	0.1	n.d.
Choline phosphoglycerides	n.d.	n.d.	0.7	n.d.	0.5	n.d.	92.2	0.4	2.4	1.4	1.2	0.9	0.5	0.2	0.1
Ethanolamine phosphoglycerides	n.d.	0.2	0.6	0.1	2.7	0.2	77.4	n.d.	8.5	2.8	2.9	1.8	1.0	1.7	0.5
Triglycerides	n.d.	n.d.	0.6	n.d.	0.2	n.d.	90.2	n.d.	4.9	1.4	1.2	1.2	n.d.	0.2	0.1

[a]L1210 cells were incubated with [1-^{14}C]palmitate or [1-^{14}C]linoleate as described in Table II. The incubations were terminated and the lipids extracted. The major lipid fractions were separated using thin layer chromatography. Each fraction was then saponified, methylated, and the fatty acids separated using gas liquid chromatography. Radioactivity was determined by liquid scintillation spectrometry. Shown are the mean of two or three determinations (mean variation 0.48%).

[b]An unidentified fatty acid chromatographing between 20:4 and 22:4.

Others have demonstrated fatty acid incorporation by transformed and neoplastic cells (12-14). It has also been shown that there is limited metabolism of the acids incorporated into the complex lipids of the cells (12,14,15). The present studies are important because they provide more quantitative data on the extent of modification of a number of fatty acids. Moreover, such information on the fatty acid metabolism of this particular cell is pertinent because of the relevance of the L1210 murine leukemia to human neoplasms. It is one of the main tumor models utilized in the preclinical screening of drugs for activity in human tumors. Most of the antileukemic drugs which are active against the L1210 model are also active against human neoplasms while many of the drugs with little clinical usefulness are inactive in this system. Therefore, the L1210 leukemia is a particularly important system for studies of lipid metabolism in the neoplastic state.

Furthermore, of particular importance are the observations of differences in modification of fatty acids destined to be esterified to various cellular lipid esters. More of the fatty acid that was subsequently incorporated into certain phospholipids was modified as compared with that incorporated into triglycerides. This might be expected since the triglycerides are predominantly present in cytoplasmic inclusions or droplets when they accumulate in cultured cells (16,17). Since they are not major components of membrane structures. changes in their fatty acyl composition may have little significance. By contrast, changes in the fatty acyl composition of phospholipids could affect membrane fluidity and, hence, important cell function (18-20). Therefore, the fatty acyl composition of the cell phospholipids may have to be more closely regulated, and more structural modification may be required if phospholipid synthesis is to continue when there is only a limited supply of fatty acid provided to the cell.

The fatty acids incorporated into ethanolamine phosphoglycerides were altered to a greater extent than those incorporated into choline phosphoglycerides. In the human erythrocyte, there is asymmetrical distribution of phospholipids within the two sides of the plasma membrane (21). Ethanolamine and serine phosphoglycerides are located preferentially on the inner lamella and choline phosphoglyceride and sphingomyelin are located on the outer lamella. It is not known whether such asymmetry exists in the L1210 plasma membrane. If it does, these locations of the phospholipids may result in differing access to the desaturases and enzymes of elongation and thereby account for the greater modification of the fatty acid incorporated into the ethanolamine phosphoglyceride fraction.

The radioactive fatty acid substrates contained small amounts of impurities. It is unlikely, however, that the modification of fatty acids in cell lipids resulted from uptake of these impurities rather than metabolic alteration. For example, the total uptake of radioactivity from incubations with [1-^{14}C] palmitate was 396 nmol/hr per 10^8 cells (Table I). Of this, 10.4% (Table III) or 41 nmol/hr per 10^8 cells was recovered in stearate. Of the 3600 nmol of labeled fatty acid initially present in the incubation medium, 0.8% or 29 nmol was in the form of stearate. Therefore, it is impossible to account for the recovery of radioactive stearate in cell lipids by uptake of the stearate impurity in the medium. Furthermore, the studies with stearate itself showed that only 9.1% was taken up. This makes it further unlikely that the impurities in the labeled palmitate account for the accumulation of the various metabolic products in the cells. When a similar analysis is made for the other major desaturation and elongation products, the accumulation of radioactivity in the fatty acid metabolites was greater than what would be anticipated from the content of the impurities in the medium. Therefore, it appears to us that many of the metabolites in which radioactivity was found were primarily due to metabolic conversion, but it is possible that some proportion of the labeled fatty acid products could have come from impurities in the medium.

The L1210 cell, like many other cancer cells (9,11,15,22), can elongate most of the physiologically important fatty acids. These cells also have some ability to desaturate as evidenced by the conversion of 18:0 to 18:1. The exact position of the double bond was not proven. However, if this labeled product is oleic acid as it seems likely, then the cells contain Δ 9-desaturase activity. The observation that only 0.6% of the radioactivity from [1-^{14}C]linoleate is recovered in 20:4 suggests limited activity of the Δ 6-desaturase. This compares to 21-36% recovered in 20:4 when cells containing Δ 6-desaturase were studied (11). Furthermore, there was no conversion of 18:1 to 18:2 or 18:2 to 18:3 although this lack of activity can be seen under certain conditions in cells containing the enzyme. Therefore, we conclude that the Δ 6-desaturase is not active or only minimally so. A deletion of Δ 6-desaturase has

been reported in hepatoma cells (23), Ehrlich ascites cells (15,24), HeLa cells (14), and three transformed cultured cell lines (11). By contrast, normal diploid cells in culture retain Δ 6-desaturase activity (11). This may represent a potentially exploitable metabolic difference between normal tissues and many types of tumors.

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Effect of Phthalate Esters on Serum Cholesterol and Lipid Biosynthesis in Liver, Testes, and Epididymal Fat in the Rat and Rabbit

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ABSTRACT

Lipid biosynthesis was studied in vitro in liver, testes, and epididymal fat obtained from rats and rabbits fed di-(2-ethylhexyl)phthalate for 4 weeks at levels of 0.5% and 1.0%, respectively. Several differences in response of the two species to DEHP feeding were observed. In rats, but not in rabbits, DEHP feeding significantly reduced the incorporation of labeled mevalonic acid into total sterols ($p < 0.02$), digitonin-precipitable sterols ($p < 0.01$), and squalene ($p < 0.05$). Inhibition of hepatic sterologenesis previously observed with DEHP feeding in the rat was also observed in the rabbit. In liver minces from the DEHP-fed rabbits, incorporation of ^3H-mevalonic acid into C_{27} sterols (cholesterol) and C_{30}sterols (lanosterol) was significantly reduced by about 40% ($p < 0.05$ and $p < 0.01$, respectively), whereas the incorporation of ^{14}C-glycerol 3-phosphate into phospholipids, and the combined fraction of monoglyceride + diglyceride, was significantly increased ($p < 0.001$ and $p < 0.01$, respectively). In studies with epididymal fat, DEHP feeding did not affect the total incorporation of ^{14}C-acetate or ^3H-mevalonate into total saponifiable and nonsaponifiable lipids of either the rat or rabbit. However, in the rat, significantly less of the ^{14}C-acetate ($p < 0.02$) and ^3H-mevalonate ($p < 0.01$) that was incorporated appeared in the combined fraction of cholesteryl ester + squalene. In addition, DEHP feeding significantly reduced serum cholesterol ($p < 0.01$) in the rat but not in the rabbit. The results of this study indicate that DEHP feeding is associated with alterations in tissue lipid metabolism and that there are species differences in the response of tissues to DEHP.

INTRODUCTION

Certain esters of phthalic acid have become widely distributed as contaminants of the environment. In fact, recent studies have shown phthalates to be present in human (1-4) and animal tissues (5-7) and to exceed the levels of DDTs (p,p′-DDT and p,p′-DDE) and polychlorinated biphenyls (PCB) in certain coastal waters and bottom sediments (8).

We have recently demonstrated that di-(2-ethylhexyl)phthalate (DEHP) has multiple effects on mammalian lipid metabolism which include inhibition of hepatic sterologenesis (9,10), inhibition of fatty acid oxidation by heart mitochondria (11), stimulation of fatty acid oxidation by hepatic mitochondria (11), and an ability to modify the pattern of circulating plasma lipoproteins (10). Dimethyl phthalate (DMP) and dibutyl phthalate (DMP) were also found to inhibit hepatic sterologenesis (10). In the present study, we examined lipid biosynthesis from radioactive acetate, mevalonate, and α-glycerol phosphate in testes, epididymal fat, and liver from rats and rabbits fed DEHP-supplemented diets. The studies indicate that the response of mammalian tissues to phthalates is variable depending upon the species.

EXPERIMENTAL PROCEDURES

Animals and Diets

Male New Zealand rabbits (2.5-3 kg) and male Sprague-Dawley rats (Upjohn Strain, 225-250 g) were used in the studies. The animals were individually housed with food and water available ad libitum. The rabbits and rats were fed for 28 days with either Purina Chow (control diet) or Purina Chow containing di-(2-ethylhexyl)phthalate (DEHP, Eastman Kodak, Rochester, NY, cat. no. 4099) at a level of 1.0% or 0.5%, respectively (12).

Tissue Preparation and Incubation

All animals were killed between 9 a.m. and 10:30 a.m. by exsanguination resulting from severence of the carotid and jugular vessels in the neck. Tissues selected for incubation were rapidly excised and rinsed in chilled 0.9% NaCl solution. Epididymal fat and liver were incubated as tissue minces (500 mg) which were prepared on a chilled watch-glass with a scalpel blade. Rat testes (right side) were decapsulated and incubated as the intact gland. Rabbit testes were decapsulated as well but incubated in sample sizes of 800 mg which were gently separated from the whole gland with the use of forceps. Sampling of the tissues for incubation was standardized to the extent that liver tissue was taken from the central portion of the large lobe, epididymal fat tissue was taken from the central portion of the epididymal fat pads, and the right testis (rat), or the central portion of the right testis (rabbits), was taken.

Incubations were performed at 37 C in 25

TABLE I

Effect of Dietary DEHP on Total Cholesterol Levels and LCAT Activity in Serum of the Rabbit and Rat[a]

Species	Diet	Serum cholesterol (mg %)	LCAT activity (% Change in free cholesterol/18 hr)[b]
Rabbit	Control	39 ± 10[c]	60 ± 8
	1.0% DEHP	34 ± 5	63 ± 6
Rat	Control	69 ± 4	28 ± 3
	0.5% DEHP	53 ± 2[d]	31 ± 2

[a]Male New Zealand rabbits (2.5-3 kg) and Male Sprague-Dawley rats (225-250 g) were fed a stock diet (Purina Chow) containing DEHP (di-2-ethylhexyl phthalate) at a level of 1.0% and 0.5%, respectively, for 28 days. Control animals received the stock diet without DEHP.

[b]LCAT (lecithin:cholesterol acyltransferase, EC 2.3.1.43) was assayed by incubating serum for 18 hr at 37 C and calculating the % loss of unesterified cholesterol from 0 time.

[c]Values are means ± SEM of six animals.

[d]Significantly different from control values ($P < 0.01$) by Student's independent t-test.

ml stoppered Erlenmeyer flasks containing 3.5-5.0 ml of Krebs-Ringer-bicarbonate buffer, pH 7.4 containing the various isotopic precursors. The isotopically labeled lipid precursors were all obtained from New England Nuclear Corp., Boston, MA, and consisted of DL-mevalonic-2-^{14}C acid, dibenzylethylenediamine salt (cat. no. NEC-166, 40.1 mC/mM), DL-mevalonic-5-^{3}H(N) acid, dibenzylethylenediamine salt (cat. no. NET-095, 5C/mM), sodium acetate-1-^{14}C (cat. no. NEC-084H, 59 mC/mM), and L-^{14}C-(U)-glycerol 3-phosphate as the disodium salt (cat. no. NEC-608, 117 mC/mM).

Lecithin:cholesterol acyltransferase (LCAT, EC 2.3.1.43) activity was measured in the serum of the rats and rabbits as previously described (13).

ANALYSES

Tissue Extraction

Following tissue incubation, tissue lipids were either extracted with chloroform-methanol as previously described (12) or obtained after digestion of the tissue with alcoholic-KOH and saponification of the lipid (9). All chloroform-methanol extracts were washed by the method of Folch et al. (14). Digestion/saponification of samples consisted of adding KOH and ethyl alcohol to the incubation mixtures to give a final concentration of 11% and 82%, respectively, and then heating the samples for 2 hr at 65 C (9). The nonsaponifiable lipid fractions (sterols and squalene) were recovered by extraction with n-hexane as previously described (9). Following extraction with n-hexane, the samples were acidified with conc. HCl to ca. pH 4 and the saponifiable lipid fraction extracted with 3 x 20 ml of diethyl ether. The hexane and ether extracts were washed with 3 x 10 ml of water and then reduced in volume under N_2 for subsequent fractionation by thin layer chromatography (TLC) and/or radioactive assay (9,12).

Thin Layer Chromatography

All fractionation of lipid extracts was performed on Silica Gel G-coated thin layer plates in a solvent system consisting of n-hexane-diethyl ether-acetic acid (146:50:4, v/v/v) (9,12). This system was used to fractionate the total lipid extracts, obtained by $CHCl_3$:MeOH extraction, into phospholipids, mono- plus diglycerides, triglycerides, and steryl esters plus squalene (12), and to fractionate nonsaponifiable lipid extracts into sterols and squalene (9). In some experiments, C_{27} and C_{30} sterols (Rf 0.12 and Rf 0.16, respectively) were isolated individually, whereas in other experiments, the zone representing Rf 0.12 and 0.16, inclusive, was taken as total sterol. Lipid bands on the thin layer plates were visualized by spraying with rhodamine 6G (0.05% in ethanol) (9). The lipid bands were scraped from the plates into vials and assayed for radioactivity by liquid scintillation counting as described previously (10).

Digitonin-precipitable sterols (those possessing a 3β-OH group) were obtained by the method of Sperry and Webb (15) and the digitonides dissolved in ca. 0.2 ml of pyridine in preparation for liquid scintillation counting (16).

Serum and tissue cholesterol were assayed by the o-phthalaldehyde method of Rudel and Morris (17).

Statistical analyses were performed using Student's *t*-test for comparing unpaired

TABLE II

Effect of Dietary DEHP on Incorporation of Labeled Mevalonic Acid into Sterols and Squalene of Rat and Rabbit Testes in vitro[a] (dpm/g wet wt)

	Rat		Rabbit	
	Control (n=6)	DEHP (n=6)	Control (n=7)	DEHP (n=7)
Total sterols	2326 ± 147	1753 ± 117[b,c]	15494 ± 925	14925 ± 2738[b]
Squalene	2124 ± 91	1488 ± 129[d]	4419 ± 556	4600 ± 513
Digitoninprecipitable sterols	1381 ± 117	1009 ± 89[e]	5512 ± 325	6646 ± 392

[a]Male Sprague-Dawley rats (225-250 g) and male New Zealand rabbits (2.5-3 kg) were fed a stock diet (Purina Chow) containing 0.5% or 1.0% DEHP (di-2-ethylhexyl phthalate), respectively, for 28 days. Testicular tissue was incubated for 3 hr at 37 C with either 2.5 μCi DL-mevalonic acid-2-^{14}C (rat) or 1.5 μCi DL-mevalonic acid-5-^{3}H (rabbit) in 5.0 ml and 3.5 ml of Krebs-Ringer-bicarbonate buffer (pH 7.4), respectively, as described under Procedures. Total sterols, squalene, and digitonin-precipitable sterols were isolated from the nonsaponifiable fraction of the tissue lipids and assayed for radioactivity, as described under Procedures.

[b]Values are the means ± SEM of the number of animals per group (given above as n).

[c,d,e]Significantly different from control values by Student's independent *t*-test (c, $P<0.02$; d, $P<0.01$; e, $P<0.05$).

samples. Significance was given by values of $p \leqslant 0.05$.

RESULTS AND DISCUSSION

In the studies presented here, rabbits were fed DEHP at a level of 1.0% in the diet whereas rats were fed DEHP at a level of 0.5% in the diet. The lower level (0.5%) of DEHP was administered to the rats because our previous studies (9,11,12) indicated that the metabolic response of rats to 1.0% DEHP was similar to the response observed at the 0.5% level of DEHP in the diet.

Serum Cholesterol and LCAT Activity

Serum total cholesterol and serum LCAT activity were measured in the rabbits and rats fed DEHP for 28 days (Table I). In rats, but not in rabbits, there was a significant ($p <0.01$) reduction in serum cholesterol which amounted to ca. 16 mg/dl. A hypocholesterolemic effect of similar magnitude was observed in our previous studies with rats in which 1% DEHP was fed (10). To our knowledge, the only other species demonstrating a hypocholesterolemic response to DEHP is the mouse (18). The failure of DEHP to reduce serum cholesterol levels in the rabbit suggests a species difference in response to DEHP particularly since the rabbits in this study received DEHP at twice the dietary level of the rats (Table I).

The activity of LCAT was also studied in vitro in sera from the rats and rabbits (Table I) because a postulated role for serum LCAT is to facilitate the removal of cholesterol from tissue compartments (19); it was reasoned that the hypocholesterolemic effect of DEHP in the rat could be secondary to an inhibitory effect on LCAT. Although the percentage of serum unesterified cholesterol that was esterified by LCAT was greater in rabbit serum than in rat serum (60% vs. 30%, respectively, in 18 hr), we did not observe any change in LCAT activity within species as a result of DEHP feeding.

In Vitro Lipid Biosynthesis

Testes: The effect of DEHP feeding on the incorporation of radioactive mevalonate into rat and rabbit testicular lipids in vitro is shown in Table II.

In rat testes, the incorporation of ^{14}C-mevalonate into the sterol percursor, squalene, was significantly reduced by ca. 30% ($p <0.01$) with DEHP feeding. The reduced incorporation of label into squalene was paralleled by significant reductions in incorporation into total sterols (25%, $p <0.02$) and digitonin-precipitable sterols (27%, $p <0.05$). In contrast to the observations in rat testes, DEHP feeding had no significant effect on the incorporation of labeled mevalonate into total sterols, digitonin-precipitable sterols, or squalene in rabbit testes (Table II).

Liver: The effect of DEHP feeding on the incorporation of radioactive mevalonate into sterols and squalene in rabbit liver minces is shown in Table III. Incorporation of ^{3}H-mevalonate into C_{27} and C_{30} sterols was significantly reduced ($p <0.05$ and $p <0.01$, respectively) by about 60% and was a reflection of the tendency for incorporation of ^{3}H-mevalon-

TABLE III

Effect of Dietary DEHP on Incorporation of DL-mevalonic-5-^{3}H Acid into Sterols and Squalene by Rabbit Liver Minces in vitro[a]

Diet	Hepatic cholesterol (mg/g tissue wet wt)	Isotope incorporation (dpm/g wet wt)		
		C_{27} Sterol	C_{30} Sterol	Squalene
Control	3.2 ± 0.2[b] ±	28890 ±6680	9116 ±2609	33079 ±11361
1.0% DEHP	3.3 ± 0.4	12138[c] ±2814	3945[d] ±654	13674 ±5489

[a]Male New Zealand rabbits (2.5-3 kg) were fed a stock diet (Purina Chow pellets) containing 1.0% di-2-ethylhexyl phthalate (DEHP) for 28 days. Liver minces (500 mg) were prepared as described under Procedures and incubated 3 hr at 37 C with 0.25 μCi DL-mevalonic-5-^{3}H acid in a total volume of 3.5 ml of Krebs-Ringer-bicarbonate buffer, pH 7.4. The incubation mixtures were saponified and the non-saponifiable lipids extracted with n-hexane and then fractionated by thin layer chromatography as described under Procedures.

[b]Values are means ± SEM of six animals.

[c,d]Significantly different from control values by Student's independent t-test (c, $P<0.05$; d, $P<0.01$).

TABLE IV

Effect of Dietary DEHP on Incorporation of L-^{14}C-glycerol 3-phosphate into Lipids by Rabbit Liver Minces in vitro[a]

Lipid fraction	Control (dpm/g wet wt)	DEHP (dpm/g wet wt)
Phospholipid	16814 ± 1029[b]	25783 ± 1394[c]
Monoglyceride + diglyceride	10000 ± 1063	17410 ± 1979[d]
Triglyceride	15244 ± 3881	24269 ± 6230

[a]Male New Zealand rabbits (2.5-3 kg) were fed a stock diet (Purina Chow) containing 1.0% di-2-ethylhexyl phthalate (DEHP) for 28 days. Liver minces (500 mg) were prepared as described under Procedures and incubated 3 hr at 37 C with 0.25 μCi L-[^{14}C (U)]-glycerol 3-phosphate in a total volume of 3.5 ml Krebs-Ringer-bicarbonate buffer, pH 7.4. The incubation mixtures were extracted with chloroform-methanol (2:1, v/v) and the lipids fractionated by thin layer chromatography as described under Procedures.

[b]Values are means ± SEM of six animals.

[c,d] Significantly different from control values by Student's independent t-test (c, ($P<0.001$; d, $P<0.01$).

ate into squalene to be lower in the DEHP group; differences in incorporation into squalene in the two groups were not statistically significant in view of the considerable variability in the control group.

The inhibitory effect of DEHP on rabbit hepatic sterologenesis observed in these studies parallels our observations in rats fed 0.5% or 1.0% in the diet and, as in the rat (9,10), the inhibitory effect of DEHP on hepatic sterologenesis is not reflected in changes in hepatic sterol content (Table III).

The inhibitory effect of DEHP feeding on hepatic sterologenesis in the rabbit was not reflected in the synthesis of the glycerol-lipids. In fact, the incorporation of L-^{14}C-glycerol phosphate into phospholipids and the combined fraction of mono- plus diglycerides was significantly increased ($p<0.001$ and $p<0.01$, respectively) in liver minces from rabbits fed DEHP (Table IV). Furthermore, there was an apparent increase in the incorporation of labeled glycerol phosphate into the triglyceride fraction in the DEHP-fed group (Table IV). The increase, however, was not statistically significant; failure of the increase to reach statistical levels of significance may have been due in part to the large degree of variability in the data. Other possible explanations are that endogenous cofactors were becoming rate-limiting as the end of the 3 hr incubation period was approached or that there are multiple pools of intracellular diglyceride which distort a precursor-product relationship between diglyceride and triglyceride because the diglyceride pools have differing turnover times (20).

TABLE V

Effect of Dietary DEHP on Incorporation (dpm/g wet wt) of Acetate-1-^{14}C and DL-mevalonic-5-^{3}H Acid into Lipids of Rat and Rabbit Epididymal Fat Tissue in vitro[a]

Species	Diet	^{14}C-Acetate			^{3}H-Mevalonate	
		NSF	SF	CE+SQ	NSF	CE+SQ
Rat	Control (7)[b]	15603[c] ±3833	832005 ±180090	1923 ±209	4873 ±549	1362 ±106
	DEHP (6)	12956 ±6126	677340 ±159345	1205[d] ±114	4028 ±516	807[e] ±78
Rabbit	Control (7)	16410 ±3696	224940 ±94260	3060 ±820	3925 ±718	2170 ±550
	DEHP (6)	24664 ±6491	388320 ±138180	4180 ±940	7316 ±2896	2870 ±690

[a]Male New Zealand rabbits (2.5-3 kg) and male Sprague-Dawley rats (225-250 g) were fed a stock diet (Purina Chow) *containing DEHP (di-2-ethylhexyl phthalate)* at a level of 1.0% and 0.5%, respectively, for 28 days. Control animals received the stock diet without DEHP. Samples of epididymal fat pad (500 mg) were incubated as tissue minces for 3 hr at 37 C in 3.5 ml of Krebs-Ringer-bicarbonate buffer, pH 7.4, containing 2.5 μCi acetate-1-^{14}C and 0.5 μCi DL-mevalonic-5-^{3}H acid. Tissue lipids were extracted with $CHCl_3$:MeOH. A portion of the lipid extract was saponified and separated into the saponifiable lipid fraction (SF) and the nonsaponifiable lipid fraction (NSF). Another portion of the lipid extract was fractionated by thin layer chromatography to achieve a separation of the phospholipids and various neutral lipids; of the lipids examined, only the combined fraction of cholesteryl esters and squalene (CE+SQ) differed significantly between control and DEHP-fed animals.

[b]Numbers in parentheses indicate the number of animals in each experiment.

[c]Values are the mean of the number of animals ± SEM.

[d,e]Significantly different from control values by Student's independent t-test (d, $P<0.02$; e, $P<0.01$).

Epididymal fat: The effects of DEHP on lipid metabolism in liver and testes (Tables II-IV) prompted our investigation of lipid biosynthesis in epididymal fat pads in the rats and rabbits; this seemed particularly pertinent in view of the reports that DEHP can accumulate in fatty tissue (1,3,21) and that ^{14}C-DEHP clearance from adipose tissue of the rat is delayed relative to other tissues (22,23).

Table V presents data on the incorporation of ^{14}C-acetate and ^{3}H-mevalonate in vitro into the lipid of epididymal fat tissue from rats and rabbits fed dietary DEHP.

Within species, DEHP feeding did not significantly affect the total incorporation of labeled acetate or mevalonate into either the saponifiable lipid fraction (SF) or the nonsaponifiable lipid fraction (NSF) of the epididymal fat. However, fractionation of portions of the tissue lipid extracts (without saponification) into the various lipid classes revealed that there was a significant reduction of incorporation of ^{14}C-acetate (37%, $p<0.02$) and ^{3}H-mevalonate (40%, $p<0.01$) into the combined fraction of cholesteryl esters and squalene (CE+SQ) in tissue from the DEHP-fed rats. In contrast, DEHP feeding did not affect incorporation of ^{14}C-acetate or ^{3}H-mevalonate into the cholesteryl ester + squalene fraction from the rabbit tissue. These results may reflect a subtle species difference in the response of epididymal fat to DEHP ingestion.

The present studies represent an extension of our previous studies on the effects of ingested DEHP on mammalian lipid metabolism.

Our previous studies provided evidence that DEHP feeding to rats results in an inhibition of hepatic sterologenesis (9,10). In view of the present data, this effect is neither species specific nor tissue specific since inhibition of sterol synthesis was also observed in the liver of DEHP-fed rabbits (Table III) and in the testes of DEHP-fed rats (Table II). Failure to demonstrate inhibition of sterologenesis in testes of DEHP-fed rabbits, however, indicates species differences in tissue susceptibility to biochemical changes resulting from phthalate feeding (Table II). Further examples of species-related differences in tissue susceptibility to DEHP feeding were observed in studies of lipid synthesis in epididymal fat from rats and rabbits (Table V) in which the total incorporation of ^{14}C-acetate and ^{3}H-mevalonate into the cholesteryl ester + squalene fraction was unchanged in DEHP-fed rabbits but significantly reduced (37-40%) in DEHP-fed rats; the change represented a redistribution of the labeled precursors that were incorporated rather than a net change in total isotope incorporation (Table V).

The rabbit differs from the rat in other responses to dietary DEHP as well. In the present study, we observed DEHP to significantly reduce ($p<0.01$) serum cholesterol levels in the rat but not in the rabbit (Table I). Furthermore, our

previous stduies indicated that DEHP-feeding has an inhibitory effect on fatty acid oxidation by rat heart mitochondria, in vitro, but not by rabbit heart mitochondria (11).

At the present time, we are unable to offer information on the mechanism by which ingested DEHP acts to produce the changes in lipid metabolism that we have observed in these studies or in our other work (9-12). It is conceivable that a metabolite of DEHP, rather than DEHP, is the causative agent. Evidence is emerging to support the view that the monoester of DEHP (MEHP) can account for some of the changes in enzyme activity and ultrastructure in tissues such as the rat liver (24-26).

The data presented here, and in previous communications (9-12,18) indicate that phthalate esters are indeed capable of modifying lipid metabolism in mammalian species such as the rat, mouse, rabbit, and pig. To our knowledge, there have been no similar biochemical studies undertaken in subhuman primates or in tissues from man. While it may be that the levels of phthalate to which man is exposed do not present a health problem, we cannot make that assumption. Unfortunately, we are unable to relate the levels of exposure to animals that we employ to those that may be encountered by man. It seems clear, however, that the widespread use of phthalates and their dissemination into the environment dictates the need for a responsible assessment of the biological effects of phthalates.

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Composition of Lipids of Bovine Optic Nerve[1]

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ABSTRACT

Lipids from bovine optic nerve were analyzed. The total content of 16.5% by weight included 27.2% nonpolar lipids, 26.1% glycolipids, and 46.7% phospholipids by weight. Free cholesterol was the major component of the nonpolar lipid fraction. The cerebrosides, 73.5% of total glycolipids, were separated by thin layer chromatography (TLC) into two bands (upper and lower) that were present in equal proportion. Cerebroside sulfates comprised about 27.5% of total glycolipids. Gangliosides were also detected in the glycolipid fraction. In order of predominance, choline glycerophospholipids, ethanolamine glycerophospholipids, ethanolamine plasmalogens, serine glycerophospholipids, sphingomyelins, and inositol glycerophospholipids were the major phospholipids. Palmitoyl (16:0), stearoyl (18:0), and oleoyl (18:1) groups were the major acyl groups in all neutral and phospholipid classes. However, ethanolamine glycerophospholipids, serine glycerophospholipids, and inositol glycerophospholipids contained a large percentage of 22:6 (docosahexaenoyl) group. The major alk-1-enyl groups of the plasmalogens were 16:0, 18:0, and 18:1. Steroyl (18:0), lignoceroyl (24:0), and nervonoyl (24:1) were the major acyl groups in all sphingolipids. Lower cerebroside band and cerebroside sulfates contained large amount of hydroxylignoceroyl (cerebronoyl) and hydroxynervonoyl groups.

INTRODUCTION

Lipids are the major constituents of optic nerve myelin (1-5). Optic nerve is an integral part of the central nervous system, and many studies have dealt with the lipid composition of other tissues associated with central nervous system (6-13) because lipids could be involved in the functional activity of these tissues. However, the detailed composition of the optic nerve lipids has not been studied. The present study reports the first comprehensive composition study of optic nerve lipids.

MATERIALS AND METHODS

Tissues

Thirty bovine eyes obtained from 3- to 4-year-old male animals at slaughter were transported on ice to the laboratory. The optic nerves were removed and processed immediately.

Lipid Extraction

Optic nerves, ca. 250-270 mg per eye, were pooled from 30 eyes to yield 5 specimens. The wet tissue was homogenized in a glass tissue grinder in cold methanol and extracted 3 times with 10 vol. chloroform-methanol (2:1, by vol) by the method of Folch et al. (14). The total lipid extract was freed from nonlipid contaminants by Sephadex column chromatography (15). A known aliquot of eluate from the sephadex column was evaporated to dryness in a tared flask and the weight of the lipid residue was determined.

Lipid Analysis

Lipids were separated into three major fractions on a silicic acid column by the successive use of chloroform, acetone, and methanol as the eluting solvents (16). The identification of various lipids in each fraction was done by the application of one- and two-dimensional thin layer chromatography (TLC) using appropriate solvent systems (17-20) and spray reagents (21-24). The chloroform fraction was mostly composed of neutral lipids, the acetone fraction mostly was composed of cerebrosides with traces of gangliosides and cerebroside sulfates, while the methanol fraction was mostly composed of phosphopholipids (including diacyl and alk-1-enyl acyl types) and cerebroside sulfates. The quantity of total nonpolar lipids was determined by evaporation of an aliquot of the chloroform eluate in a tared container. A preparative silica gel thin layer chromatograph was done with the chloroform and methanol fractions in order to isolate individual nonpolar and phospholipid classes, respectively (17,18). The quantity of free cholesterol and total cholesterol was determined in the total chloroform eluate by adopting the procedure of Courchaine et al. (25). Triglycerides and free fatty acids isolated from TLC plates were assayed by the methods of Van Handel and Zilversmit (26) and Novak (27), respectively.

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TABLE I

Composition of Bovine Optic Nerve Lipids

Total lipids	16.50 ± 0.45[a]
Total nonpolar lipids	4.48 ± 0.12
Total phospholipids	7.72 ± 0.63
Plasmalogens (acid-labile phospholipids)	2.50 ± 0.05
Total glycolipids	4.30 ± 0.16

[a]Mean g/100 g of wet tissue ± SD of five analyses of six samples each.

TABLE II

Percent Composition of Neutral Lipids

Parameters	% Composition
Cholesteryl esters	Trace
Cholesterol (free)	86.1 ± 4.3[a]
Triglycerides	7.9 ± 0.9
Free fatty acids	6.0 ± 0.4

[a]Mean ± SD of five analyses of six samples each.

TABLE III

Percent Composition of Glycolipids

Parameters	% Composition
Upper cerebroside band	36.8 ± 3.3[a]
Lower cerebroside band	36.7 ± 3.2
Cerebroside sulfate	26.5 ± 2.9
Ceramides	Trace
Gangliosides	Trace
Others (unidentified)	Trace

[a]Mean ± SD of five analyses of six samples each.

TABLE IV

Percent Composition of Phospholipids

Parameters	% Composition
Choline glycerophospholipid	40.3 ± 2.5[a]
Inositol glycerophospholipid	2.2 ± 0.3
Sphingomyelin	4.8 ± 0.7
Ethanolamine glycerophospholipid (total)	39.8 ± 2.0
Ethanolamine plasmalogens	29.4 ± 1.7
Serine glycerophospholipid	12.9 ± 1.2
Lysocholine glycerophospholipid	Trace

[a]Mean ± SD of five analyses of six samples each.

The quantity of total cerebrosides in the acetone fraction was determined by the method of Dubois et al. (28). The quantity of total cerebroside sulfate was determined by measuring the sulfur content in an aliquot of both acetone and methanol eluates (29). The quantity of total phospholipids in the methanol eluate and individual phospholipids isolated from the TLC plates was determined by measuring the amount of phosphorus according to the procedure of Bartlett (30). The alk-l-enyl group content of the methanol eluate was measured by the method of Gottfried and Rapport (31). The quantity of total glycolipids was obtained by measuring the weight of the residue obtained after evapoaration of an aliquot of the acetone solution in a tared container and adding to it the value of cerebroside sulfate eluated in the methanol fraction. The relative distribution of the two cerebroside bands as separated by TLC from the acetone eluate was determined by adopting the charring transmission densitometric method of Rouser et al. (21). A two-dimensional TLC method (1) with cleavage of phosphoglyceride alk-l-enyl groups by HCl to aldehyde was used for the quantitative determination of the ethanolamine plasmalogen. The aldehyde released from the acid-labile ethanolamine plasmalogens was isolated from the TLC plates and quantitated by the phenylhydrazine method of Ferrel et al. (32).

Preparative silica gel TLC (33), using the solvent systems of Mangold and Malins (17), Parker and Peterson (18), and Berra and Zambotti (13), was done to isolate individual nonpolar lipids, phospholipids, and glycolipids, respectively, for the purpose of estimating their acyl group composition. The acyl group composition of individual nonpolar lipids was determined by gas liquid chromatography (GLC) of their fatty acid esters (34) according to the conditions described earlier (35). For the determination of acyl group composition of individual phospholipids by GLC, the phospholipids were subjected to acidic methanolysis, and the fatty acid methyl esters were separated from the dimethyl acetals by preparative TLC (36). For the determination of the alk-1-enyl group composition of ethanolamine plasmalogens, the two-dimensional TLC method (1) was used. The aldehydes released from the acid-labile ethanolamine phosphoglyceride by HCl fumes were isolated from the TLC plate and converted to cyclic acetal derivative for GLC analysis according to the conditions of Sun and Horrocks (36). For the determination of acyl grup compositions of sphingolipids, individual lipid residues, containing ca. 0.4-0.5 μmols of fatty acids, were subjected to methanolysis with 1 ml of 0.75N methanol-HCl (37). The tube was sealed and heated at 80 C for 24 hr. After cooling, the tube was opened, and the fatty acid methyl esters were extracted with heptane (38). Normal fatty acid esters were separated on a Packard Model 7400 gas chromatograph as described earlier (35). For the identification of the presence of hydroxy

TABLE V

Acyl Group Composition of Nonpolar and Phospholipids

	14:0[a]	16:0	16:1	18:0	18:1	20:0	20:1	20:4	22:4	22:5	22:6
Triglycerides	3.9 ± 0.2[b]	42.7 ± 1.5	3.2 ± 0.2	22.9 ± 0.5	23.7 ± 1.2	0.8 ± 0.0	2.8 ± 0.1	Tr	Tr	Tr	Tr
Free fatty acids	3.5 ± 0.1	32.3 ± 1.6	3.6 ± 0.1	14.8 ± 0.6	41.6 ± 1.8	3.2 ± 0.0	1.0 ± 0.0	Tr	Tr	Tr	Tr
Phosphatidyl choline	2.1 ± 0.1	34.4 ± 1.4	3.7 ± 0.0	12.6 ± 0.4	33.5 ± 1.6	1.1 ± 0.0	1.5 ± 0.0	3.7 ± 0.0	3.5 ± 0.0	2.1 ± 0.0	1.8 ± 0.2
Phosphatidyl inositol	0.2 ± 0.0	4.4 ± 0.0	0.4 ± 0.0	31.8 ± 1.5	28.8 ± 1.2	1.3 ± 0.0	1.8 ± 0.1	11.9 ± 0.6	2.9 ± 0.1	0.9 ± 0.0	15.6 ± 1.2
Phosphatidyl ethanolamine	0.9 ± 0.0	8.9 ± 0.1	0.7 ± 0.0	23.9 ± 0.9	22.5 ± 1.1	0.4 ± 0.0	1.6 ± 0.0	12.9 ± 0.3	3.2 ± 0.2	2.1 ± 0.2	22.9 ± 1.3
Phosphatidyl serine	0.8 ± 0.0	4.6 ± 0.1	1.0 ± 0.0	35.8 ± 1.7	25.2 ± 1.8	0.3 ± 0.0	1.3 ± 0.0	4.9 ± 0.0	2.8 ± 0.0	0.5 ± 0.0	22.8 ± 1.6

[a]No. of carbon:no. of double bonds.

[b]Each value is the mean (weight % of total fatty acids) ± SD of five analyses of six samples each.

TABLE VI

Alk-l-enyl Group Composition of Ethanolamine Plasmalogens

Alk-l-enyl Group	Weight percentage
14:0[a]	1.1 ± 0.0[b]
16:0	24.1 ± 1.1
16:1	1.2 ± 0.0
18:0	27.2 ± 1.2
18:1	44.6 ± 2.1
18:2	Trace
20:0	1.0 ± 0.0
20:4	Trace

[a]No. of carbon:no. of double bonds.

[b]Mean ± SD of five analyses of six samples each.

fatty acids, if any, the total fatty acid methyl ester solution was treated with TMS_i reagents (pyridine-hexamethyldisilazane-trimethylchlorosilane, 9:3:1) (39) and analyzed on a 3% SE-30 column with a temperature program of 10 C/min from 180-250 C and at a nitrogen flow rate of 30 ml/min. When necessary, fractions isoalted by GLC were identified after hydrogenation (40) and rechromatography of the saturated derivatives.

RESULTS

The data on the amount of total lipids, total nonpolar lipids, total phospholipids, total plasmalogens, and total glycolipids are given in Table I. The total content included 27.2% nonpolar lipids, 26.1% glycolipids, and 46.7% phospholipids by weight. Plasmalogens constituted about 32.4% of total phospholipids.

The data on the composition of nonpolar lipids are given in Table II. Free cholesterol was the major component of nonpolar lipids, while triglycerides and free fatty acids were the minor components. Cholesteryl ester was present only in trace amount.

The composition of glycolipids is shown in Table III. Cerebrosides and cerebroside sulfates were the major glycolipids. The cerebrosides, 73.5% of total glycolipids, were separated by TLC into two bands (upper and lower) that were present in equal proportion. Ceramides, gangliosides, and other unidentified glycolipids were present in trace amounts.

The data on the composition of phospholipids are given in Table IV. In order of predominance, choline glycerophospholipids, ethanolamine glycerophospholipids, serine glycerophospholipids, sphingomyelins, and inositol glycerophospholipids were the major phospholipids. Lysocholine glycerophospholipids were present only in trace amount. Ethanolamine plasmalogens constituted 90.7%

TABLE VII

Acyl Group Composition of Sphingolipids

	Sphingomyelin	Cerebrosides		Cerebroside sulfate
		Upper band	Lower band	
14:0[a]	0.2 ± 0.0[b]	0.5 ± 0.0	0.4 ± 0.0	1.1 ± 0.0
16:0	4.9 ± 0.1	2.3 ± 0.0	2.6 ± 0.0	1.9 ± 0.0
16:1	0.8 ± 0.1	Trace	Trace	0.4 ± 0.0
18:0	23.9 ± 1.2	12.5 ± 0.6	12.1 ± 0.4	14.1 ± 0.7
18:1	5.8 ± 0.3	0.7 ± 0.0	0.8 ± 0.0	0.9 ± 0.0
18:2	Trace	Trace	Trace	Trace
20:0	1.7 ± 0.0	0.8 ± 0.0	0.2 ± 0.0	1.8 ± 0.1
20:4	Trace	Trace	Trace	Trace
22:0	4.1 ± 0.1	5.6 ± 0.1	5.8 ± 0.1	4.8 ± 0.0
22:1	0.9 ± 0.0	1.1 ± 0.0	0.7 ± 0.0	0.5 ± 0.0
23:0	---	3.8 ± 0.1	1.9 ± 0.1	1.1 ± 0.0
23:1	---	0.6 ± 0.0	0.4 ± 0.0	1.8 ± 0.0
23h:0[c]	---	Trace	2.1 ± 0.0	1.8 ± 0.1
23h:1	---	Trace	0.6 ± 0.0	1.6 ± 0.0
24:0	28.6 ± 1.3	30.3 ± 2.1	14.4 ± 0.7	18.3 ± 0.8
24:1	28.5 ± 1.2	31.4 ± 1.9	16.8 ± 0.5	20.2 ± 1.2
24h:0	---	Trace	15.2 ± 0.5	8.2 ± 0.7
24h:1	---	Trace	16.3 ± 0.5	9.1 ± 0.4
25:0	---	2.2 ± 0.0	0.9 ± 0.0	1.8 ± 0.0
25:1	---	1.9 ± 0.0	0.8 ± 0.0	0.9 ± 0.1
25h:0	---	Trace	1.1 ± 0.0	1.2 ± 0.0
25h:1	---	Trace	0.7 ± 0.0	1.3 ± 0.0
26:0	0.6 ± 0.0	2.8 ± 0.0	1.4 ± 0.0	1.6 ± 0.1
26:1	---	3.5 ± 0.1	2.1 ± 0.0	1.7 ± 0.0
26h:0	---	Trace	1.5 ± 0.0	1.8 ± 0.0
26h:1	---	Trace	1.2 ± 0.0	2.1 ± 0.1

[a]No. of carbon:no. of double bonds.
[b]Mean percent of total acyl group ± SD of five analysis of six samples each.
[c]Hydroxy.

of total plasmalogens, and 73.9% of the total ethanolamine glycerophospholipids contained alk-l-enyl groups. The alk-l-enyl group contents of other phosphoglycerides were not determined.

The data on the acyl group composition of individual nonpolar lipids and phospholipids of bovine optic nerve are given in Table V. Palmitoyl (16:0), stearoyl (18:0), and oleoyl (18:1) were the major acyl groups in all lipid classes. Most phospholipids, except choline glycerophospholipids contained unusually high amount of polyunsaturated acyl groups such as 20:4 (eicosatetraenoyl), 22:4 (docosatetraenoyl), and 22:6 (docosahexaenoyl) groups. The data on the alk-l-enyl group composition of ethanolamine plasmalogens are given in Table IV. The major alk-l-enyl groups were 16:0, 18:0, and 18:1. The data on the acyl group composition of individual sphongolipids are given in Table VII. Stearoyl (18:0), lignoceroyl (24:0), and nervonoyl (24:1) groups were the major acyl groups in all sphingolipids.

DISCUSSION

The bovine optic nerve contains unusually high amount of lipids which are predominantly composed of cholesterol, glycolipids, and phospholipids. The minor components of optic nerve lipids are the nonpolar lipids such as triglycerides, free fatty acids, diglycerides, monoglycerides, and cholesteryl esters. The free fatty acid content is rather high, perhaps because of the lapse of time between death and extraction (41).

The major lipids of the optic nerve are of structural types and are related to high myelin content. The composition is mostly that of myelin with dilutions by other glial and anonal membranes (oligodendroglia, astroglia, neuronal perikarya). Similar types of structural lipids have been isolated from bovine optic nerve myelin (1), rat optic nerve myelin (4,5), kitten optic nerve (3), chick sciatic nerve (42), bovine white matter (10), and superior cervical ganglion (43).

The optic nerve contains a high amount of glycolipids. Major glycolipids appear to be cerebrosides and cerebroside sulfate. Similar types of glycolipids are known to be present in white matter of human brain in large amount (44). The upper band of cerebrosides contain only trace amount of hydroxy fatty acid, while

the lower band of cerebrosides contain significant amount of hydroxylignoceroyl and hydroxynervonoyl groups. To our knowledge, no other report is available on the acyl group composition of the lower and upper cerebroside bands.

The major phospholipids of the optic nerve are choline glycerophospholipids, ethanolamine glycerophospholipids, ethanolamine plasmalogens, serine glycerophospholipids, sphingomyelins, and inositol glycerophospholipids. Similar types of phospholipids have been found to be the major components of the optic nerve myelin (1,4,5) and retina (6,8,9). It should be noted that the optic nerve contains the myelinated axons of retinal ganglion cells (45). Phospholipids such as choline glycerophospholipids, ethanolamine glycerophospholipids, and serine glycerophospholipids are the major components of the photoreceptor membranes of bovine retina (7) and these phospholipids contain a large percentage of a long chain polyunsaturated acyl group, docosahexaenoyl group. It has been suggested that the electron-dense polyunsaturates are responsible for packaging of a lipoprotein complex required for the formation of visual membranes (46). Very high 22:6 in rod outer segments may be involved in transduction of light and subsequent electrical signal. Since bovine optic nerve phospholipids contain a large percentage of long chain polyunsaturated acyl groups such as eicosatetraenoyl, docosatetraenoyl, and docosahexaenoyl groups, it remains to be seen whether these polyunsaturates in bovine optic nerve are also responsible for binding with protein and thereby involved in transmission of electrical signal. Similar types of polyenoic fatty acids are also present in significant amount in brain phospholipids (9). It should be noted here that 22:6 is characteristic of neurons, 20:4 and 22:4 are characteristic of both neurons and glia (47,48).

Inositol glycerophospholipid has been isolated from bovine optic nerve. It is not known at this time whether it exists as mono-, di-, tri-, or polyphosphoinositides. It is most likely that it is monophosphoinositide because polyphosphoinositides may not be extracted by the procedure adopted by us. Polyphosphoinositides are present in large amount in myelin-rich particles of guinea pig forebrain (49). It has been suggested that inositol phospholipids in membrane have important roles in the selective control of movement of ions and other solutes, packaging and translocation of macromolecules, grouping and orientation of vectorially directed enzyme systems, and transmission of extracellular information to the cell interior (50-52).

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Sterol-Polyene Antibiotic Complexation: Probe of Membrane Structure

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ABSTRACT

Polyene antibiotics are useful tools for studying the role of sterols in biological membranes. The interaction of polyene antibiotics with membrane-bound sterols in artificial membrane systems, prokaryotic and eukaryotic cells, and lipid-containing viruses is reviewed. The pentaene macrolide, filipin, is shown to serve as a probe of phosphatidylcholine-sterol interaction and of the localization of cholesterol in the membrane of mycoplasmas.

Since the discovery of the first polyene macrolide antibiotic, nystatin, in 1950 (1), a great deal of information has been gathered concerning the effects of polyene antibiotics on sterol-containing organisms. There is abundant evidence, originally circumstantial in nature (2,3) and later direct (4,5), that these antifungal agents produced by *Streptomycetes* interact preferentially with cells whose membranes contain 3β-hydroxyl sterols. Nonspecific incorporation into membranes and cell walls is, of course, possible, but it is well established that, at low polyene concentrations (and high membrane sterol concentrations), the biological properties of these antibiotics result primarily from their ability to interact with sterols in cell membranes, leading to disruption of membrane function and ultimately to leakage of cellular constituents (cf. 6). Conceptual models of the aggregation of the initially formed polyene–sterol complexes into nonstatic transmembrane aqueous pores (7-9) have been proposed. The increase in conductance and permeability of ions and small nonelectrolytes in black lipid membranes depends on the polyene antibiotic concentration to a high power (4 to 12, varying with membrane composition), implying the involvement of a multimolecular array of polyene–sterol complexes in channel formation. Essential low-molecular-weight metabolites and constituents can be released from the cell via pores resulting from amphotericin action. Indeed, efflux of potassium and other intracellular markers has been used by many investigators as an indication of the functional integrity of membranes exposed to polyene antibiotics (e.g., 10). On the other hand, in some cell lines, growth promotion has been observed on treatment with a polyene antibiotic, presumably because of uptake of small nutrients from the growth medium into transport-deficient cells (11).

FIG. 1. Structures of filipin (top) and amphotericin B (bottom).

Considerable chemical interest was generated in this group of macrolides soon after the discovery of nystatin from *Streptomycetes noursei* (12). All of the antibiotics have a macrolide polyhydroxylic lactone ring of more than 23 atoms, with four to seven conjugated double bonds. The pentaene filipin has been the subject of extensive structural studies, and its structure has been revised (13) because the original work was done on a mixture of filipins (now referred to as the filipin complex) consisting of more than four components (14). Amphotericin B, a conjugated heptaene, and nystatin, whose chromophore is a conjugated tetraene distant by two methylene groups from a diene, are structurally similar. Both have a carboxyl group and an aminoglucoside (mycosamine) group linked to the macrocyclic ring, and probably exist in the hemiketal form, at least in the crystalline state (15-17). The structures of filipin and amphotericin B are shown in Figure 1.

The biological activity of these antibiotics includes the capacity to control serum choles-

terol levels (18,19) and prostate malfunction (20) in dogs and to exert larvicidal and chemosterilant activity in some insects (21,22). In addition to their ability to inhibit the sporulation and growth of yeast and other fungi (23), some polyene antibiotics possess antiprotozoal (24) and antiviral (25-27) activities and, despite their ability to bind vertebrate cell sterols, they have clinical applicability. In fact, amphotericin B is currently the most effective agent in antifungal chemotherapy. Administration of amphotericin B by the intraperitoneal route to patients with various fungal infections is associated with severe nephrotoxicity. Efforts to reduce the total dose of the polyene antibiotic include (a) combined therapy with the antimetabolite 5-fluorocytosine [e.g., in the treatment of systematic candidiasis and cryptococcosis (28-30)] and (b) combinations with antioxidants, which extend the duration of polyene activity (31). Addition of protecting ions during treatment of systemic infections with amphotericin B may also decrease the side effects of the polyene by maintaining intracellular cation concentrations. In certain strains of yeast, the fungicidal activities of polyenes are lowered by addition of K^+ and Mg^{2+} (32,33). The methyl ester of amphotericin B is less toxic than the parent compound, probably because it is more water soluble (less lipophilic), while retaining antifungal and antiviral activities (24,25,27,34).

Potentiation of the intracellular action of metabolic inhibitors has been achieved in many eukaryotic cells by addition of polyene antibiotics. For example, some polyenes have been used to circumvent the permeability barrier of fungal cells, L929 and 3T3 mouse fibroblasts, HeLa cells, and mouse leukemic cells, resulting in incorporation of nonpolyene antibiotics which are normally not taken up by the cells (e.g., 35-37). Several polyene antibiotics enhanced interferon production in L929 cells, possibly by increasing the penetration of poly (I) poly (C) (38).

The membrane-perturbing activity of polyene antibiotics has been used to probe various aspects of membrane organization and function. Amphotericin B and nystatin, for example, have been used to alter the membrane permeability of skeletal muscle fibers (39) and to modify the ion gradients of erythrocytes (40). Filipin has been used to establish the osmotic permeability properties of lipid-enveloped viral particles (41), the transport mechanisms for entry of phosphate and succinate into spermatozoa (42), the role of Ca^{2+} in flagellar activity of spermatozoa (43), the mechanisms of intestinal uptake of Ca^{2+} mediated by vitamin D (44), and the relationship between hormone–receptor binding and adenylate cyclase activation in beef thyroid membranes (45) and pigeon erythrocyte membranes (46).

The binding of filipin and amphotericin B to sterol-containing membranes is easily monitored by absorbance and fluorescence intensity measurements (47-49). Large changes in the fluorescence polarization (48) and circular dichroism (50) of filipin accompany its association with cholesterol and ergosterol. Hydrophobic forces are involved in maintaining the complex (47,50-52). From the dependence of the absorbance and fluorescence polarization of filipin on the sterol concentration in phosphatidylcholine (PC) vesicles, apparent binding constants were calculated (53). In vesicles prepared from mixtures of sterols and egg PC, the binding of filipin to cholesterol is tighter than to epicholesterol (K_d = 0.80 ± 0.14 compared with 13 ± 0.5), which is consistent with observations of surface pressure increases in PC-sterol monolayers on addition of filipin (47). The binding of filipin to ergosterol in vesicles is strong (K_d = 2.47 ± 0.05, but approximately three times weaker than that to cholesterol) (53). This is consistent with the report that addition of cholesterol simultaneously with filipin decreases the fungicidal activity of the antibiotic more effectively than does exogenous ergosterol (54). Filipin and amphotericin B differ markedly in their ability to discriminate among sterols incorporated into vesicles, with respect to equilibrium (48,50) and kinetic (55,56) properties. Amphotericin B is incorporated relatively rapidly into vesicles devoid of sterols, whereas the rapid association of filipin with vesicles requires sterol. The requirements for strong filipin–sterol binding are the same as those for sterols to exert a regulatory effect in artificial membranes and *Acholeplasma* membranes, i.e., a planar steroid nucleus, an unesterified 3β-hydroxyl group, and an apolar side chain at C-17; thus, the optical properties of filipin provide a convenient means by which phospholipid–sterol interactions may be examined (48,50,57).

Filipin is not a typical probe in that it causes membrane perturbation, the extent of which depends on the conditions (antibiotic to sterol molar ratio, period of exposure of the membrane to the antibiotic, temperature) (50). Some of these perturbations are particularly striking and involve lateral redistributions of membrane components, as in the filipin-induced morphological alterations in the envelope of vesicular stomatitis (VS) virions (26). Along the periphery of the filipin-treated particles, a series

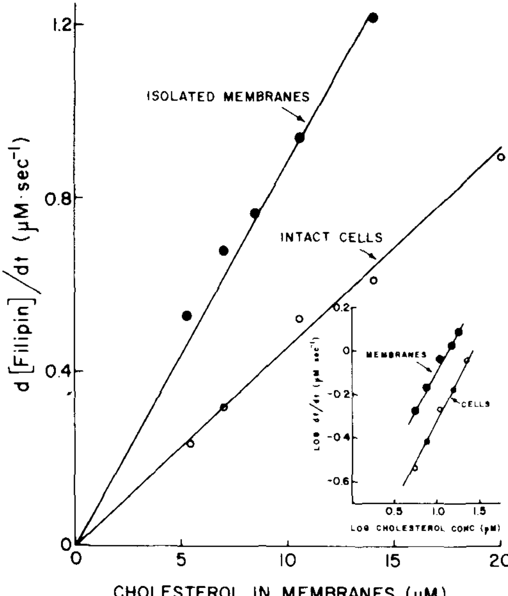

FIG. 2. Initial rate of disappearance of free filipin at 10 C on binding to varying concentrations of unesterified cholesterol in intact *M. gallisepticum* cells (o) and unsealed isolated membranes (•). Insert: A plot of the logarithm of the initial rate vs. the logarithm of cholesterol concentration, showing that the binding reaction is first order with respect to cholesterol in both cells and membranes. The indicated cholesterol concentrations represent the final concentration of unesterified cholesterol after mixing of equal volumes of antibiotic with cells or membranes in the stopped-flow spectrophotometer in 0.4 *M* sucrose containing 10 *mM* sodium phosphate (pH 7.2) and 20 *mM* $MgCl_2$ (68).

of depressions and ridges occurred, with a periodicity of 15 to 20 nm; glycoprotein spikes were attached only to the ridges. Morphological alterations produced by filipin in the envelopes of influenza and Rauscher leukemia virions differed from those seen in VS virions. Amphotericin B also caused redistribution of VS viral membrane components, but in a manner different from that found with filipin. The "pits" observed in sterol-containing membranes treated with filipin (50,58) may result from the formation of a planar filipin-cholesterol complex similar to that proposed for amphotericin B and nystatin (7,9), but with a diameter of 15 to 25 nm (determined by the geometry of the filipin and sterol molecules). The curvature of the VS virion may preclude the formation of a planar complex in this viral membrane. An elongated structure of the same diameter may form the series of ridges and depressions, with the glycoproteins redistributed into regions between filipin-cholesterol complexes. These alterations, which result from the binding of ca. 1 mole of filipin per mole of cholesterol, occur without detectable dissociation of lipids or proteins from the viral membrane (26).

Stopped-flow kinetic measurements of filipin-cholesterol association represent a means by which filipin-induced membrane disruption can be minimized, especially if high cholesterol to filipin molar ratios and low temperatures are used together with the very short reaction times. In vesicles prepared from mixtures of saturated phosphatidylcholines and cholesterol, the association process follows second-order kinetics (first order in filipin, first order in cholesterol) (55). The second-order rate constant for filipin-cholesterol complexation was essentially constant when the mole percent of cholesterol was varied over a wide range. The unusually high activation energy (6.8-14.3 kcal mol-1, depending on the fatty acid composition) may be attributed to insertion of filipin into the vesicle bilayer. It is of interest that no discontinuity in the Arrhenius activation energy was detected at the lipid phase transition temperature. This is consistent with the coexistence of two phases in dimyristoyl PC bilayers containing 25 (or less) mole percent cholesterol (59). Since filipin is selectively taken up by cholesterol, the failure to observe a transition in the association reaction suggests that a cholesterol-rich domain is present. The transition of gel to liquid crystal that is detected by differential scanning calorimetry in dimyristoyl PC-cholesterol (0.25 mole fraction) vesicles must, therefore, reflect the existence of a relatively pure PC domain. Kinetic study of the filipin-cholesterol interaction (55) thus provides support for the idea that lateral phase separation takes place in PC-cholesterol mixtures.

Information about the transbilayer distribution of the components of a biological membrane is a prerequisite for detailed knowledge of the function of the membrane. Rapid kinetic studies of filipin-cholesterol complexation in membranes where both sealed and unsealed preparations can be generated in a pure state provide a measure of the cholesterol distribution in the inner and outer halves of the lipid bilayer. The mycoplasmas are a simple system for the study of cholesterol localization and movement in a biological membrane. These microorganisms are the only prokaryotes that require cholesterol (or other sterols having a planar steroid nucleus, free 3β-hydroxyl group, and an apolar side chain at C-17) for growth. It has been suggested that their lack of a cell wall, coupled with their inability to regulate membrane fluidity by modifying fatty acids, accounts for the requirement of cholesterol for growth (60). In most

TABLE I

Second-Order Rate Constants for Filipin Binding to Free Cholesterol in Intact Cells and Isolated Membranes of *M. gallisepticum* and *M. capricolum*

Orbanism	$k^a/M^{-1}\ sec^{-1}$		
	Cells	Membranes	Cell/membrane ratio
M. gallisepticum	$7.8\cdot10^3 \pm 0.1\cdot10^3$	$15.3\cdot10^3 \pm 0.4\cdot10^3$	0.51
M. capricolum	$14.3\cdot10^3 \pm 0.4\cdot10^3$	$21.7\cdot10^3 \pm 0.6\cdot10^3$	0.66

[a]Values of *k* were calculated from each experimental point as described in the text. Each value of *k* is the mean ± the standard error of determinations made with at least 5 cholesterol concentrations. At each cholesterol concentration, 6 to 8 measurements of the initial rate were made. Rate constants are given in terms of binding to free cholesterol; for *M. capricolum* grown with 10% horse serum, the molar ratio of free to esterified cholesterol is 1.1, assuming an average molecular weight of 626 for the cholesteryl esters. In *M. gallisepticum* (A5969) cholesteryl esters comprised only about 2% of the total cholesterol.

strains, free cholesterol is incorporated from the medium into the membrane without any alteration (61). Cholesteryl esters apparently originate in mycoplasma membranes by incorporation from the growth medium. Membranes obtained from mycoplasma cells by sonication or osmotic lysis are known to be largely unsealed, based on studies of morphology (62), enzymatic activity (63), and transport (64). The initial rates of filipin-cholesterol association were significantly lower with intact mycoplasma cells than with isolated membranes (Fig. 2). Although the cholesterol taken up from lipoproteins or any other exogenous source is first incorporated into the outer half of the lipid bilayer of the mycoplasma membrane, it is distributed in both halves of the bilayer of cells grown to mid-exponential phase, as shown by these kinetic studies. Since the initial rate of interaction of filipin and cholesterol is sensitive to sterol accessibility and concentration (53,55), the ratio of the second-order rate constants in the unsealed isolated membranes relative to the intact cells is a measure of the cholesterol distribution (65). These rate constants indicate a symmetrical distribution of cholesterol in thetwo leaflets of the *Mycoplasma gallisepticum* membrane; however, in *Mycoplasma capricolum,* about two-thirds of the free cholesterol is localized in the outer half of the lipid bilayer (Table I). In confirmation of these results obtained with filipin, exchange studies of ^{14}C-cholesterol between resting *M. gallisepticum* cells and high density lipoproteins (HDL) (Fig. 3) show the existence of two different cholesterol environments, with ca. 50% in the inner half of the bilayer of *M. gallisepticum* (66). About 50% of the labeled free cholesterol in intact cells is readily exchanged with the exogenous HDL cholesterol, whereas more than 90% of the free cholesterol in isolated membranes is exchanged in a single kinetic process. It is, therefore, likely that the two environments in intact cells represent the inner and

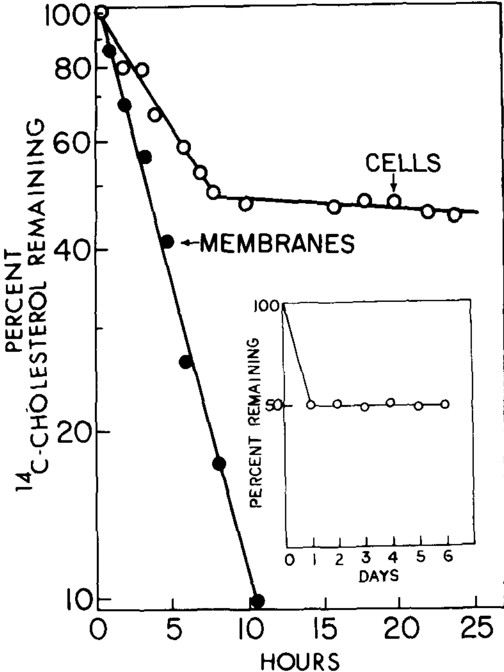

FIG. 3. Exchange of ^{14}C-cholesterol from intact cells and unsealed isolated membranes of *M. gallisepticum* with unlabeled cholesterol of high density serum lipoproteins. Radioactive cholesterol was added to the growth medium. Incubation of cells or membranes with a large excess of high density lipoproteins (about 100 times more unesterified cholesterol than was present in the membranes) was carried out at 37 C in 0.4 *M* sucrose containing 10 *mM* sodium phosphate (pH 7.2) and 20 *mM* $MgCl_2$. Cholesterol in one environment of the intact cells, representing ca 50% of the total unesterified cholesterol, is readily exchanged, with a half-time of about 4 hr at 37 C.

TABLE II

Second-Order Rate Constants for Association of Filipin and Free Cholesterol in Intact Cells and Isolated Membranes of the *M. capricolum* Adapted Strain, Transferred to a Cholesterol-Rich Medium[a]

Time of incubation in 10% fetal-calf serum (hr)	kCells[b] (M^{-1} s^{-1})	kMembranes[b] (M^{-1} s^{-1})	kCells/kMembranes[c]
0	5.0×10^4	7.1×10^4	0.73 ± 0.08 (14)
1	4.3×10^4	8.3×10^4	0.53 ± 0.04 (9)
2	4.0×10^4	8.8×10^4	0.45 ± 0.06 (16)
4	4.4×10^4	9.1×10^4	0.45 ± 0.07 (16)

[a]The initial rates of filipin-free cholesterol association were measured at 10 C at various cholesterol concentrations in intact cells and in isolated membranes obtained from at least nine different cell cultures, each incubated with medium containing 10% fetal-calf serum for the indicated period of time. The number of cell cultures investigated is indicated in parentheses.

[b]A representative example of second-order rate constants analyzed from one culture.

[c]Average ratio of second-order rate constants. Error limits of the kinetic data are standard errors of the mean.

outer halves of the bilayer. Figure 3 shows that transbilayer movement of cholesterol from the inner to the outer half of the bilayer in resting cells is very slow. However, the experiments on rapid filipin binding (Fig. 2, Table I) indicate that cholesterol translocation from the outer to the inner halves of the bilayer occurred within the 18- to 24-hr growth period. Evidence that rapid translocation of cholesterol from the outer to the inner surface takes place in growing *M. capricolum* cells has recently been obtained by measuring the initial rates of filipin association with membrane cholesterol (67). Transfer of a growing cholesterol-depleted adapted strain of *M. capricolum* to a cholesterol-rich medium containing fetal-calf serum resulted in an approximately sixfold enhancement in free cholesterol content of the membrane within 4 hr of incubation. The second-order rate constants for filipin-cholesterol association indicated that the transbilayer distribution of cholesterol was essentially invariant after only 1 hr of incubation (Table II). When growth is inhibited, translocation becomes much slower, and cholesterol accumulates in the outer half of the bilayer. The continued application of the techniques of rapid kinetics to the filipin-sterol interaction in this and other biological membranes may lead to an improved understanding of the factors that influence cholesterol accumulation in membranes and its distribution and movement within the lipid bilayer.

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The Steric Requirements for Sterol Inhibition of Tetrahymanol Biosynthesis

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ABSTRACT

Many naturally occurring sterols are accumulated and metabolized by *Tetrahymena pyriformis.* In most cases, the sterols are desaturated to give $\Delta^{5,7,22}$-derivatives. Compounds with an ethyl, but not with a methyl, substituent at C-24 are dealkylated. Exposure of the ciliates to the appropriate sterol sharply curtails the synthesis of the native pentacyclic triterpenoid alcohols, tetrahymanol and diplopterol. An analysis with modified sterols has revealed several additional features that are required for desaturation at C-7,8 and C-22,23 and for inhibition of tetrahymanol biosynthesis. The presence of a *trans*-17(20)-double bond, which eliminates free rotation at C-20 and fixes C-22 to the right of the nucleus, does not interfere with desaturation, while the *cis*- or left-handed isomer is not metabolized. The *cis*-$\Delta^{17(20)}$-isomer is, however, an effective inhibitor of tetrahymanol biosynthesis, although less so that the *trans*-counterpart. When a methyl or hydroxyl group at C-20 protrudes to the front of the molecule in the right-handed conformation, metabolism is reduced or abolished. Shortening (by one C-atom) or lengthening of the sterol side chain has little effect on the ability of the compounds to inhibit tetrahymanol biosynthesis or to support growth, as long as the overall length of the side chain does not exceed seven carbons from C-20. The presence of a 7α-, 7β-, 20α-, 20β-, or a 25-hydroxy group in the cholesterol molecule sharply inhibits desaturation and curtails the effectiveness of the compound as an inhibitor of tetrahymanol biosynthesis. The 7- or 22-keto derivatives seem to act in a fashion similar to the hydroxy derivatives, but these compounds show greater inhibition of growth. 20-Methylcholesterol, however, is a potent inhibitor of synthesis, which suggests that the polarity of the substituent of C-20 is more important than bulk. Many sterols can effectively replace tetrahymanol in the membranes of these ciliates. However, several of the compounds, which inhibit synthesis, appear to be physiologically inappropriate, and poor growth results. An example of the latter class is 20-methylcholesterol. Finally, a class of sterols, represented by 20α-hydroxycholesterol and 7-ketocholesterol, does not severly inhibit tetrahymanol synthesis but leads to growth inhibition and surface abnormalities. These sterols apparently lead to a disordered membrane, even in the presence of tetrahymanol.

The ciliated protozoan, *Tetrahymena pyriformis* W, has no sterol nutritional requirement (1), in contrast to members of the genus *Paramecium* (2-4). The tetrahymenids do not have the capacity to synthesize sterols (5,6), but they do synthesize the pentacyclic triterpenoid alcohols, tetrahymanol (gammaceran-3β-ol) and diplopterol, by a direct proton-induced cyclization of all-*trans* squalene (7-10). 2,3-Squalene epoxide, which is a precursor for sterol biosynthesis, is not utilized by these cells (11). Tetrahymanol is localized in the cellular membrane systems and is especially enriched in the limiting organelle that is analogous to the plasma membrane of multicellular systems (12,13).

Cholesterol addition to cultures of *Tetrahymena* results in a series of events that reflect a change in the morphology, metabolism, and physiology of the cell. The volume is decreased by approximately one-third (14), tetrahymanol biosynthesis is curtailed (6), the sterol is metabolized to 7,22-bisdehydrocholesterol (15), and the fatty acyl composition of the polar lipids is modified (14).

A number of sterols other than cholesterol have been observed to give rise to comparable phenomena. Cholestanol, ergosterol, stigmasterol, and fucosterol, to name a few, are effective agents (16). A series of investigations has been directed toward elucidation of the mechanisms involved in the desaturation of the side chain and the B ring of the sterols (17,18). The stereochemistry of dehydrogenation at (C-5,6), (C-7,8), and (C-22,23) has been established (19-22). It has been demonstrated that a planar configuration of the side chain is an essential requirement for desaturation at C-22 (17). Further, the requirement for a "right-handed" side chain for not only the C-22,23, but also the C-7,8 desaturation has been shown. (*E*)-17(20)-Dehydrocholesterol, which has C-22 fixed to the right of the ring structure (*trans* with respect to C-13), was desaturated in a manner similar to cholesterol, while (*Z*)-17(20)-dehydrocholesterol, which has C-22 fixed to the left (*cis* with respect to C-13), was found to be essentially inert metabolically (18).

TABLE I

Sterols That Inhibit Tetrahymanol Biosynthesis, Support Growth of *Tetrahymena pyriformis*, and are Metabolized to 7,22-Bisdehydrocholesterol or Its 24-Dehydro-, 24-C-, or 24-C_2-Derivatives

Cholesterol	Brassicasterol
7-Dehydrocholesterol	22,23-Dihydrobrassicasterol
22-Dehydrocholesterol	Campesterol
Lathosterol	Stigmasterol
Cholestanol	Sitosterol
Desmosterol	Fucosterol
22,24-Bisdehydrocholesterol	Stigmastatrienol
7,22-Bisdehydrocholesterol	Ostreasterol
Ergosterol	

The impact of a series of sterols on tetrahymanol biosynthesis and on cellular morphology and growth has been assessed. This report will detail the stereochemical requirements for inhibition of the synthesis of pentacyclic triterpenoid alcohols, contrast these requirements to those for desaturation, and note those compounds that are inhibitory to growth or lead to abnormal morphology.

Cells were grown in a peptone-based culture fluid, supplemented with ca. 25 μM sterol, for 21 hr at 28.5 C (15). Cell numbers were estimated by the use of a Model A Coulter Counter, equipped with a 200-μm orifice. The ciliates were observed with a Wild-Heerburgh inverted phase-contrast microscope at a magnification of 300x. The cells were harvested and lyophilized, and the neutral lipid fraction was isolated by procedures described elsewhere (14).

The neutral lipid fraction was saponified overnight in a nitrogen atmosphere at 37 C with 10% KOH in 80% methanol. The resultant ether-soluble fraction, which contained the free sterol and tetrahymanol, was not purified further. The tetrahymanol content of each fraction was determined by gas liquid chromatographic (GLC) analysis on a 0.75% SE-52 Gas-Chrom P column with a Hewlett Packard instrument Model No. 402, and estimated by comparing peak weights to those of standard tetrahymanol solutions.

The purity of most of the sterols employed was ascertained by gas chromatography-mass spectrometry, argentation thin layer chromatography, or GLC, on a 0.75% SE-52 Gas-Chrom P and a QF-1 column (16-18). *cis*-22-Dehydrocholesterol was a gift of J.A. Svoboda.

All sterols assayed that lack further oxygenation and have a C-27 skeleton in the same overall configuration as cholesterol inhibit tetrahymanol biosynthesis to a significant degree. The same is true for sterols with a methyl, methylene, ethyl, or ethylidene substituent at C-24 (16-18) (Table I). The extent of inhibition depends on the concentration of the sterol in the medium and on the length of incubation. Inhibition is virtually complete when an initial cell density of ca. 8 x 10^3 ciliates/ml, a concentration of 25 μM sterol, and a growth period of 21 hr at 28.5 C is employed. This uniformity of inhibition is not surprising, since compounds such as cholestanol (Δ^0), lathosterol (Δ^7), sitosterol (Δ^5), and stigmasterol ($\Delta^{5,22}$) are metabolized ultimately to 7,22-bisdehydrocholesterol (16). In contrast to 24-ethylsterols such as stigmasterol, which are dealkylated at C-24, the 24-methylsterols are not demethylated (16,23,24). The 24-methyl- and 24-ethylsterols as well as their $\Delta^{24(28)}$-derivatives, fucosterol and isofucosterol, are desaturated; however, the ethylidene substitutent is not removed (19).

A 3β-hydroxyl group is required for both the inhibition of tetrahymanol biosynthesis and for desaturation to occur. Cholestane (no hydroxyl), epicholesterol (3α-hydroxyl), and coprostanol (*cis*, A-B ring fusion) are ineffective substrates.

Compounds with a 4,4-*gem*-dimethyl substitution are without effect on pentacyclic alcohol synthesis (Table II, Fig. 1). The most striking example is the native alcohol, tetrahymanol, which does not regulate its own biosynthesis (16), nor is it desaturated or degraded (12). Others in this groups include β-amyrin, cycloartenol, lanosterol, and 24,25-dihydrolanosterol. The same situation would appear to exist for the 4-monomethyl derivatives, since lophenol was not altered when added as a supplement to the ciliates (25).

Sterols with a double bond in the side chain provide valuable information with regard to the specificity for both desaturation and inhibition. In the *trans*-17(20)-dehydrocholesterol molecule, the side chain is rigidly fixed to the right of the nucleus (*trans* with respect to C-13). This compound can be desaturated (18)

FIG. 1. Schematic diagram of the side chains of some of the sterols employed in these studies. I: Cholesterol, II: Halosterol, III: 27-Desmethylcholesteryl, IV: 26-*n*-Ethyl-27-desmethylcholesterol, V: 26-*n*-Butyl-27-desmethylcholesterol.

and is effective as a tetrahymanol synthesis inhibitor. Desaturation occurs to the same extent, and curtailment of triterpenoid synthesis is nearly equivalent to that observed for cholesterol. A double bond at C-24(25) (desmosterol), at *trans*-C-20(22) (*trans*-20(22)-dehydrocholesterol) and either at *cis*- or *trans*-C-22 (*cis*-22-dehydrocholesterol, *trans*-22-dehydrocholesterol) has no influence on the extent of inhibition. A conjugated diene sequence at C-22 and C-24 (22,24-bisdehydrocholesterol) does not interfere with the inhibition process.

Sterols with a left-handed configuration of the side chain, by virtue of a *cis*-17(20)-double bond (*cis*-17(20)-dehydrocholesterol), and sterols with an inverted configuration at C-20 (20-isocholesterol, 20-iso-norcholesterol) are less effective, but still are potent blocking agents for tetrahymanol biosynthesis (Table III). In contrast, none of these sterols was metabolized to a significant degree.

Sterols with a polar group in the side chain (20α-hydroxycholesterol, 20β-hydroxycholesterol, 25-hydroxycholesterol, and 22-ketocholesterol) do not inhibit tetrahymanol biosynthesis appreciably (Table II). These compounds also yield little in the way of desaturation products, and only the 22-keto compound depresses cell growth.

An exceptional behavior is found in 20-methylcholesterol (Table III). This molecule has a right-handed side chain and a methyl group at C-20 which replaces the hydrogen that projects to the front of the molecule. Although this compound shows virtually no desaturation at C-22,23 or C-7,8 (18), it brings about nearly complete inhibition of tetrahymanol biosynthesis. This behavior is in contrast to that of 20α-hydroxycholesterol (hydroxy projects forward), where the inhibition is insignificant.

A series of sterols with either longer or shorter side chains than cholesterol was investigated. Cholesterol analogs with radicals on C-20 that did not exceed seven C atoms suppressed synthesis and supported growth to degree equivalent to that of cholesterol (Table III). However, the analog with nine C atoms in that radical (Table II) was relatively ineffective as an inhibitor. Terminal branching had little effect on either inhibition or growth support.

Three examples of the introduction of a polar group into the B-ring have been examined (7-ketocholesterol, 7α-hydroxycholesterol, and 7β-hydroxycholesterol in Table II). These compounds are not metabolized, and only the 7-ketocholesterol causes any appreciable inhibition of tetrahymanol biosynthesis. Once again, the keto compound leads to a slower rate of cell growth.

In summary, the most effective inhibitors of tetrahymanol biosynthesis have a 3β-hydroxyl group, a sterol skeleton lacking methyl groups at C-4, and no polar groups in the side chain or at C-7 in the B ring. Free rotation around C-20 is not essential, and variation in the length of the side chain has little or nor effect on tetrahymanol inhibition until the chain length exceeds seven carbons from C-20.

Several of the same conditions apply to desaturation at C-7,8 and C-22,23. A 3β-hydroxy group is obligatory, as is the absence of methyl groups at C-4. In addition to these structures, the side chain must be "right-handed" and planar for Δ^{22}-introduction and

TABLE II

Sterols and Other Polyisoprenoid Derivatives That Do Not Inhibit Tetrahymanol Biosynthesis

7α-Hydroxycholesterol	Coprostanol
7β-Hydroxycholesterol	Lophenol
20α-Hydroxycholesterol	Dihydrolanosterol
20β-Hydroxycholesterol	Lanosterol
25-Hydroxycholesterol	Cycloartenol
7-Ketocholesterol	β-Amyrin
22-Ketocholesterol	Tetrahymanol
26-*n*-Butyl-27-desmethylcholesterol[a]	Cholestane

[a]See Figure 1.

TABLE III

The Effects of Certain Modifications of Cholesterol on Sterol Desaturation, Inhibition of Tetrahymanol Biosynthesis, and Growth

Sterol	Desaturation[a]		Inhibition[b]	Growth[c]
	C-7	C-22		
Cholesterol	++	++	++	++
20-Isocholesterol	0	0	+	+
20-Methylcholesterol	±	0	++	+
trans-17(20)-Dehydrocholesterol	++	++	++	+
cis-N(20)-Dehydrocholesterol	0	0	+	+
Halosterol[d]	++	0	++	++
20-Isohalosterol	0	0	+	+
27-Desmethylcholesterol[d]	++	++	++	++
26-n-Ethyl-27-desmethylcholesterol[d]	++	0	++	++

[a]++ is greater than 50% desaturation; ± is 0-3% desaturation.

[b]++ is 85-100% inhibition of tetrahymanol biosynthesis; + is 50-85% inhibition.

[c]++ is 85-120% of the cell numbers of cultures grown without sterols; + is 50-85%.

[d]See Figure 1.

for extensive Δ^7-desaturation. Further, a group larger than a hydrogen atom on the front face of C-20 prevents Δ^{22}-introduction and diminishes or prevents metabolic changes at Δ^7 (17).

The steric requirements for desaturation are more stringent than for inhibition of tetrahymanol biosynthesis. 20-Methylcholesterol undergoes minimal desaturation, yet leads to complete inhibition. Inversion of the configuration at C-20 almost elimininates desaturation, while only partially reducing synthesis. A polar group in the B ring gives rise to the same situation. In general, it can be stated that all sterols that are desaturated are effective inhibitors of tetrahymanol biosynthesis. However, all inhibitors of tetrahymanol biosynthesis are not desaturated. The major difference appears to reside in the side chain where the requirements are strict for desaturation and are less rigid for tetrahymanol inhibition.

The act of desaturation and the process of inhibition of tetrahymanol biosynthesis appear, on the surface, to be unrelated. However, there may be an event common to both. Sterol carrier protein (SCP) has been noted in this ciliate (25) and may be involved in the ultimate fate of the sterols and the organism. Liver SCP is known to bind a number of sterols and is believed to be a necessary component in many of the oxidation-reduction reactions that occur during the biosynthesis of cholesterol. The protozoal SCP has been reported to be a necessary ingredient in the Δ^5, Δ^7, and Δ^{22} desaturase reactions in the ciliate (25-27). Initial binding of the sterol to the protein may be important for both of the physiological processes followed in our studies. If a compound does not bind to the protein, then metabolism of the molecule or inhibition of tetrahymanol synthesis would not be anticipated. In this context, it is of importance to note that tetrahymanol does not bind to SCP. If a sterol is bound, the lower-order specificity seen for the inhibition of tetrahymanol biosynthesis could be explained by removal of the protein as a consequence of the formation of a sterol complex. This, in turn, could reduce the availability of SCP molecules to form a substrate complex necessary in the tetrahymanol biosynthetic scheme. An alternative interpretation is that the sterol-protein complex apparently acts as a negative effector on the activity of one or more of the enzymes involved in tetrahymanol biosynthesis.

There appears to be a major difference between the mammalian and the protozoal systems with regard to their response to various classes of sterols in the regulation of the pathway leading to squalene biosynthesis. The oxygenated sterols are much more effective than cholesterol in mammalian systems (28-34), while the opposite is true in *Tetrahymena.* Kandutsch et al. (35) have provided evidence for an oxygenated sterol-binding protein that is distinct from a cholesterol-binding component in mouse L-cells. It would be of interest to determine whether the oxygenated sterols are bound by any of the protozoal proteins. A lack of binding would provide presumptive evidence suggesting a reason for the ineffectiveness of the steroidal derivatives.

The higher-order specificity noted for sterol desaturation could be explained by the steric requirements of the desaturases. A sterol-binding protein complex may be formed that does not have the proper configuration for the

enzymes so that no further metabolism occurs. This premise would be in accord with the observation that all sterols that are metabolized also inhibit tetrahymanol synthesis, but that not all sterols that inhibit synthesis are desaturated.

All sterols added to the cells are accumulated and concentrated by a factor of at least 6-fold and often 30-fold. Many of these, however, have detrimental effects on cell growth and a few lead to marked abnormalities in morphology. Examples of growth-inhibitory compounds are: *trans*-20(22)-dehydrocholesterol, *trans*-17(20)-dehydrocholesterol, *cis*-17(20)-dehydrocholesterol, 20-isocholesterol, 20-isonorcholesterol, 20-methylcholesterol, 20α-hydroxycholesterol, and 7-ketocholesterol. All but the last two severely restrict tetrahymanol biosynthesis and may be viewed as compounds that do not serve as proper replacements in the maintenance of the appropriate membrane characteristics. The last two do not lead to marked tetrahymanol inhibition but do cause extensive surface abnormalities. This may indicate a disruptive influence of these sterols on an otherwise stable tetrahymanol-containing membrane.

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Steryl Glycoside Biosynthesis

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ABSTRACT

The existence of steroid glycosides has been known for many years and, more recently, their derivatives have been described. Steroid glycosides have been isolated from a number of organisms, however, the largest number of these compounds are found in plants. As to glycoside biosynthesis, the sterols are the most extensively studied steroid group. Of the sterols, only the 4-demethyl sterols have been isolated as glycosides. The glycosidic bond formation is mediated through nucleotide sugars, and UDP-glucose appears to be the most active glycosyl donor. In cell-free studies, the pH of the incubation medium is quite critical and depends on the tissue under investigation, but generally the optimum is near pH 7.0. Formation of steryl glycosides is particulate in nature and is stimulated by ATP, Ca^{2+}, and Mg^{2+}. Similar results are obtained, regardless whether the sterol or the sugar moiety is labeled. Formation of acylsteryl glycosides could occur via two pathways: through the acylation of steryl glycosides or through the transfer of an acylglycosyl unit to a sterol moiety. Results from in vitro experiments suggest that acylsteryl glycoside formation occurs via steryl glycosides. Two acyl transfer reactions have been demonstrated; one is microsomal in nature and involves phosphatidylethanolamine, while the other reaction involves a soluble enzyme and requires galactolipids. In vivo experiments, however, indicate that a second pathway may also exist. If cholesterol-4-^{14}C is used as substrate, a highly radioactive component can be isolated which is readily converted to acylsteryl glycoside, but not to free sterol or steryl glycoside. It is suggested that this component is an intermediate in acylsteryl glycoside biocynthesis. At present, the nature of the component is unknown. It is quite stable, and acid hydrolysis produces free sterol. Saponification produces two products which in thin layer chromatograms closely resemble acylsteryl glycoside.

INTRODUCTION

The number of steroid glycosides found in nature is quite large, and most of these constituents occur in plants. However, more recently, steroid glycosides have also been found in mycoplasma (1), yeast (2), slime mold (3), sea cucumber (4), starfish (5), toad (6), and mammals (6). Without knowing any of the chemistry, the ancient Egyptians and Romans used preparations from plants, *Scilla maritima,* as cardiac stimulants and diuretics. In 1785, William Withering, a Scottish physician, used extracts from dried seeds and leaves of purple foxglove, *Digitalis purpurea,* in the successful treatment of heart problems. We now know that these plant extracts contain steroid glycosides. Natives of Africa and South America have used similar extracts of various plants as arrow poison.

At the present, at least four groups of steroid glycoside are recognized: the cardiac glycosides, the saponins, the steroid glycosides, and the estrogenic glycosides. However, a number of other steroid glycosides that do not fit into these groups are known, e.g., dendrosteroside and ochreasteroside (7). Medically and historically the cardiac glycosides attract the largest interest. Over 50 cardiac glycosides from about 12 plant species have been isolated (8). The biological activity of these compounds resides in the aglycone, and two aglycone groups are recognized: the cardenolides and the bufadenolides. These two aglycones are very similar in structure except that the bufadenolides have a six-membered C_{17} lactone side chain, whereas it is five-membered in the cardenolides. The sugar moieties of cardiac glycosides are mostly 6-deoxy and 2-deoxy sugars, but some novel sugars have also been reported. Up to five sugars may form a straight chain, and the linkage to C_3 of the aglycone is always β-oriented. The most frequently studied cardiac glycosides are digitoxigenin, digoxigenin, and gitoxigenin, all found in *Digitalis.* The cardiac glycosides are found in all tissues of the plant, but the quantities may vary with stage of development, ecological factors, time of collection, and sample handling.

The saponins are steroid glycosides which are widely distributed, especially in the monocots, e.g., Liliaceae, Amaryllidaceae, and Dioscoreacea. The sugars of the saponins generally form branched chains and are attached to the C_3 position of the aglycone. The sapogenins are C-27 steroids with generally one to three hydroxyl groups.

An estrogenic glycoside, 17α-estradiol glucoside, has been isolated from glucuronidase-treated urine extracts of rabbits that received large doses of estrone benzoate (6). In this isolation, the glucose was attached at the 17α-hydroxyl of the steroid moiety. However, the C-3 glycosides of estadiol and estrone have also been reported (9). Estrogenic glycosides have not yet been isolated from plants.

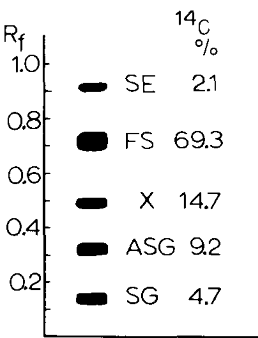

FIG. 1. Cholesterol-^{14}C incorporation into various sterol fractions of tobacco. TLC on Silica Gel G with Solvent System I (see text). SE = steryl ester; FS = free sterol; ASG = acylsteryl glycoside; SG = steryl glycoside; and X = unknown radioactive component.

Probably the best known and most often studied group of steroid glycosides are the steryl glycosides (SG). The first SG was isolated in 1913 from plants (10). It took another 50 years before it was discovered that two types of SG exist: the nonacylated and the acylated form (11). Since then, the structure of both glycosides has repeatedly been confirmed (12-15). The sterol moieties of the glycosides are the sterols normally found in the same plant (16). SG have also been isolated from certain strains of bacteria (1) and yeast (2).

The naturally occurring SG are 3β-hydroxy glycosides where C-1 of the carbohydrate forms the glycosidic linkage. The steroid moiety of the glycosides can be any 4-demethyl sterol. Glycosides of the 4,4-dimethyl and 4α-methyl sterols have not yet been reported and may not exist. The sugar most often reported in SG is glucose (11,12,17), although mannose (18), galactose (19,20), and gentiobiose (21) have also been found. The dominant carbohydrates in starfish steroid glycosides are glucose, quinovose, and fucose. Glucose is the carbohydrate linked to the steroid moiety, and fucose is at the nonreducing end of the oligosaccharide (5). The acylated steryl glycosides (ASG) are 6-*0*-acyl-β-D-glycosides. The most frequent fatty acid moieties are palmitate, stearate, oleate, linoleate, and linolenate (11).

SG probably occur in all plants. The occurrence of these glycosides in other organisms is less explored. Investigations of various plant tissues indicate that SG occur in all of them, and probably all tissues have the capability of synthesis (16). At the cellular level, chloroplasts appear to have the lowest quantity of SG (22) and the microsomes the highest concentration (23,24). ASG are quantitatively highest in the mitochondria (24).

EXPERIMENTAL PROCEDURES

Plant Material

We have used two types of plant material: 6-day-old tobacco seedlings, *Nicotiana tabacum*, and etiolated 3-day-old barley roots, *Hordeum vulgare*. The tobacco seedlings were obtained by germinating seeds in water on Whatman No. 1 filter paper in a germination box under constant light, as previously described (25). The barley roots were harvested from etiolated plants, grown on 0.5 *mM* $CaSO_4$ at room temperature. The excised roots were washed with 0.5 *mM* $CaSO_4$ for 30 min and rinsed with distilled water.

Chemicals

All solvents were reagent grade and redistilled if needed for chromatography. Cholesterol-4-^{14}C (34.8 mCi/*mM*) and sitosterol-4-^{14}C (61 mCi/*mM*) were used in all experiments, and β-D-cholesteryl-4-^{14}C glucose and 6-*0*-palmitoyl-β-D-glucosyl cholesterol-4-^{14}C were synthesized as previously described (26).

In vivo Experiments

Tobacco seedlings (5-6 g) and filter paper were transferred to Petri dishes, which contained the sterol or the appropriate glycoside in 20 ml of distilled water with Tween 20. With barley roots, the tissue (10-20 g) was incubated in 500 ml of water containing 1 ml of ethanol, which was used to dissolve the steroid. The roots were aerated throughout the experiment, and excised roots and tobacco were incubated in the light. Generally, experimental periods were 1 to 2 hr, but longer and shorter periods were also used.

At the end of the incubation period, the tissues were harvested, drained on a Büchner funnel, and washed with at least 10 *l* of water. The tissues were ground in acetone and extracted as reported elsewhere (25).

Chromatography

The thin layer chromatography (TLC) plates (Silica Gel G) were developed with either chloroform-methanol-acetic acid-water (90:8:1:1, Solvent System I), or diisobutyl ketone-acetic acid-water (80:50:1, Solvent System II). Rhodamine 6G and α-naphthol were used to visualize the sterols and glycosides. Radioactivity was detected by radioautography and determined by liquid-scintillation counting of the isolated zones.

For column chromatography, 30 g activated (24 hr at 110 C) Silica Gel 60 (70-230 mesh)

TABLE I

Column Chromatography of Acetone Extract from Barley Root[a]

Eluant	ml	Sterol[b]	Incubation, hr			
			0.1		2	
			cpm/g	%	cpm/g	%
C_6H_{14}	100	---	2	0	8	0
40% C_6H_6 in C_6H_{14}	100	SE	42	1.2	82	0.6
C_6H_6, then $CHCl_3$	50 + 100	FS	934	25.9	4,739	36.8
2% CH_3OH in $CHCl_3$	100	ASG	1,559	43.1	5,120	39.7
5% CH_3OH in $CHCl_3$	100	SG	333	9.2	1,304	10.1
CH_3OH	150	---	745	20.6	1,646	12.8

[a]The roots were incubated in an aqueous medium containing 5μCi of cholesterol-4-^{14}C.
[b]For abbreviations, see Figure 1.

was packed with hexane into a column, 1.9 cm id, which was eluted with 100 ml of each, hexane, 40% benzene in hexane, benzene, chloroform, 2% then 5% methanol in chloroform, and finally methanol. The ASG was eluted with 2% and the SG with 5% methanol in chloroform. The flow rate was about 2 ml/min.

The second type of column employed was activated (24 hr at 110 C) silicic acid SIL-LC (Sigma, St. Louis, MO.), 325 mesh (20 g), which was eluted with 40% benzene in hexane, benzene, chloroform, and 0.5%, 1.0%, and finally 1.5% methanol in chloroform. Flow rate was about 1.0 ml/min.

RESULTS

The acetone extract from tobacco seedlings which were incubated with cholesterol-4-^{14}C for 30 min gave, by TLC with Solvent System I, five radioactive zones (Fig. 1). The Rf values of the five zones correspond to the free sterol (FS), steryl ester (SE), ASG, SG, and an unknown component (26). The FS, SE, SG, and ASG fractions combined contained over 80% of the total radioactivity, and the unknown fraction generally had 10 to 15% of the activity. Isolation by TLC of larger quantities of the unknown component for further analysis was unsuccessful. However, during separate experiments with excised barley roots, it was noted that a very similar fraction could be recovered by elution of a silica gel column with 2% methanol in chloroform. It appeared to occur in higher concentration and was quite radioactive, even after only a 6-min incubation period (Table I). It was known that the 2% methanol in chloroform fraction also contains ASG. To determine whether the radioactivity was entirely due to ASG, an aliquot was saponified with 5% KOH in methanol and analyzed by TLC with Solvent System II (Fig. 2). The 2% methanol fraction migrated with ASG, but after saponification no SG could be recovered. Instead, the saponified fraction gave a broad peak in the area of FS. This would indicate that the major portion of the radioactivity is not in ASG. It was, therefore, decided to determine, by digitonin precipitation, whether a free sterol component might be contaminating the fraction, even though good separation seemed to occur (see Fig. 3). In the presence of carrier cholesterol, the digitonide pellet contained about 40% of the ^{14}C activity (Table II). The soluble fraction (supernatant) thus obtained was hydrolyzed with acid (refluxed with 0.5% H_2SO_4 in methanol [v/v] for 12 hr) or base (refluxed with 5% KOH in methanol [w/v] for 30 min), and after addition of carrier cholesterol, it was precipitated with digitonin (Table III). Acid

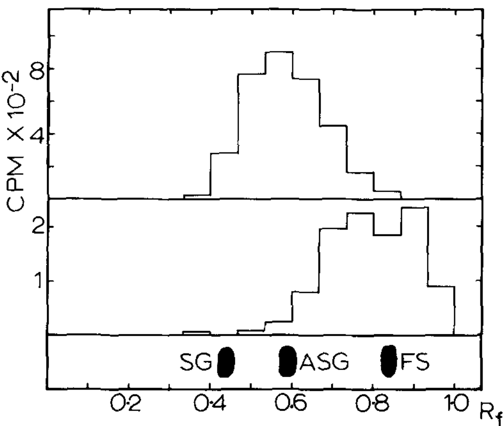

FIG. 2. Cholesterol-^{14}C incorporation into the 2% methanol in chloroform fraction obtained by column chromatography (see Table I). TLC on Silica Gel G with Solvent System II (see text). Top: 2% methanol in chloroform fraction; middle: same fraction after saponification; bottom: reference compounds, labeled as in Figure 1.

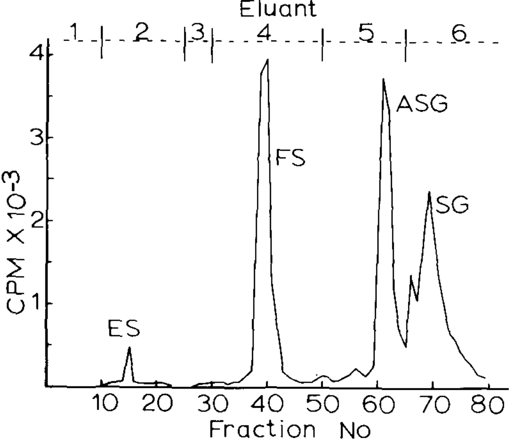

FIG. 3. Acetone extract from barley tissues incubated with cholesterol-^{14}C for 2 hr. Silica Gel 60 column eluted with: 1 = hexane, 2 = 40% benzene in hexane, 3 = benzene, 4 = chloroform, 5 = 2% methanol in chloroform, and 6 = 5% methanol in chloroform;10 ml fraction; flow rate, 2 ml/min. Reference compounds labeled as in Figure 1.

TABLE II

Digitonin Precipitation of the 2% Methanol in Chloroform Fraction as Eluted from a Silica Gel Column (cf. Fig. 2)[a]

Fraction	cpm	Percent
Soluble fraction	94,282	56
Ether wash (3)	1,659	1
Digitonide precipitate	72,404	43

[a]Carrier cholesterol was added.

hydrolysis yielded a digitonide pellet with only 10% of the radioactivity. This would indicate that the fraction is low in ASG. Digitonin precipitation after base hydrolysis, however, yielded 30% of the radioacitivity. Obviously, the barley root extract must be a mixture of several components and needs further investigation. Our preliminary data indicate that this barley fraction does not appear to be the same as the tobacco extract mentioned earlier, because this fraction, upon acid hydrolysis, yielded free sterol (26).

A chromatographic system has now been devised, based on a column of Silica Gel SIL-LC, which appears to isolate a highly radioactive component from tobacco extracts (Fig. 4). The unknown component is eluted in Fractions 50 through 55, and it has a very similar migration rate in TLC to X in Figure 1.

DISCUSSION

The first glycosylation reaction of sterols was demonstrated with a particulate preparation isolated from immature soybean (27). Formation of SG has since been confirmed by a number of laboratories with several species by using radioactive carbohydrates (28,29) and sterols (25) as substrates. Presently, the reaction can be summarized as follows:

$$\text{Sterol} + \text{UDP-glucose} \xrightarrow[\text{Ca}^{++} \text{ or Mg}^{++}]{\text{glucosyltransferase}} \text{SG} + \text{UDP}$$

The most effective hexose donor is UDP-glucose; TDP-glucose is less effective, and ADP-, GDP-, CDP-, IDP-glucose, UDP-galactose, UDP-mannose, and UDP-glucoronic acid are essentially inactive (27,30) With mitochondria, UDP-galactose is incorporated into SG. However, no galactolipid is formed suggesting that an epimerase converts UDP-galactose to UDP-glucose (29,31).

In vivo, free sterol disappearance studies indicate no sterol specificity (25). However, a number of other investigations indicate a sitosterol preference (20,32). The question of sterol specificity requires more thorough investigation, because in many cases the endogenous free sterol concentration has not been considered (33).

TABLE III

Digitonin Precipitation of the Soluble Fraction (see Table II) after Acid and Base Hydrolysis.[a]

Treatment	Soluble fraction cpm	Soluble fraction percent	Digitonide precipitate cpm	Digitonide precipitate percent
Cholesterol-4-^{14}C (standard)	162	0.7	23,859	99.3
Acid hydrolysis	6,582	90.1	727	9.9
Base hydrolysis	1,429	69.4	629	30.6

[a]Carrier cholesterol was added.

The pH optimum for SG formation is critical and appears to depend upon the tissue under investigation, but generally it is near the neutral point. A pH range of 6.5 to 8.5 has been reported (25,27,28). Transglycosylation is stimulated by Ca^{++} and Mg^{++} (31,34,35) and in many cases also by ATP (25,27,33). It has been suggested that ATP stimulates acylation of SG and thus influences glycosylation (30).

The glycosyltransferase activity is membrane bound and has been demonstrated in chloroplast, mitochondria (29), and microsomes (36). The enzyme activity, however, can be separated from these particulate fractions and it appears to be due to contamination with Golgi membranes (37-39). Identification of the Golgi apparatus as the center of glycosyltransferase activity would correspond to animal systems, where this membrane fraction is involved in the synthesis or modification of glycoprotein. Glycosyltransferase activity has been found in mycoplasma (1) and fungi (2), and a similar enzyme appears to exist also in mammalian tissue (9,40).

From a mechanistic point of view, ASG can be formed via two pathways as follows (27,41):

SG + acyl donor ⟶ ASG　　I

UDP-glucose+acyl ⟶ acylglucosyl+sterol ⟶ ASG

UDP-glucose + acyl donor ⟶
acylglucosyl + sterol ⟶ ASG　　II

Formation of ASG from SG would appear to be the simpler of the two pathways. However, the second pathway cannot be rejected offhand, because acylated sugars have been found in microorganisms (42,43). Most of the acyltransferase studies have been carried out by following the radioactivity of the sugar moiety. Kinetic studies with particulate enzyme preparations indicate that SG is the precursor for ASG (27,29,32,33,41). Similarly, in vivo experiments with UDP-glucose-^{14}C show a substrate-product relationship for SG and ASG (26). The particle-bound acyltransferase utilizes phosphatidylethanolamine and to a lesser degree phosphatidylcholine (36,44,45). The transfer of the acyl group has been demonstrated with acyl-^{14}C labeled phospholipid (44,46). Galactolipids were not utilized for acylation by this enzyme.

A second, soluble acyltransferase has been isolated. This enzyme utilizes galactolipids but not phospholipids as the acyl donor (46,47). With semisynthetic galactolipids, it was found that the acyl groups of C_1 and C_2 of mono- and

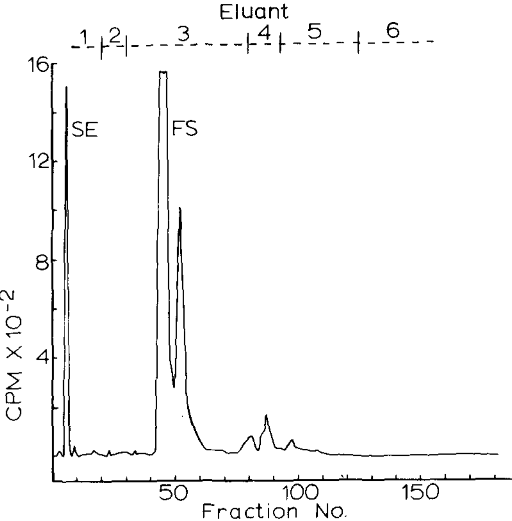

FIG. 4. Extract from tobacco tissues, incubated with sitosterol-^{14}C. Silica Gel SIL-LC column eluted with: 1 = 40% benzene in hexane, 2 = benzene, 3 = chloroform, 4 = 0.5% methanol in chloroform, 5 = 1.0% methanol in chloroform, 6 = 1.5% methanol in chloroform; 15 ml fraction; flow rate, 1.0 ml/min. Reference compounds labeled as in Figure 1.

digalactosyl diglyceride could be transfered to SG. The in vivo involvement of galactolipids has been questioned (48).

If radioactive sterol is used in in vitro experiments, glycosylation is rapid and up to 60% of the added sterol can be recovered from the SG fraction (25). The amount of SG and ASG formed depends upon the pH. Generally, SG accumulation is favored at pH values above 7.0, while ASG accumulation is higher under more acidic conditions.

In in vivo experiments, however, only 20 to 30% of the accumulated sterol is recovered as the glycoside, and ASG has a higher specific radioactivity than SG (25). These results have been interpreted to mean that ASG formation can also proceed without going through SG. If cholesteryl-^{14}C glucoside is supplied to tissue, about 10% of the radioactivity is recovered from ASG. However, the FS has twice the ^{14}C activity (Table IV). These data could be interpreted to mean that ASG is formed from SG. Since formation of ASG from FS is quite rapid, it could also mean that SG gave rise to FS, which in turn was converted to ASG. In vivo, similar metabolic experiments with SG, in which the carbohydrate was radioactive, showed a conversion of SG to ASG of 4 to 50% (41,46,49). The latter results have been cited as proof that ASG is formed from SG, but in these experiments the formation of free carbohydrate

TABLE IV

Metabolism of Various Sterols by Intact Tobacco Seedling[a]

Sterol supplied	Percent conversion[b]			
	SG	ASG	FS	SE
Cholesteryl-4-^{14}C glucoside	62	11	24	3
Palmitoyl cholesteryl-4-^{14}C glucoside	53	18	27	2
Cholesterol-4-^{14}C	5	19	73	3
X in Figure 1	7	89	4	<1

[a]Incubation of seedling is described in Methods. TLC with solvent system I was used for separation.
[b]For abbreviations see Figure 1.

was not determined. Furthermore, these results were obtained with enzyme preparations which required Triton X-100 for transacylation to occur (44).

In experiments (26) with intact tissues and sterol as the substrate, a highly radioactive component (R_f 0.45 to 0.50) was found, which is not ASG (Fig. 1). This radioactive component was initially isolated from tobacco seedlings. The unknown fraction gave a positive test for carbohydrate (aromatic amine reagent) and for fatty acids (hydroxylamine-ferric chloride reagent). Metabolism studies with intact tissues have shown that this component is preferentially converted to ASG (Table IV). It is suggested that this constituent is a metabolic intermediate in the formation of ASG (26). A sterol component somewhat similar in nature has also been isolated from barley roots. However, unlike the tobacco component, the barley constituent upon acid hydrolysis did not give FS (Table III). Base hydrolysis of the barley component did not give SG (Fig. 2), indicating that we are not dealing with ASG, but a certain quantity of FS was released (Table III). At the present time, it is not known whether the tobacco and barley components are the same, but the barley fraction is easier to work with because larger quantities can be recovered.

In summary, on the basis of in vivo experiments, it is postulated that ASG can be formed via two pathways. The pathway through SG would be reversible, while the second pathway through an unknown intermediate would not be reversible. The significance of these two pathways in the intact system will have to await further experimentation.

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Inhibition of Cholesterol Synthesis by Oxygenated Sterols

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ABSTRACT

Sterols derived from cholesterol by introducing a ketone or hydroxyl function in the 6, 7, 15, 20, 22, 24, or 25 positions are known to be potent inhibitors of sterol synthesis in cell cultures. To gain more information regarding structural requirements for inhibitory activity, inhibitory potencies were determined for a series of 18 C_{27}-steroids with various combinations of ketone and hydroxyl functions substituted in positions 3, 4, 5, 6, and 7, or with a single ketone or hydroxyl function in one of these positions. The effects of nuclear double bonds upon inhibitory activity were also examined. A ketone or hydroxyl function in position 3 and a second ketone or hydroxyl function in position 6 or 7 was required for inhibitory activity with two kinds of cell culture. A 3β5α6β-triol was not more inhibitory than a comparable 3β,6β-diol. Cholestane-3β 5α-diol inhibited sterol synthesis in L cells but not in liver cell cultures. The inhibitory activities of 7-oxygenated sterols were not markedly affected by the presence of a double bond in position 4 or 5. Current knowledge of the mechanism through which the oxygenated sterols suppress cholesterol synthesis is reviewed.

The results of studies of the regulation of cholesterol synthesis in cultured cells differ from those obtained with intact animals in one important respect. In animals, dietary cholesterol inhibits hepatic cholesterogenesis by suppressing 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, although it has little effect upon high rates of sterol synthesis in intestine and skin (Ref. 1 for review). In contrast, the addition of pure cholesterol to the medium of liver cell cultures and to cultures of other cell types has little or no effect on the enzyme (2-6). Certain oxygenated sterols are, on the other hand, potent suppressors of HMG-CoA reductase activity in cultured cells (2-8) and are also capable of inhibiting sterol synthesis in liver and intestine in vivo (9-11). Some of these oxygenated sterols, i.e., 7α-hydroxycholesterol, 20α-hydroxycholesterol, and 24β-hydroxycholesterol, are normal metabolites of cholesterol in liver, adrenal glands, and brain, respectively (reviewed in Ref. 12). It is, therefore, reasonable to suggest that sterol synthesis in vivo may be controlled by specific oxygenated sterols, the structures of which may be different in different cells or tissues. Two important questions which are related to this hypothesis are: (a) What structural features of the steroid molecule are required for inhibitory activity? and (b) Through what mechanism do the inhibitory sterols act? Both are difficult questions, and final answers to them are still lacking. However, relevant information is accumulating rapidly, and we will try to indicate some directions in which it is leading.

On the basis of studies already published (2-8), some general structural requirements for inhibitory activity seem apparent: (a) all of the inhibitory sterols can be identified as derivatives of 5α-cholestane; (b) all have a hydroxyl, methoxyl, or ketone function at position 3 and a ketone or hydroxyl function at position 6, 7, 15, 20, 22, 24 or 25; (c) a complete side chain is necessary for full inhibitory activity. Sterols that meet the above general requirements vary greatly in their inhibitory potencies, depending upon the position of the second ketone or hydroxyl function, upon the orientation of the hydroxyl functions, and upon ancillary features of the molecule. The results presented in Figures 1-4 represent an effort to gain more detailed information regarding relationships between structural features in rings A and B and inhibitory potency. Further studies of the effects of detailed structural modifications on the inhibitory potencies of sterols oxygenated in the 14, 15, and 32 positions and in the side chain are being carried out in collaboration with Dr. George J. Schroepfer, Jr., and his associates, Rice University, Houston, TX, and with Mme. M. Wilpart-Cocu, Catholic University, Brussels, Belgium.

We have previously described the tests we employ for measuring the potency of specific suppressors of HMG-CoA reductase and sterol synthesis (2). Briefly, the potency and specificity of the inhibitory activity of a compound was determined by testing its effects upon rates of acetate metabolism to sterols, fatty acids, and CO_2 in monolayer cultures of L cells (mouse fibroblast). If the compound specifically inhibited sterol synthesis, a second experiment was carried out to determine its potency as a suppressor of HMG-CoA reductase activity. These two assays were then repeated with primary cultures (monolayers) of fetal mouse liver in order to detect cell-specific responses to the sterols. In each test, the con-

STEROL	CONCENTRATION (μM) REQUIRED FOR 50% INHIBITION			
	L-CELL CULTURE		PRIMARY CULTURES OF LIVER CELLS	
	STEROL SYNTHESIS	HMG-CoA REDUCTASE	STEROL SYNTHESIS	HMG-CoA REDUCTASE
5-Cholestene-3β,7α-diol	2.5	10.0	32.0	50.0
5-Cholestene-3β,7β-diol	2.0	5.7	8.8	6.3
3β-Hydroxy-5-cholesten-7-one	3.0	3.0	21.3	11.3
3β-Hydroxy-5α-cholestan-7-one	1.3	2.0	3.5	8.5
7α-Hydroxy-4-cholesten-3-one	>15.0	>15.0		

FIG. 1. Steroids oxygenated in positions 3 and 7. Data for the sterols with double bonds in positions 4 and 5 have been presented (2) and are included for comparison. 3β-Hydroxy-5αcholestan-7-one was purchased from Steraloids. Wilton, N.H.

STEROL	CONCENTRATION (μM) REQUIRED FOR 50% INHIBITION			
	L-CELL CULTURE		PRIMARY CULTURES OF LIVER CELLS	
	STEROL SYNTHESIS	HMG-CoA REDUCTASE	STEROL SYNTHESIS	HMG-CoA REDUCTASE
5α-Cholestane-3,6-dione	1.8	7.0	4.5	11.2
3β-Hydroxy-5α-cholestan-6-one	2.1	1.5	9.0	6.3
5α-Cholestane-3β,6β-diol	14.0	9.0	8.8	4.8
4-Cholestene-3,6-dione	1.9	5.8	9.0	5.5
Cholestane-3β,5α,6β-triol	6.0	13.3	25.0	11.5
3β-Hydroxycholestane-5α,6α-epoxide	9.3	>25.0 [a]	17.3	7.5

[a] RESULTS OF 2 OR MORE EXPERIMENTS

FIG. 2. Steroids oxygenated in positions 3 and 6, or 3,5, and 6. The preparation of 5α-cholestane-3β,6β-diol has been described (5). 3β-Hydroxy-5α-cholestan-6-one was from Sigma St. Louis, MO. The remaining steroids were from Steraloids.

centration of sterol required to suppress sterol synthesis of HMG-CoA reductase activity by 50% was determined from a plot of activity vs. sterol concentration. Steroids were recrystallized to constant melting point shortly before they were assayed for inhibitory activity.

The inhibitory potencies of some sterols oxygenated at C-7 are shown in Figure 1. The inhibitory activity of 7β-hydroxycholesterol was greater than that of the 7α-epimer and was approximately equal to that of the corresponding 7-ketone. Reduction of the Δ^5-bond had little effect upon the inhibitory potency of the 7-ketone. Oxidation of the 3-hydroxyl function of the relatively weak inhibitor, 7α-hydroxycholesterol, with migration of the double bond to the 4 position to give 7α-hydroxy-4-cholesten-3-one resulted in a loss of all activity at the concentrations tested. As can be seen in Figure 2, 5α-cholestane-3,6-dione and 4-cholestene-3,6-dione suppressed HMG-CoA reductase activity approximately as effectively as did 3β-hydroxy-5α-cholestan-6-one, suggesting that a 3-hydroxyl function may not be required for inhibitory activity. Reduction of the 3-ketone may, however, occur in the cells, because 3β-dehydrogenases capable of reducing a wide variety of 3-keto cholestane derivatives are present in liver (13). In previous studies, the introduction of a third hydroxyl function into an inhibitory diol did not increase its activity and in some cases suppressed it (7,8). As shown in Figure 2, cholestane-3β,5α,6β-triol exhibited weak inhibitory activity, although in liver cell cultures it was less potent than the corresponding 3β,6β-diol. 3β-Hydroxycholestane-5α,6α-epoxide was not inhibitory in L cells but was approximately as effective as the triol in liver cell cultures.

Insofar as reasonable comparison can be made, 3β-hydroxy sterols oxygenated in the 6- or 7-position appeared to be approximately equally effective as suppressors of HMG-CoA reductase activity. In contrast, a sterol with adjacent hydroxyl functions, 5-cholestene-3β,4β-diol, was completely inactive in L cells and in liver cell cultures (Fig. 3). Results obtained when the two hydroxyls were separated by one carbon atom, as in cholestane-3β,5α-diol, differed from those seen with any other diol tested so far. Cholestane-3β,5α-diol was a potent suppressor of HMG-CoA reductase in L cells but was inactive in liver cell cultures. Further studies are needed before a general con-

STEROL	CONCENTRATION (μM) REQUIRED FOR 50% INHIBITION			
	L-CELL CULTURE		PRIMARY CULTURES OF LIVER CELLS	
	STEROL SYNTHESIS	HMG-CoA REDUCTASE	STEROL SYNTHESIS	HMG-CoA REDUCTASE
Cholestane-3β,5α-diol	1.0	0.8	>75.0	>75.0
5-Cholestene-3β,4β-diol	>15.0		>75.0	

FIG. 3. Hydroxylation in positions 4 and 5. The 3β,5α-diol was a gift from Dr. George J. Schroepfer, Jr., Rice University, Houston, TX. 5-Cholestene-3β,4β-diol was from Steraloids.

STEROL	CONCENTRATION (μM) REQUIRED FOR 50% INHIBITION			
	L-CELL CULTURE		PRIMARY CULTURES OF LIVER CELLS	
	STEROL SYNTHESIS	HMG-CoA REDUCTASE	STEROL SYNTHESIS	HMG-CoA REDUCTASE
5α-Cholest-2-en-6-one	>15.0			
1,4-Cholestadien-3-one	>20.0	>25.0		
4,6-Cholestadien-3-one	15.0	>25.0	52.0[a]	>75.0
4,7-Cholestadien-3-one	2.5[a]	9.5	11.0[a]	>75.0[b]
3,5-Cholestadien-7-one	15.0		57.0	60.0

[a]INHIBITED FATTY ACID AND CO_2 PRODUCTION
[b]RESULTS OF 2 OR MORE EXPERIMENTS

FIG. 4. Unsaturated steroids with one functional group a ketone) in positions 3,6, or 7. The preparation of 4,7-cholestadien-3-one has been described (14). 3,5-Cholestadien-7-one was from Kurt Spira and Co., 4,6-cholestadien-3-one was from Schwartz/Mann Orangeburg, NY, and 5α-cholest-2-en-6-one and 1,4-cholestadien-3-one were from Sigma.

clusion can be drawn regarding the inhibitory activities of 5-oxygenated sterols.

Figure 4 shows that cholestane derivatives with a single ketone function at position 3, 6, or 7 in combination with one or more double bonds were essentially inactive. The slight inhibition obtained with 4,7-cholestadien-3-one and 3,5,-cholestadien-7-one may have been due to the generation of inhibitory autoxidation products during the incubation of the sterones with the cultures. These compounds and 4,6-cholestadien-3-one appeared to autoxidize easily as evidenced by a detectable lowering of the melting point within one week after they were purified. In tests by Bell et al. (15), 4,6-cholestadien-3-one appeared to inhibit cholesterol synthesis in rat hepatoma cell cultures, whereas the data in Figure 4 show it to be inactive. These contrasting results may be attributable to the ease with which the compound undergoes autoxidation, although the possibility that the hepatoma cells metabolized 4,6-cholestadien-3-one to a more inhibitory compound is not excluded. It is apparent from this discussion that our ability to define the precise structural features of the sterol molecule that are required for inhibitory activity is, to some degree, limited by knowledge of the uptake and intracellular metabolism of the administered compounds. Furthermore, we do not yet know whether all of the various oxygenated sterols suppress HMG-CoA reductase activity through one common mechanism, or whether two or more mechanism are involved.

Presently we know little of the detailed process through which MHG-CoA reductase activity and, hence, sterol synthesis is regulated. There is good evidence that at least some regulatory fluctuations in HMG-CoA reductase activity are brought about by alterations in the rate at which the enzyme is synthesized. Cyclic changes in enzyme activity as well as induction of activity following the ingestion of cholestyramine or the injection of Triton WR 1339 appear to involve this mechanism (reviewed in Ref. 1). In addition, the results of some studies were interpreted as suggesting that feeding cholesterol to rats (16) or adding 25-hydroxycholesterol to rat hepatoma cell (HTC) cultures (17) resulted in increased degradation or inactivation of HMG-CoA reductase as well as in suppression of its synthesis. However, the latter investigations involved the use of antibodies prepared against HMG-CoA reductase preparations which, as recent studies suggest, may have been grossly impure (18). Interpretation of these results is, therefore, in doubt. Other support for a physiological control mechanism other than the regulation of the synthesis of HMG-CoA reductase comes from studies of factors that affect the activity of the enzyme in vitro. A major fraction of the HMG-CoA reductase present in a liver homogenate appears to be inactive and is activated when the homogenate is incubated under appropriate conditions (19-21). Furthermore, the microsome-bound enzyme is rapidly inactivated by ATP through some unknown process which appears to require Mg^{2+} and a cytoplasmic

protein (20-25). Evidence for at least two different cytosolic activating proteins has been presented. One protein is thought to reactivate the HMG-CoA reductase that has been inactivated by ATP (21). Activation of microsomal HMG-CoA reductase by the other cytoplasmic protein is blocked by certain lipids (25). The activities of solubilized enzyme preparations are also influenced by ATP and by association with lipids (18,21,24). Based upon these and related observations, two separate posttranscriptional mechanisms for regulating the activity of HMG-CoA reductase have been postulated, one involving ATP, and the other involving alterations in the lipid milieu of the enzyme (20-27).

Current evidence indicates that the oxygenated sterols do not inactivate HMG-CoA reductase directly because they did not affect its activity when they were added to the enzyme incubation mixture (2,3,7). If the oxygenated sterols do not interact directly with the enzyme, then it seems reasonable to postulate that they affect its activity by binding to another macromolecule which is involved either in the regulation of enzyme synthesis or degradation, or in the inactivation of the enzyme. In support of this hypothesis, we have demonstrated that 25-hydroxycholesterol is taken up by cultured cells and bound to some cytoplasmic protein(s) that are different from the cholesterol-binding protein(s) (28). In further, but still incomplete, tests of the specificity of the binding proteins, various unlabeled sterols were preincubated various unlabeled sterols were preincubated with the cultures to allow them to "compete" for binding sites with 25-hydroxycholesterol-[26,27-^{3}H]. Six inhibitory oxygenated sterols tested were "competitive," i.e., were bound to the cytosolic 25-hydroxycholesterol-binding protein(s). Three noninhibitory sterols, on the other hand, did not affect the binding of 25-hydroxycholesterol.

The identification of a binding protein with specificity for at least some oxygenated sterols opens new avenues for investigating both the inhibitory sterols and the control mechanism. Correlations between the affinities of the oxygenated sterols for the binding protein and their potencies as suppressors of HMG-CoA reductase may provide evidence for or against the participation of binding protein(s) in the regulatory system. Such studies may also indicate whether all of the oxygenated sterols act through a common mechanism and, ultimately, may provide a detailed rationale for the structural features of inhibitory sterols.

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Cyclopamine and Related Steroidal Alkaloid Teratogens: Their Occurrence, Structural Relationship, and Biologic Effects

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ABSTRACT

A spontaneous congenital deformity is produced in lambs whose dams consume *Veratrum californicum* on the 14th day of gestation. The deformity is generally expressed as cyclopia, cebocephaly, anophthalmia, or microphthalmia. This teratogenic effect is produced by certain steroidal alkaloid teratogens from the plant – most notably the compound cyclopamine. Cyclopamine is a C-nor-D-homo steroid with fused furanopiperidine rings E and F at right angles to the plane of the steroid because of spiro attachment at C-17 of the steroid. Among veratrum alkaloids, only those with an intact furan ring E were teratogenic in sheep, whereas those in which the piperidine ring is not rigidly positioned at right angles to the steroid were not. Many ruminants and laboratory animals are susceptible to the teratogen. It has wide species and tissue specificity and appears to have a direct effect on the embryo, not as a consequence of metabolic alteration of its structure nor as an indirect effect through a maternal influence. Other plant sources, notably potatoes, tomatoes, and eggplant contain related spirosolane steroidal alkaloids. Among naturally occurring spirosolanes, solasodine is teratogenic in hamsters, but neither tomatidine not diosgenin, the non-nitrogen containing analog of solasodine, is teratogenic. Results of these and other studies suggest that a basic nitrogen positioned α with respect to the steroidal plane and at appropriate distance beyond the D ring confers the teratogenicity on the molecule. Potato sprouts with high alkaloid content are teratogenic in hamsters, but tubers and peels are not.

HISTORICAL ASPECTS

During the first half of the century, a significant percentage of lambs born in certain parts of Idaho were congenitally deformed (1-3). The deformity was restricted nearly exclusively to the head, and in its most severe expression was a true cyclopia (Fig. 1). This problem was experienced only on certain ranches in Idaho. Owners of the ranches where the condition existed reported that incidence of the disease was restricted geographically to certain forest areas grazed by some of their sheep. All of the range areas were in localized parts of the Boise, Sawtooth, and Challis National Forests of Idaho.

By the 1950s some operators were experiencing an incidence of up to 25% deformed lambs in bands of ewes from these areas (1-3). Deformities in livestock were universally attributed to genetic causes at that time, but because of breeding practices, a genetic cause did not seem likely for this condition. Considerable genetic variation existed among affected animals from ranch to ranch and from year to year, and no correlation with incidence was apparent. Incidence in a given ranch appeared to be related to area grazed rather than to identity of rams or ewes.

FIG. 1. Cyclopic congenital deformity in a lamb.

Workers in the Poisonous Plant Research Laboratory became interested in the problem at about that time and began experimental work that has finally culminated in a good understanding of the disease. They were able to rule out, in part, a genetic basis for the disease (1) by a breeding experiment involving ewes that had given birth to deformed lambs on a previous occasion when bred to rams that were sons of these ewes. Next, no unusual elemental composition was detected in flora, soil, or water from the affected areas, and preliminary feeding trials of many indigenous plants recognized as poisonous failed to reproduce the condition (1).

But certain plants were common to all the ranges where ewes that later gave birth to deformed lambs had grazed. Among these plants, attention was focused on *Veratrum californicum,* and extensive feeding trials were begun. Pregnant sheep gavaged dried or fresh plant for up to the first 30 days of gestation gave birth to deformed lambs (4). The experiments suggested that the embryonic

susceptibility period was between the 8th and 17th days of gestation (4). The precise insult time proved to be the 14th day of gestation (5). This information provided ranchers with a suitable practical solution to their problem. They could keep their pregnant ewes away from the plant until they had passed the 14th day of gestation. The use of this information alone now keeps the incidence of the disease very low.

JERVINE R_1=OH, R_2=O
CYCLOPAMINE R_1=OH, R_2=2H
CYCLOPOSINE R_1=GLU, R_2=2H

FIG. 2. The teratogenic veratrum alkaloids cyclopamine, jervine, and cycloposine.

ISOLATION AND STRUCTURAL DETERMINATION OF THE TERATOGEN

However, for reasons that will become apparent, we continued to study this problem. We speculated in 1964 (6) that the *Veratrum* teratogen might be one of the many steroidal alkaloids reported from members of the *Veratrum* genus (7). This seemed likely because of reported cell division or growth inhibitory properties of some of these alkaloids (8-10). In feeding trials with pregnant sheep, preparations were tested from a variety of alkaloid extraction methods applied to *V. californicum* plant material. Some of the offspring were deformed, suggesting that the teratogen was extractable and was probably a glycoside or parent alkamine among one or more of the 50-60 ester, glycoside, and parent steroidal alkaloids reported in other members of the *Veratrum* genus (7). By 1967, we tested 13 of these alkaloids, four of which proved teratogenic to a greater or lesser degree (11,12). The compound we named cyclopamine appeared to be the teratogen of principal importance in the plant. Only 3 (Fig. 2) of the 4 proved in the last analysis to be teratogenic in pure form. They are cyclopamine, jervine, and a compound that appeared to be the glycoside of cyclopamine, which we designated cycloposine (13).

Structural analysis demonstrated the close similarity of the three compounds. Jervine is a compound whose structure has been known for many years (7), and cyclopamine bears many similarities to jervine in nuclear magnetic resonance (NMR), infrared (IR), mass spectrometry (MS), and other methods of analysis. Cyclopamine proved to be 11-deoxojervine, contrary to our suppositions in 1966 based on IR analysis of an impure preparation (11). In 1964 the compound 11-deoxojervine had been isolated from *Veratrum grandiflorum* and characterized by Masamune and others (14) in Japan.

Our isolated cyclopamine (15) had a mass of 411, as expected of 11-deoxojervine. It had 1050-1057 cm^{-1} absorption in the IR, suggesting a Δ^5, 3β-ol steroidal system, about the same

FIG. 3. Congenital double-globe cyclopia produced by maternal cyclopamine ingestion.

3500 cm^{-1} absorption as that of jervine and about one-half that of veratramine, suggesting the expected single hydroxyl group. Peaks at 927, 984, and 1118 cm^{-1}, also found in jervine, were consistent with a ring oxygen system (the furan ring). As expected, there was no evidence of aromatic ring absorption in the UV region. Elemental analysis suggested an empirical formula of $C_{27}H_{41}O_2N$, as would be expected of 11-deoxojervine. NMR suggested 41-42 protons with signals for 12 protons, consistent with 4 methyl groups at $C_{18,19,21}$, and $_{26}$. There were recognizable signals for 2 other protons assignable as the C-6 olefinic and C-3 protons. The NMR results were, therefore, consistent with an identity of cyclopamine as

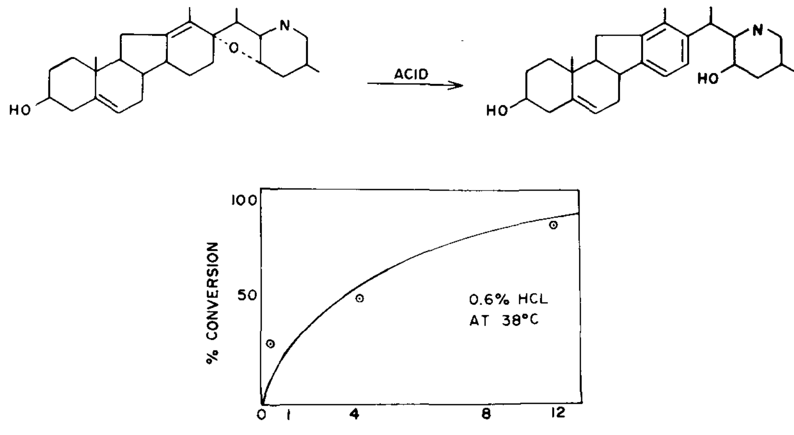

FIG. 4. Conversion of cyclopamine to veratramine.

11-deoxojervine (14). MS fragmentation results were also consistent with the assignment. There was a recognizable parent ion at 411 m/e and fragments at 396, 340, 326, 310, 298, 125, 124, and 110 m/e, as expected on the basis of the reported fragmentation of jervine (16). Acetylation or acid aromatization produced the expected N,O-diacetyl derivative or veratramine (15). Finally, Wolf-Kishner reduction of jervine to produce 11-deoxojervine yielded a product indistinguishable from our isolated cyclopamine (15). Thus, the isolated cyclopamine was indeed 11-deoxojervine.

Analysis of the other teratogen, cycloposine, established (17) that it was 3-glucosylcyclopamine, a compound not previously reported. Thus, all 3 active compounds were closely related structurally (Fig. 2).

ANATOMICAL DEVIATIONS PRODUCED IN SHEEP BY CYCLOPAMINE

When sheep voluntarily eat *V. californicum* on the 14th day of gestation (5), or when the plant or the pure teratogen (5,13) are administered on that day, a variety of deformities can result. The 14th day is the primitive streak/neural plate stage of embryonic development in sheep. The required oral dose of cyclopamine to produce effects in sheep is about 30 mg/kg. Many of the deformed offspring (2,3) are either single- or double-globe cyclopics (Fig. 3), usually with a proboscis above the eye. Some are microphthalmic or anophthalmic, with or without a proboscis. All of the above generally have a pronounced mandible that curves up over an absent or a vestigial maxilla or premaxilla. The Basque sheepherders call the condition "monkey face" lamb disease (1) – a term derived from the appearance of the double-globe cyclopics. Less severe expression varies from a slight shortening of the premaxilla to a more typical cebocephalic appearance with a defective nose. Affected brains vary from slight fusion of cerebral hemispheres to the absence of all but a rudimentary cerebrum. The pituitary gland is usually absent in severe cases, and only a single optic nerve is present in cyclopics.

SPECIES SPECIFICITY AND DIRECT ACTION OF CYCLOPAMINE ON THE EMBRYO

Until about 1969, all of our work including isolation and characterization of cyclopamine and the other teratogens, had been done with sheep as the assay animal. The gestation period in sheep (and, therefore, the duration of each assay) is 145 days. We found that gram quantities of each isolated alkaloid were required per sheep. Those whose assays take minutes or hours and require milligram quantities of precious compounds, will recognize certain logistic difficulties with our assay. Despite this, we had not wanted to seek a different assay for fear that this might lengthen rather than shorten the overall period required to arrive at a definitive answer on teratogen identity for sheep. We recognized the risk of extending conclusions from one species to another. We did manage to shorten the assay period in sheep considerably by killing each treated ewe on the 50th day of gestation, a time when deformities could be readily recognized in the fetus (12). This maneuver allowed us to identify the teratogen as described above.

With the answer at hand for sheep, we began a search for susceptible laboratory animals for future studies, and we concurrently examined susceptibility in other classes of livestock.

In early experiments, published some years later, cattle and goats as well as sheep proved susceptible to the teratogenic action of orally administered *V. californicum* (18). These 3 classes of livestock are ruminant animals, and we wondered whether rumen microorganisms might enhance or reduce the potency of the plant by metabolizing the teratogen. Was cyclopamine the teratogen, or was a metabolic product of that compound the true teratogen? Assessing the susceptibility of non-ruminant laboratory animals would answer that question.

Rabbits proved susceptible to the teratogenic action of cyclopamine (19) when the compound was administered orally at 33-45 mg/kg. However, on the basis of clinical toxicity observations and on in vitro assay, there was some aromatization of the administered cyclopamine to the non-teratogenic, but very toxic veratramine as a result of the lability of cyclopamine to stomach acid (Fig. 4). That conversion could be prevented by simultaneous administration of $CaCO_3$ to buffer the stomach acid (19). The gestational insult period proved to be day 7 in the rabbit (20), which is the primitive streak/neural plate stage of embryonic development in that species. Anatomical deviations included double globe cyclopia and cebocephalia with a single or closely spaced double nostril (19,20).

Cyclopamine also produced deformities in rats, mice, and hamsters gavaged during the primitive stread/neural plate stage of development with as little as 240, 180, and 170 mg/kg, respectively (21), but cyclopics were not evident. In rats, microphthalmia and cebocephalia predominated; in mice, a few exencephalics resulted; whereas in hamsters, cebocephalia, encephalocele (cranial bleb), exencephaly, and hare lip resulted (21).

Clearly, conversion of cyclopamine by rumen microorganisms was not essential. Indeed, ruminant sheep and non-ruminant rabbits were about equally susceptible to cyclopamine on a body weight basis. But the small total dose of 20-30 mg required for hamsters, their short 16-day gestation period, the extremely high incidence of deformities in treated animals, and their general ease of handling have made hamsters the assay animal of choice (12).

Meanwhile, results of 2 additional experiments lent support to the likelihood that cyclopamine, and not a metabolic conversion product from maternal rumen or liver metabolism, was the true teratogen, i.e., that cyclopamine has a direct effect on the embryo. Malformations in hatched chicks resulting from direct application of 1-2 mg of cyclopamine to the embryonic shield of windowed chicken eggs (22,23) showed that maternal metabolic alteration of cyclopamine was not necessary. Further, in other experiments in sheep, intrauterine injection of as little as 1-2 mg of cyclopamine produced deformities. Thus, the dam could be bypassed, and direct treatment of the embryo with cyclopamine produced teratogenic effects (23).

FIG. 5. Congenital reduction in length of metacarpal and metatarsal bones from maternal ingestion of cyclopamine.

EMBRYONIC SPECIFICITY OF THE ACTION OF CYCLOPAMINE

Thus far we have considered effects produced by cyclopamine when the embryonic insult is during the primitive streak/neural plate stage. In the deformities observed in sheep under natural conditions, most deviations from normal could be attributed to embryonic insult at that time. But a few cases of cleft lip/cleft palate or limb bone shortening (Fig. 5) were observed. Results in feeding trials with plant material showed that these conditions were produced by insult on about the 25-30th days of gestation (18). Again, cyclopamine proved to be the active principle from the plant (24). The results suggested that, in addition to having broad specificity as far as susceptible animal species was concerned, cyclopamine also had broad specificity regarding susceptible embryonic age or tissue. Cyclopamine is an almost "universal" teratogen.

STRUCTURAL SPECIFICITY IN SHEEP AMONG VERATRUM TERATOGENS

So, species and tissue specificity of cyclopa-

FIG. 6. Comparison of the structures of cyclopamine, cycloposine, jervine, veratramine, and muldamine.

mine action was broad, but what of chemical structural specificity – was specificity broad there also? Did the general class of steroidal alkaloids represent a teratogenic hazard for man as well as animals? Veratrum steroidal alkaloids are found in a variety of non-food plants. These genera include *Veratrum, Zygadenus,* and *Schoenocaulon* (7). Related steroidal alkaloids are found in some genera, including a variety of human foods, such as certain *Solanum spp.* (both eggplant and potato) as well as *Lycopersicon* (tomato) (25). We had reported preliminary experiments in 1970 designed to test whether potatoes and tomatoes with high alkaloid content could produce the same effects in sheep as those produced by cyclopamine (26). The results were negative.

Although the question of specificity among all steroidal alkaloids still interested us, our data at that time allowed inference on specificity only for the veratrum alkaloids. There did appear to be considerable structural specificity among the veratrum alkaloids for teratogencity in sheep (26). Much could be adduced from consideration of the structures of 5 of the major alkaloids of veratrum tested in sheep (Fig. 6), cyclopamine, cycloposine, jervine, muldamine, and veratramine. The similarities among the 3 active compounds, cyclopmaine, cycloposine, and jervine, were previously mentioned. All are C-nor-D-homo steroids with a fused furanopiperidine functional group attached spiro at C-17 of the steroid. Muldamine is a principal alkaloid of *V. californicum,* which we isolated and found to be non-teratogenic in sheep and which we believe to have the structure shown in Figure 6 (27). Muldamine differes from cyclopamine in having no ether bridge giving rise to a furan ring, no unsaturation in ring D, but having an O-acetate function on C-11. Veratramine, having a hydroxyl group on ring F and a fully unsaturated ring D, also produced no cyclopia in sheep. The teratogenic and structural relationship of these 5 compounds suggested, among other things, that something about the presence of the furan ring was important – either the ring itself or configurational aspects conferred on the molecule by the presence of that ether bridge (26).

COMPARISON OF VERATRUM AND SOLANUM STEROIDAL ALKALOIDS

Our early interest in the question of whether solanum alkaloids might be teratogenic was further stimulated in 1972 by a publication by Renwick (28) hypothesizing that consumption of blighted potatoes by pregnant women was responsible for congenital spina bifida and anencephaly (ASB) in their babies. His hypothesis was based on what he interpreted as a correlation between ASB and blight from epidemiologic evidence. If his hypothesis, since then largely discredited (29), had merit, perhaps a solanum steroidal alkaloid might be responsible (30).

The USPHS provided funds through an interagency agreement between USPHS and the United States Department of Agriculture to

FIG. 7. Projection drawing of the structures of solasodine, tomatidine, diosgenin, cyclopamine, and solanidine.

study the relationship between veratrum and alleged potato teratogens. Funding from that agreement and from USDA was provided, in part, to prepare analogs of veratrum and solanum alkaloids with altered or eliminated functional sites in an attempt to elucidate the essential features of steroidal alkaloid teratogens. Meanwhile, before analogs were made, testing was started on the teratogenicity in experimental animals of certain commercially available compounds and also various blighted and non-blighted potato preparations.

In testing compounds related to cyclopamine, our first inclination was to examine those with the closest structural resemblance to it. There are no solanum alkaloids with a furan ring E fused to a piperidine and attached spiro at C-17 of a C-nor-D-homo steroid like the veratrum teratogens. But spirosolane solanum alkaloids from such plants as potato, tomato, and eggplant have a furan ring E fused to a conventional steroid and attached spiro to a terminal piperidine ring (25). The configuration is similar to that of cyclopamine, with piperidine more or less at right angles to the plane of the steroid.

Rats were gavaged in late 1972 with cyclopamine, or with the spirosolane alkaloids solasodine or tomatidine at the primitive streak/neural plate stage of embryonic development at 240 mg/kg (30). Cyclopamine produced terata, but neither solasodine nor tomatidine was teratogenic, although in retrospect we wonder whether the dosages were too low. By 1973 it had become apparent that hamsters were a better assay animal, and the experiments were repeated with that species.

We compared the teratogenicity of solasodine and tomatidine with that of diosgenin, the non-nitrogen containing analog of solasodine, and with that of cyclopamine, the veratrum teratogen (Fig. 7) (31). Solasodine was teratogenic and produced deformed offspring from dams gavaged at 1200-1600 mg/kg – a dose nearly 10 times that required to produce terata with cyclopamine. Neither diosgenin nor tomatidine was teratogenic at double the dose. We subsequently learned from a 1975 *Chemical Abstracts* entry that Russian workers had found solasodine to be teratogenic in rats (32).

Projection drawings of the tested compounds suggest a number of conclusions (31). Clearly, neither the C-nor-D-homo steroid skeleton nor the fused furanopiperidine is essential to teratogenicity. Presence of a nitrogen in ring F is essential, as is the configurational position of that nitrogen conferred by virtue of the spiro connection between rings D and E or E and F. In both cyclopamine and solasodine (teratogenic compounds), the essential nitrogen projects α with respect to the plane of the steroid. In tomatidine (non-teratogenic), the nitrogen of the piperidine ring projects in the β direction with respect to the plane of the steroid, but notice the difference in potency of the 2 teratogens. Cyclopamine is almost 10 times as teratogenic as solasodine. The data suggested (31) as one possibility that teratogenicity was a function of the extent of the α projection of the nitrogen, inasmuch as the extent of α projection is much greater in cyclopamine than in solasodine. Alternatively, potency might have been related to basicity, because the basicity of the nitrogen of solasodine is less than that of cyclopamine (31). Possibly, the nitrogen must be positioned favorably for interaction with an unidentified active site in the susceptible embryo. Thus, the importance

TABLE I

Susceptibility of Pregnant Simonsen S_1 Hamsters to Potato Sprouts and Steroidal Alkaloids[a]

Group	Total no. litters[a]	Percent malformed litters	Malformations—statistical significance[c]
H_2O controls	522	1.34	
'Kennebec' sprouts	181	25.4	$P < .0005$
'Russet Burbank' sprouts	70	25.7	$P < .0005$
'Pioneer' sprouts	60	8.3	$P < .0005$
'Targhee' sprouts	58	10.4	$P < .0005$
'Sebago' sprouts	52	13.5	$P < .0005$
'Nampa' sprouts	55	18.2	$P < .0005$
'Norchip' sprouts	46	8.7	$P \approx .0005$
Jervine	26	73.1	$P < .0005$
Cyclopamine	17	47.1	$P < .0005$
Solasodine	105	26.7	$P < .0005$

[a]Single dose levels fed by gavage to 135 ± 23-g pregnant hamsters: 500 mg sprouts, 150-200 mg solasodine, 20 mg jervine or cyclopamine.
[b]Carried to term or resorbed (10-40% overdose dam death).
[c]Statistical significance of the difference between the incidence of malformations in control and treated groups.

of that ether bridge (the furan ring) in cyclopamine began to be apparent – to provide some molecular rigidity to position the nitrogen appropriately with respect to the overall shape of the molecule.

Brown prepared a number of analogs of cyclopamine or jervine and of solanidine which were tested in the hamster assay (33). Parent compounds are shown in Figure 7. The fact that cyclopamine and jervine analogs devoid of the 5-6 or 12-13 double bonds were teratogenic suggests that neither of the double bonds was essential for activity. N-methyljervine was as active as jervine, suggesting that a free electron pair on a tertiary nitrogey was all that was required for interaction with the active site in the susceptible embryo. N-butyljervine was less active than jervine, probably because of substituent bulk. N-acetyljervine was even less active, perhaps because of reduction in nitrogen basicity. The solanidane analogs prepared were R-S isomers at C-22 and C-25, providing a series of conformational isomers with the lone electron pair on the nitrogen projecting in either the α or β direction with respect to the steroid plane. In the 22R,25S-isomer, the lone electron pair projects β whereas, in the 22S,25R-isomer, the pair projects α. The 22R,25S-isomer with its β-projecting pair was not tertogenic in hamsters, but the 22S,25R-isomer was. Naturally occurring solanidine, the aglycone of the common potato alkaloidal glycoside solanine, is the 22R,25S or non-teratogenic form (33). The data from the solanidan epimers suggest that neither the C-nor-D-homo steroid ring system nor the furan ring of cyclopamine is an essential feature for conferring teratogenicity. Even in the much more structurally remote solanidans, the requirement may center on the presence of a lone electron pair on a basic nitrogen projecting α with respect to the plane of the steroid, positioned more or less rigidly at an appropriate distance from the steroidal end of the molecule.

TERATOGENICITY OF POTATO PREPARATIONS

The testing for teratogenicity in laboratory animals of certain potato preparations proved very interesting to us. In our early experiments (34), preparations tested included blight-infected tuber material and tuber material aged to enhance alkaloid level about 10 times. Specifically, 'Russet Burbank' tubers were either sliced and aged or were experimentally infected with either *Phytophthora infestans* or with *Alternaria solani*, the late and early blight organisms. Incubated tissue was harvested, lyophilized, and fed by gavage to rats, mice, hamsters, and rabbits at levels near 1/50 the body weight at the primitive streak/neural plate stage of embryonic development. None of the preparations produced teratogenic effects.

In later experiments, sprout material from the 'Kennebec' potato produced terata in offspring from hamster dams gavaged with that material (35). The alkaloid concentration was usually up to 1,000 times higher in sprouts than in tuber material. Sprouts were fed by gavage to pregnant hamsters with the expectation that if an alkaloid from potatoes were teratogenic, their offspring would be deformed. Recently (36), we reported that sprouts from 7 potato varieties produced terata in a particularly

sensitive hamster strain; the incidence of affected litters was 8.5-25.7% (Table I). But neither tuber nor peel material from these 7 potato varieties produced deformed offspring at doses up to 4 times as high as those used with sprouts. We have not yet established whether the effect is due to alkaloids present in the sprouts, but preliminary experiments suggest that this is possible. The dosage of the dried sprout material required to produce the deformities was very high – 2,500-3,500 mg/kg.

Meanwhile, this evidence of teratogenicity of potato sprouts for one hamster strain does not seem to bear any relationship to the alleged potato-related congenital defects in humans hypothesized by Renwick (28), because the required dosages were so high (equivalent to over 1,000 g wet weight of sprouts in a single dose for a woman of average weight), because the edible parts of the plant (the tubers and peels) were not teratogenic even at 4 times that dose, and because sprouts are not generally consumed at all by humans, the alkaloids rendering the sprouts extremely bitter (36).

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Sex Hormones of the Aquatic Fungus *Achlya*

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ABSTRACT

Fungi in the order Saprolegniales are known to contain varying proportions of sterols, such as cholesterol and fucosterol. In the case of *Achlya,* it has been found that fucosterol, the major sterol component, serves as the biosynthetic precursor of the hormones, antheriodiol and the oogoniols. Antheridiol is secreted by female strains of *Achlya* and induces the formation of antheridial hyphae in male strains. It also causes the male to secrete the oogoniols which induce the formation of oogonial intitials in female strains. Antheriodiol is responsible for the chemotropic growth of the antheridial hyphae to a developing oogonium which results in sexual conjugation. The structures, biosynthesis, and functions of these hormones are discussed in this paper.

INTRODUCTION

The functions of sterols in plants have been extensively investigated (1), and their role as components of membranes is well documented (2). There has been great interest in possible functions of mammalian steroid hormones in plants, and studies have been carried out on the effect of estradiol and of testosterone, for example, in promoting germination of seeds, growth stimulation, and flower induction (3).

Of particular interest to us is the effect of sterols on sexual reproduction in fungi belonging to the Oomycetes. Two genera, the plant pathogens *Pythium* and *Phytopthora,* are exceptional in that they are unable to synthesize sterol. They apparently do make squalene but lack the ability to transform this to sterol. Without sterol the growth of these organisms remains purely vegetative. In the presence of sterol, growth is stimulated and, more significantly, sexual reproduction can take place. 29-Carbon sterols, fucosterol, 28-*iso*-fucosterol, sitosterol, and stigmasterol, have been found to be most active in promoting development of the sexual reproductive organs, antheridia and oogonia, and the subsequent formation of oospores (4). These sterols are not believed to be the true hormones controlling the sexual reproductive process but rather precursors of the hormones. The precursors could be metabolized in the organism to more active substances by oxidative reactions. Such a process would be analogous to the biosynthesis of mammalian sex hormones from cholesterol. An even closer analogy is the biosynthesis of the hormones that control sexual reproduction in *Achlya,* which, like *Pythium* and *Phytopthora,* is a member of the Oomycetes. Unlike the latter, however, *Achlya* can synthesize sterol, and the mycelium of several species has been found to contain fucosterol (~0.1%) together with smaller quantities of 24-methylene cholesterol and cholesterol (5). Fucosterol has recently been shown to be the biosynthetic precursor of antheridiol and the oogoniols, hormones which control sexual reproduction in *Achlya.*

Like other members of the order Saprolegniales, *Achlya* is a water mold or aquatic fungus which occurs most commonly in fresh water. Species of *Achlya* are saprophytic, living on dead plant or animal remains, or they are obligate parasites of fish and various small aquatic animals. In its sexual cycle, *Achlya* is basically similar to *Pythium* and *Phytopthora* (order Peronosporales).

ISOLATION AND STRUCTURES OF ANTHERIDIOL AND THE OOGONIOLS

The discovery of the sex hormones of *Achlya* was made many years ago by Raper (6,7), but definition of their chemical nature has come only recently. Sexual reproduction is initiated by the vegetative female mycelium, which secretes the first hormone ("hormone A") into the surrounding water. In response to hormone A, male hyphae in the vicinity react by putting out numerous thin lateral branches, which elongate into thin, much-branched contorted filaments. These are the antheridial hyphae.

Hormone A induces the male hyphae to secrete another hormone ("hormone B") which induces the development of spherical oogonial initials on female mycelium. Oogonial initials are believed to secrete relatively large amounts of hormone A, which directs the growth of the antheridial hyphae to the developing oogonium, leading to conjugation of the sex organs. A crosswall near the tip of the antheridial hypha delimits a multinucleate antheridium, and shortly after, the oogonium is delimited by a crosswall at the top of the stalk of the initial. Within the oogonium, protoplasm reorganizes

Antheridiol

23-Deoxyantheridiol

Figure 1.

to form between one and twenty mononucleate eggs. Fertilization is accomplished by passage of male nuclei through fertilization tubes that extend from the antheridium to each egg. Meiosis is believed to take place in the antheridium and the oogonium, and the fertilized egg or oospore is thus diploid. On germination, the thick walled resistant oospore develops into the hypha of a new plant.

Early attempts to isolate hormone A were made by Raper and Haagen-Smit (8). This involved solvent partition and selective adsorption of the hormone from culture liquids of *Achlya bisexualis* and gave a small amount of solid material with high biological activity. However, isolation of pure hormone A was not achieved until many years later in our laboratory at the New York Botanical Garden (9). Extracts of culture liquids of *A. bisexualis* supplied by Alma Barksdale, (New York Botanical Garden) were chromatographed on silica gel with ethyl acetate as solvent, followed by thin layer chromatography with chloroform-methanol. Isolation of the hormone was guided by a bioassay developed by Barksdale. In this way, about 1 mg of crystalline hormone was isolated from 85 *l.* of culture liquid in 1965. The pure hormone, which was renamed antheridiol, was found to induce branching in the male *Achlya ambisexualis* E87 at a concentration as low as 10 pg/ml.

The structure of antheridiol was determined from mass spectral studies of Biemann and Arsenault and other spectral evidence on the hormone and its derivatives (10). It was confirmed by a synthesis of antheridiol carried out by Edwards and co-workers at the Syntex Corporation in California (11). Although the stereochemistry of the side chain was not defined, they were able to show that it had the *erythro* rather than *threo* configuration at C-22, C-23.

Culture liquids of female strains of *Achlya* were found to contain another steroid, 23-deoxyantheridiol, which is closely related to antheridiol (Figure 1). The structure was confirmed by synthesis of the C-22 epimer by Green et al. (12). The stereochemistry at C-22 was established by comparison of the circular dichroism curve of 23-deoxyantheridiol with those of lactones of established absolute configuration. This allowed the absolute configuration of antheridiol to be assigned as 22*S*,23*R*. The stereochemistry was confirmed in later synthetic studies of antheridiol by Edwards and co-workers (13).

Synthesis of antheridiol in reasonable yield has been achieved in our laboratory (14), and the method has been used to prepare labeled hormone (22-^{3}H, 23-^{3}H antheridiol) (15). We have recently completed a synthesis of 23-deoxyantheridiol also, and we have thus been able to show that this steroid is weakly active in inducing branching in *A. ambisexualis* E87 (16).

Hormone B, the second hormone discovered by Raper, is secreted by male strains of *Achlya* on stimulation by antheridiol. In our first attempts at isolation of this hormone, antheridiol was added to cultures of *A. ambisexualis* E87, and the culture liquids were later examined for the presence of hormone B. Barksdale had developed an assay for hormone B, which indicated that very small amounts of hormone were being produced. Chromatography of active extracts showed that hormone B was slightly less polar than antheridiol, and mass spectral data suggested that it might be a steroid.

Examination of a hermaphroditic strain of *Achlya (A. heterosexualis)* revealed that this strain secretes hormone B without prior stimulation by exogenous antheridiol. The strain was used in subsequent isolation experiments (17,18). Chromatography of methylene chloride extracts of culture liquids gave a biologically active crystalline fraction. The yield was ca. 1 mg from 20 *l.* of culture liquid, i.e., about the same order of magnitude as the yield of antheridiol.

This crystalline fraction has proved to be a complex mixture, consisting of seven or more

Oogoniol (R = H)
Oogoniol-1 (R = $(CH_3)_2CHCO$)
Oogoniol-2 (R = CH_3CH_2CO)
Oogoniol-3 (R = CH_3CO)

24(28)-Dehydro-oogoniol (R = H)
24(28)-Dehydro-oogoniol-1 (R = $(CH_3)_2CHCO$)

Figure 2.

steroids. Two sharply melting substances were first isolated and named oogoniol-1 and -2. Spectral analysis on the oogoniols and their derivatives led to structures which, in some respects, are similar to that of antheridiol (19). Thus, they both have 29-carbon skeletons and a Δ^5-7-ketone chromophore. However, the oogoniols possess ester functions at C-3 (isobutyrate and propionate) and C-11α- and C-15β-hydroxyl substituents. Also, there is no unsaturated lactone function on the side chain, but a primary hydroxyl group is present. The latter was at first though to be at C-26, but recent evidence from ^{13}C NMR spectra of model 3,26- and 3,29-dihydroxy 5-stigmastenes show that the primary hydroxyl is at C-29 (20). The stereochemical problem on the side chain in the revised structure is thus simplified, since there is only one chiral center which needs to be defined.

We have recently succeeded in doing this by preparing 29-hydroxy sitosterol and its C-24 epimer, 29-hydroxy clionasterol. The two epimers can be distinguished by their 2200 MHz NMR spectra. In the spectrum of the former, the C-26 and C-27 methyl protons appear as two doublets (centered at δ 0.84 and 0.85) while, in the spectrum of 29-hydroxy clionasterol, they appear as a triplet (due to coincidental overlap) at δ 0.84. This is exactly the pattern observed for the C-26 and C-27 methyl protons in the spectra of oogoniol-1 and -2 and indicates that the oogoniols have the clionasterol carbon skeleton (Figure 2).

The 220 MHz spectrum of oogoniol-1 shows two doublets (at δ 0.95 and 1.01), which were assigned originally to C-27 and C-21 methyl protons. The doublet at δ 1.01 has now been found to be due to the presence of another steroid, actually 24(28)-dehydro oogoniol-1. Although the sample of oogoniol-1 appeared to be a single compound on the basis of thin layer chromatography (TLC) in several solvent systems, it has been resolved into two components by high pressure liquid chromatography (HPLC). The major component (85%) has spectral properties fully consistent with the structure proposed for oogoniol-1. Significantly, the 220 MHz spectrum does not have a doublet at δ 1.01. The minor component (15%) has spectral properties which are in complete agreement with a 24(28)-dehydro oogoniol-1 structure. In particular, the 220 MHz NMR spectrum shows a strong doublet at δ 1.01 which is assigned to the C-26 and C-27 methyl protons. The methylene protons at C-29 appear together with the C-11β proton as a multiplet at δ 4.1, and the C-28 vinyl proton as a triplet at δ 5.3. The NMR spectrum also indicates the same substitution pattern on the tetracyclic nucleus as in oogoniol-1.

The mass spectrum of oogoniol-1 has an intense peak at m/e 458, which results from loss of isobutyric acid from the molecular ion. In the dehydro analog, there is no peak at m/e 458 but, instead, one at m/e 456 (M^+-88) and at m/e 438. These and others in the spectrum are in agreement with the proposed structure. The stereochemistry of the side chain double bond is probably the same as that in fucosterol (E) on biogenetic grounds. NMR spectral evidence supports this, because of the absence of C-25 proton resonance at δ 2.8, which is expected for the 24(28) Z-configuration.

Analysis of a sample of oogoniol-2 by HPLC indicated that the corresponding dehydro analog was also present. There is also evidence for the presence of dehydro analogs in samples of oogoniol-3, which was obtained as a mixture with the C-15 ketone corresponding to oogoniol-2, and of oogoniol. Thus, all samples of oogoniols so far isolated by TLC, irrespective of the species of *Achlya* which produces them, have been found to be mixtures of steroids possessing saturated and unsaturated side chains. The proportion of dehydroanalog varied with dif-

Fucosterol

Figure 3. Proposed biosynthetic pathways to sex hormones of *Achlya.*

ferent isolates and in some cases was as high as 25%.

BIOSYNTHESIS OF ANTHERIDIOL AND THE OOGONIOLS

Investigation of the biosynthesis of the sex hormones of *Achlya* has revealed much about the pathways by which these compounds are derived in the organism. In common with other members of the Saprolegniales and with the brown algae, *Achlya* contains fucosterol which, from a consideration of its structure, appears to be a likely precursor of antheridiol. This was first pointed out by Popplestone and Unrau (21) who were then able to demonstrate the incorporation of exogenous ^{3}H-fucosterol into antheridiol in cultures of the female strain of *A. bisexualis* (22). Feeding experiments with 5,22(*E*),24(28)-stigmastatriene-3β-ol and 3β-hydroxy-5,22(*E*),24(28)-stigmastatriene-29-oic acid showed that these steroids were intermediates in the biosynthesis. However, 3β-hydroxy-5,24(28)-stigmastadien-29-oic acid was incorporated only poorly into antheridiol. Thus, they propose that, in the conversion of fucosterol to antheridiol, the sequence of reactions involves dehydrogenation at C-22,C-23, followed by oxidation at C-29 and hydroxylation to form the hydroxy butenolide side chain (Figure 3).

Because the first oogoniols to be identified had a saturated side chain, we considered sitosterol or clionasterol as possible precursors. However, feeding experiments with these steroids showed poor incorporation into the oogoniols (5). Fucosterol, however, was found to be efficiently converted to the oogoniols by *A. heterosexualis*. Recent experiments with [CD_3]-methionine indicate that fucosterol is oxidized at C-29 to the aldehyde level (with the loss of two of the four deuterium atoms incorporated at C-28 and C-29) (23). Since the oogoniols contain a C-29 hydroxyl, a reversible dehydrogenase may be operating. Reduction of the "aldehyde" could occur at this stage or per-

haps after modification of the tetracyclic nucleus.

The identification of 24(28)-dehydro oogoniols suggests that the hydroxylation at C-11, and C-15, the oxidation at C-7, and the esterification of the C-3 hydroxyl take place before reduction of the C-24,C-28 double bond. Fucosterol itself is not reduced in *Achlya*, for neither clionasterol nor sitosterol could be detected in the mycelium (5). Hydroxylation at C-29 must apparently take place before reduction of the double bond can occur. It is interesting to note that the stereochemical outcome, i.e., production of a clionasterol rather than a sitosterol skeleton, is the same as that observed in algae such as *Ochromonas danica* (24). Our present knowledge concerning the biosynthesis of steroid hormones in *Achlya* can be summarized as shown in Figure 3.

BIOASSAY FOR ANTHERIDIOL AND THE OOGONIOLS

The production of antheridial hyphae in the strong male *A. ambisexualis* E87, after treatment with dilute solutions of antheridiol, can be readily demonstrated by Barksdale's method (25). The lowest concentration of antheridiol which will cause the formation of branches is ca. 10 pg/ml of solution. The concentration of antheridiol determines both the number of branches initiated and the time that elapses between the addition of hormone and the appearance of branches. The number of branches increases with increasing concentration of hormone until an upper limit is attained. The length of time before branches appear decreases with increasing concentration until a minimum of 40-45 min is reached. Branching also depends on the presence of nutrient, i.e., amino acids (edamin) and glucose. Without these, very little branching occurs, even with high concentrations of antheridiol. Indeed, Barksdale has suggested that hormone A′ might be an amino acid mixture (26). Raper had proposed that this factor is secreted by male mycelia, because filtrates from the male strain were found to enhance the effect of antheridiol (27). The properties reported for hormone A′ are consistent with those expected for an amino acid mixture.

Antheridiol is almost completely insoluble in water, but it is so potent that even very dilute solutions will produce a biological effect. Treatment with dilute acid or base rapidly inactivates antheridiol. The C-3 hydroxyl is readily lost by acid- or base-catalyzed elimination. Also, the hydroxy butenolide system on the side chain is very sensitive to base which causes isomerization of the double bond and ring opening.

Modifications in the structure of antheridiol give compounds with sharply reduced activity. Thus, the structure lacking the C-7 ketone has about 5% of the activity of antheridiol. Structures with a different configuration at C-22 or C-23 have activities which are 0.1% or less of that of antheridiol. 23-Deoxyantheridiol and the trienoic acid, which is a biosynthetic intermediate (see Figure 3), each possess about 0.1% of the activity of antheridiol.

The bioassay for the oogoniols can be demonstrated with the female strain *A. ambisexualis* 734 (17). Addition of a dilute solution of the hormone to a culture of this strain causes the formation of oogonial initials, which become visible after about 12 hr and reach a maximum number in 24-48 hr. Like antheridia, the number of oogonial initials increases with increasing concentration. They have a distinct spherical shape and are easily counted. In a strongly positive reaction, there are well over fifty in any field near the edge of the mycelial mat. The protoplasm in the developing oogonia may be quite dense compared to that in the hyphae. However, protoplasm does not differentiate to form oospheres, as is observed when male and female strains are grown together and conjugation of antheridial hyphae and oogonia occurs. In some cases, the oogonia put out branches which develop into additional oogonia.

The lowest concentration at which oogoniol-1 exhibits biological activity was first reported to be 620 ng/ml (19). The sample tested was actually a mixture of two compounds, oogoniol-1 and 24(28)-dehydro oogoniol-1. Recent tests show that the latter compound elicits a positive reaction in the female strain at a minimum concentration of ca. 50 ng/ml. The lowest concentration at which oogoniol-1 shows biological activity is about 5 μg/ml. Thus, the dehydro analog is 100 times more active and appears to be the true hormone. Reduction of the 24(28)-double bond is accompanied by a sharp drop in biological activity which is consistent with the idea that oogoniol-1 may be a metabolic product of the hormone.

MECHANISM OF ACTION OF ANTHERIDIOL

The biochemical events which are brought about by antheridiol in *Achlya* parallel closely what is known to take place in mammalian target tissue in response to steroid sex hormones. A number of investigators have shown that antheridiol stimulates the synthesis of ribosomal ribonucleic acid (RNA) and poly-

adenylic acid-containing RNA (presumptive mRNA) and protein. The synthesis of the latter occurs just prior to sex organ initial formation. Inhibitors of RNA synthesis such as actinomycin D, *p*-fluorophenylalanine, and cordycepin affect not only the accumulation of mRNA but prevent antheridial branch formation when added at the same time as antheridiol. By about 3 hr after addition of the hormone, no further RNA synthesis is required for branch initiation to occur. Protein synthesis, however, is required virtually throughout development. Silver and Horgen (28) and Timberlake (29) have proposed that changes in RNA synthesis induced by antheridiol are responsible for the production of new proteins required for differentiation of the male sex organs.

Groner et al. have reported a study on the induction of specific proteins in *A. ambisexualis* by antheridiol (30). They detected an induced protein, with a molecular weight of 69,000, 1 hr after addition of the hormone. The synthesis of this protein precedes the microscopic appearance of antheridial initials. Pulse labeling with L ^{3}H-leucine showed that synthesis of the 69,000 MW protein decreased after about 5 hr. Proteins excreted into the culture medium were also examined. Mycelium, induced with antheridiol for 1 hr, excreted proteins migrating at 40,000 MW and smaller amounts at 29,000 MW, 64,000 MW, and 88,000 MW. Increasing the labeling time to 3 hr or more resulted in excretion of a complex pattern of proteins, different from that found inside the mycelium and, therefore, not attributable to cell lysis.

The specific functions of proteins induced by antheridiol have also been investigated. Thomas and Mullins were the first to demonstrate that there is increased production of cellulase enzymes accompanying morphological expression of antheridial hyphae (31,32). Experiments with cycloheximide and actinomycin D indicated that both translation and transcription are required for the induction of cellulase. Hormone-treated mycelia previously treated with cycloheximide or actinomycin D were still capable of showing a production of antheridial hyphae and cellulase when the inhibitors were removed by washing.

Cellulase is believed to cause a localized softening of the hyphal wall which produces weak spots that are blown out into lateral blebs by turgor pressure. Electron microscope studies by Mullins and Ellis (33) and by Nolan and Bal (34) indicate that the cellulase is contained in vesicles that are localized at points of branch formation. The cell wall is known to contain ca. 15% cellulose and 85% of an amorphous noncellulosic polysaccharide complex. Interestingly, antheridiol does not stimulate cellulase secretion in female mycelia.

Another function of proteins induced by antheridiol appears to be the synthesis of the oogoniols from fucosterol. Barksdale and Lasure (17) found that the strong male *A. ambisexualis* E87 will not secrete the oogoniols unless grown together with a female, *A. ambisexualis* 734, or grown in the presence of antheridiol. Recently, we showed that ^{3}H-fucosterol is gradually taken up by the mycelium of *A. ambisexualis* E87 over a period of several hours during which time it is diluted by endogenous fucosterol. Addition of antheridiol will induce the synthesis of radioactive oogoniols, which can be detected in the culture liquid about 1 hr later. The amount increases steadily during the next several hours and then levels off (5). It appears that, on adding antheridiol, the enzymes required for the synthesis of the oogoniols are rapidly induced concurrently with enzymes required for development of antheridial hyphae. As expected, addition of cycloheximide at the same time as antheridiol completely inhibits production of oogoniols.

Very little is known about the biochemical processes which are involved in chemotropic growth of antheridial hyphae to the oogonial initials. Raper postulated that this directional growth was caused by a hormone distinct from hormone A (antheridiol), but Barksdale later showed that antheridial hyphae were attracted to particles of plastic previously treated with a solution of hormone A (35). We felt that it would be worthwhile to repeat the experiment, using synthetic antheridiol so as to eliminate any possibility that the chemotropic effect might have been caused by another substance present in Barksdale's sample. Silica gel particles (200 mesh) on which antheridiol was adsorbed were sprinkled on a culture of *A. ambisexualis* E87 and were observed to cause a strong chemotropic effect on antheridial hyphae, thus confirming Barksdale's conclusions.

There are two other effects which have been attributed to antheridiol. One is the formation of crosswalls (septa), which delimit the antheridia from the antheridial branches. A higher concentration (about 50-fold) of antheridiol is required for delimitation than for induction of branching (9). The other effect is the inhibition of formation of oogonial initials and also the degeneration of oogonial initials when added to mycelium having both male and female structures (36).

It is reasonable to assume that the hormonal interactions which have been demonstrated in

Achlya also take place in closely related organisms; for example, in members of the Saprolegniales and in the Peronosporales, if supplied with sterol. It is possible that *Pythium* or *Phytopthora* possess hormones analogous to antheridiol and the oogoniols and that they are formed from a precursor such as fucosterol (37). In view of the widespread occurrence of sterols in plants and fungi, it would not be surprising if these sterols were used for elaboration of hormones specific to particular genera.

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The Interaction of 1,25-Dihydroxyvitamin D_3 with Its Intestinal Mucosa Receptor: Kinetic Parameters and Structural Requirements

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ABSTRACT

Vitamin D_3 and its metabolites comprise an endocrine system which plays a critical role in calcium homeostasis. The active form of vitamin D_3 is 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$]. Chromatin localization of $1,25(OH)_2D_3$ and sucrose density gradient centrifutation have demonstrated the presence of an intestinal mucosa cytosol receptor which specifically binds $1,25(OH)_2D_3$. The kinetic parameters of $1,25(OH)_2D_3$ binding to its receptor have been determined by hydroxylapatite and reconstituted chromatin cytosol assays. Utilization of these assays has also permitted a determination of the precise structural requirements of the vitamin D ligand for the intestinal receptor. Furthermore, it has been possible to propose two receptor-ligand models which are capable of accommodating the conformationaly modile A ring of the vitamin D seco-steroids.

Vitamin D_3 is the naturally occurring form of calciferol and is normally derived by exposure to sunlight of the precursor 7-dehydrocholesterol (1) which is present in the skin. The primary biological functions of vitamin D in animals are to mediate intestinal calcium absorption and to permit normal skeletal development.

A formal definition of a "vitamin" is that it is a trace dietary constituent required to affect normal functioning of a physiological process. Emphasis here is on *trace* and the fact that the vitamin *must* be supplied dietarily; this implies that the body is unable to synthesize the vitamin in question. Thus, calciferol is only a vitamin when the animal does not have access to sunlight or ultraviolet light. Under normal physiological circumstances, all mammals, including man, can generate, via UV photolysis adequate quantities of vitamin D.

Chemically, vitamin D is a steroid and, in fact, its formal chemical name is based on the rules of nomenclature for steroids. It is largely due to a historical accident that calciferol has been classified as a vitamin rather than as a steroid hormone. While chemists have certainly appreciated the structural similarity between vitamin D and other steroids (1,2), this correlation has never been widely acknowledged in the biological/clinical or nutritional sciences. Most investigators continue to classify calciferol as a vitamin.

Until recently, little progress was made in understanding the mode of action of calciferol. Much work focused on the nutritional and putative cofactor functions of the molecule. However, since 1968 there has emerged a new model for the mechanism of action of this important seco-steroid. The model is based on the concept that, in terms of both chemical structure and mode of action, vitamin D is similar to other steroid hormones such as estradiol, testosterone, hydrocortisone, aldosterone, or ecdysone (3).

In fact, it is now recognized that there is an endocrine system for converting the prohormone vitamin D into its hormonally active form(s) (see Fig. 1). It is now agreed that the biologically active form of vitamin D, particularly in the intestine, is 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] (4,5). The endocrine gland which produces that biologically active form(s) of vitamin D is the kidney (6,7). After metabolic conversion of vitamin D_3 to 25-hydroxyvitamin D_3 [$25(OH)D_3$] by a liver mitochondrial enzyme (8), this circulating form of the seco-steroid serves as substrate for either the renal 25(OH)D 1-hydroxylase or the 25(OH)D 24-hydroxylase. Both enzymes (9,10) are located in the mitochondrial fraction of the kidney cortex. In fact, the 1-hydroxylase has been shown to be localized in the kidney of members of every vertebrate class from teleost through amphibia, reptila and aves to mammals, including primates (11). It has been shown that the 1-hydroxylase enzyme system is a classical mixed function steroid hydroxylase, similar to the hormone hydroxylases located in the mitochondria of the adrenal cortex. The 1-hydroxylase is a cytochrome P-450 containing enzyme (10) which involves an adrenodoxin component and which incorporates molecular oxygen into the 1α-hydroxyl functionality (5).

In recent years, a number of laboratories have directed their efforts toward the elucidation of the mechanism by which steroid hormones interact with their target tissues. For each of the different classes of steroid hormones, a similar mode of action has been demonstrated. Figure 2 shows a schematic representation of this general mechanism. The

FIG. 1. Metabolic pathway for the production of the hormonally active form of vitamin D_3, 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$].

distinguishing feature of a target tissue, enabling it to respond to a specific steroid hormone, is the presence of a receptor system in the target cell for that hormone. Upon entering the cell, the steroid is bound specifically and with high affinity to a cytoplasmic receptor. This complex subsequently becomes "activated," enters the nucleus of the cell, and becomes tightly associated with the chromatin fraction. In an as yet undefined manner, the interaction of the steroid-receptor complex with chromatin results in the induction of new proteins.

If the biological effects of 1,25$(OH)_2D_3$ are mediated through a classical steroid hormone mechanism of action, then one would expect its target tissues to possess a specific receptor system for this steroid. Not only must the presence of a receptor be demonstrated, but it must also exhibit the extreme specificity, high affinity, and low capacity for 1,25$(OH_2D_3$ that other steroid receptors exhibit for their respective hormones.

1,25$(OH)_2D_3$ stimulates intestinal calcium absorption (12,13), and thus the intestinal mucosa is a obvious target tissue. Early in vivo studies with radioactive vitamin D_3 or 1,25 $(OH)_2D_3$ demonstrated that there is a specific accumulation of 1,25$(OH)_2D_3$ in the chromatin fraction of chick intestinal mucosa cells (12,14), which suggested the presence of a receptor system. More direct evidence for the existence of a mucosal cytosol receptor has been obtained by means of sucrose density gradients. In this technique, two incubations of a low, but saturating, concentration of $[^3H]$-1,25$(OH)_2D_3$ are carried out with mucosa cytosol. The two incubations are identical, except for an excess of nonradioactive 1,25$(OH)_2D_3$, which is added to one. When these two incubates are subsequently centrifuged through 5-20% sucrose gradients, the presence of a receptor will be observed as a peak of radioactivity that is absent in the incubate to which excess nonradioactive ligand was added. This is due to the high affinity and low capacity of a true steroid hormone receptor. The top panel of Figure 3 demonstr-

ates this phenomenon for the cytoplasmic receptor for $1,25(OH)_2D_3$. The bottom panel of this figure shows that the chromatin-extractable complex also migrates which a sedimentation coefficient of ca. 3.7S in these gradients.

Although this technique demonstrates the existence of a steroid receptor complex, it must be further examined to show definitively specificity, high affinity, and low capacity. These properties can best be studied in a receptor binding assay. The assay we have found most useful for studying the binding of $1,25(OH)_2D_3$ to its receptor is a hydroxylapatite assay, developed in this laboratory (15). In this assay, bound and free steroid are separated by binding of the steroid-receptor complex to hydroxylapatite. Steroid that is nonspecifically adsorbed to the resin is removed by successive washes with a 0.5% Triton X-100 buffer. Specific protein-bound steroid is determined by performing the assay in the presence and absence of excess nonradioactive steroid. The experimentally determined equilibrium dissociation constant, association rate constant, and dissociation rate constant are summarized in Table I, along with some physical characteristics of the mucosa cytosol $1,25(OH)_2D_3$ receptor.

We have also extensively utilized a reconstituted chromatin-cytosol system to examine some of the properties of the receptor system (17). Figure 4 shows the saturation of the reconstituted system by $1,25(OH)_2D_3$. From this figure, it is clear that the receptor is of low capacity. From the level of saturation in Figure 4, we have calculated that there are approximately 2400 molecules of receptor per mucosa cell (based on a 1:1 stoichiometry). This value is in excellent agreement with that determined from the x-intercept of the Scatchard analysis of the binding of $1,25(OH)_2D_3$ to chick intestinal cytosol by the hydroxylapatite assay (Table I).

From the results presented here, it is clear that the receptor system for $1,25(OH)_2D_3$ from chick intestinal mucosa possesses all of the requirements of a classical steroid hormone receptor system. It is specific for $1,25(OH)_2D_3$, binds this ligand with high affinity, and is present in low concentration in each mucosa cell.

Although the chemical structure of vitamin D was determined in the Thirties, and the x-ray crystallographic analysis of crystalline form of the seco-steroid was reported in 1948 (18), it was not until 1974 that the truly unique structural aspects of the molecule became apparent. Wing et al. (19) reported that in

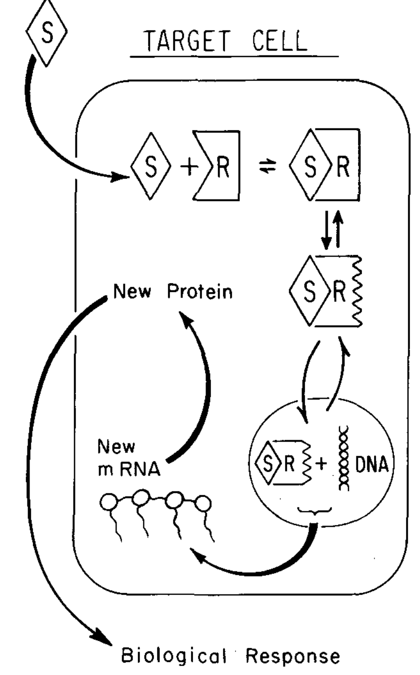

FIG. 2. Generalized model of steroid hormone action. S = steroid, R = cytosol receptor.

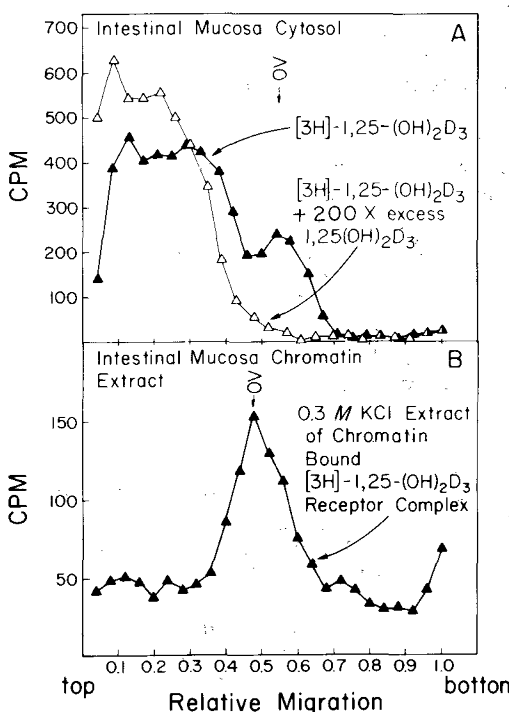

FIG. 3. Sucrose density gradient sedimentation profiles for intestinal mucosa cytoplasmic (A) and chromatin extractable (B) $1,25(OH)_2D_3$ receptors.

TABLE I

Properties of the Chick Intestinal Mucosa $1,25(OH_2D_3$ Cytosol Receptor

Property	Value
Ligand specificity[a]	$1,25(OH)_2D_3$
Sedimentation coefficient[b]	3.7 S
Molecular weight (daltons)[c]	65,000
Stokes molecular radius (Å)[c]	33.5
Molecules of receptor per cell	2500
Equilibrium dissociation constant (M)[d]	2.3×10^{-10}
Association rate constant ($M^{-1}min^{-1}$)[d]	7×10^6
Dissociation rate constant (min^{-1})[d]	4×10^{-5}
Half-life for dissociation (hr)[d]	290

[a]Determined by competition binding studies (16,17).
[b]Determined by sucrose density gradient sedimentation analysis (4).
[c]Determined by analytical gel filtration.
[d]Determined by a hydroxylapatite batch assay at 0.4 C (15).

solution vitamin D, all its metabolities, and any seco-B analogs have a high degree of conformational mobility. Through the use of high-resolution, 300 mHz, NMR spectroscopy, it was possible to demonstrate the rapid interconversion of the two A ring chair conformers. The existence of these ring conformers is a direct consequence of the fact that vitamin D is a seco-steroid. Clevage of the 9,10 carbon bond of ring B results in "unlocking" the A ring, which is fixed in other steroids, thus permitting a dynamic chair-chair interconversion many thousands of times per second.

One of the important consequences of this rapid conformational acitivity is that, for each chair-chair interconversion, every equatorial position becomes axial and every axial position becomes equatorial (19). Further, it is known from other chemical studies (19) that the equilibrium constant between the two conformers depends upon the nature and location of the substitutent groups on the conformationally mobile A ring. Experimental analysis of the conformational ratio of $1,25(OH)_2D_3$ indicates an equational-to-axial (e/a) ratio of 55:45 for the 1α-hydroxyl, while for the analogous 3-deoxy-1α,25-dihydroxyvitamin D_3 [3-D-$1,25(OH)_2D_3$] the e/a ratio for the 1α-hydroxyl is 70:30. Thus, it is now clear that the molecular shape and conformation of the seco-steroid, vitamin D_3, and of its hormonally active form, $1,25(OH)_2D_3$, has certain unique properties not shared by other steroid hormones.

In order to provide a detailed molecular description of the action of steroid hormones, it is mandatory, as one aspect of that analysis, to describe in detail the molecular architecture and functional groups of the steroid hormone which confer upon it the ability to recognize and interact with only its own receptor(s). In principle, all receptors in the vitamin D endocrine system must have the capability of accommodating to the conformational mobility of the A ring of vitamin D seco-steroids. We have attempted to relate the advantages, disadvantages, and consequences of the rapid chair-chair equilibration for the intestinal receptor system for $1,25(OH)_2D_3$ to its function (16,20,21).

Over the last 5 years, we have employed a number of strucural analogs of $1,25(OH)_2D_3$ in the reconstituted chromatin-cytosol assay to examine the features of the $1,25(OH)_2D_3$ molecule that are required for the optimal interaction with its receptor system. We have done this by using a competitive binding assay in which increasing amounts of nonradioactive competitor are incubated with a fixed concentration of tritated $1,25(OH)_2D_3$. The decrease in the amount of $[^3H]$-$1,25(OH)_2D_3$ bound to chromatin is subsequently measured. This ability to compete with the normal ligand for interaction with the receptor system can be related to the structural differences between the analog and the normal ligand. A greater ability of an analog to compete is an indication of the tolerance of the receptor for the particular structural modification in the analog. Analogs that are very poor competitiors possess structural modifications which prevent them from binding to the receptor.

The competitive binding results obtained with the reconstituted chromatin cytosol assay have been verified with the recently developed hydroxylapatite assay (15). This assay measures binding of $1,25(OH)_2D_3$ to the cytosol receptor without subsequent binding of the steroid-receptor complex to chromatin. Table II gives a summary of the relative competitive effectiveness of vitamin D structural analogs for the $1,25(OH)_2D_3$ receptor, expressed as a

relative competitive index (RCI).

We have found that a basic requirement for interaction of a steroid with the intestinal receptor system is that it must be a 9,10-seco-steroid. This implies that the receptor has a structural organization which can accommodate the conformationally mobile A ring. In addition, we have reported (16,21) that the relative importance of the individual hydroxyl groups is 1α> 25 >3β; th combined absence of the 1α- and 25-hydroxyl groups, as in vitamin D, abolishes interaction. Thus, 25(OH)D$_3$[1] and 1α (OH)D$_3$ were only 1/1000 as effective as 1α, 25(OH)$_2$D$_3$ in binding to the intestinal receptor, while 3-D-1α,25(OH)$_2$D$_3$ and 3-D-1α(OH)D$_3$ were only 1/16 and 1/1500 as effective, respectively. The intestinal receptor is very specific with regard to the functionalities on the A ring of the seco-steroid.

A second part of the 1,25(OH)$_2$D$_3$ molecule in which the intestinal receptor is very intolerant of change is the length of the 8-carbon 25-hydroxylated side chain. Lengthening the side chain of 25(OH)D$_3$ by one carbon or shortening it by one or two carbons while maintaing the tertiary hydroxyl functionality [as in 24-*homo*-25(OH)D$_3$, 24-*nor*-25(OH)D$_3$ or 23;24-*dinor*-25(OH)D$_3$] yields analogs that are only 1/400, 1/400, or >1/10,000, respectively, as effective as 25(OH)D$_3$ in interacting with the intestinal receptor. Similarly, the analog 24-*nor*-1,25-(OH)$_2$D$_3$ is only 1/2 as effective as 1,25(OH)$_2$D$_3$ (20,21).

Apparently, for the intestinal receptor for 1,25(OH)$_2$D$_3$ and probably also for other receptors in the vitamin D endocrine system, the two chair conformers of the A ring are not topologically equivalent. Therefore, it is interesting to answer two questions (a) when 1,25(OH)$_2$D$_3$ exists as a steroid-receptor complex, does the A ring of 1,25(OH)$_2$D$_3$ continue to oscillate between the two conformers; or (b) if in the steroid receptor complex, the A ring of 1,25(OH)$_2$D$_3$ is not oscillating, i.e., when it is "frozen out" by suitable ligand interaction with functional groups on the protein receptor, will only one or both conformers bind to the receptor? The experimental data cited above indicate that the intestinal receptor system is very specific with regard to the structure of the A ring as well as the length of the side chain. This indicates that the A ring does not continue to oscillate in the

[1]The structures of the parent metabolites of the above-mentioned analogs can be seen in Figure 1. The carbon skeleton is numbered so that the structural modifications that result in each analog can be visualized.

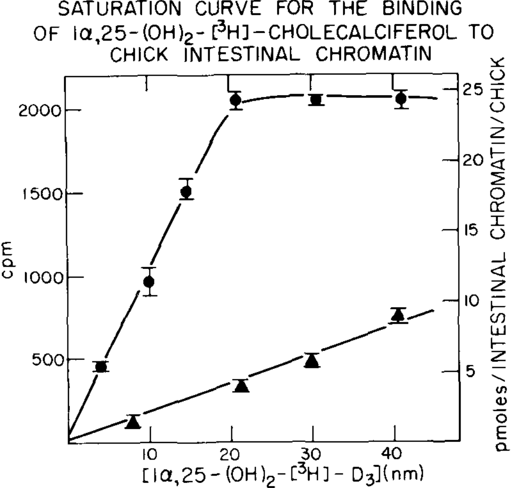

FIG. 4. Saturation of reconstituted chromatin cytosol system from intestinal mucosa by 1,25(OH)$_2$D$_3$ at 23 C (●) and at 0 C (▲).

TABLE II

Relative Competitive Index (RCI) Values for Vitamin D$_3$ Metabolites and Analogs[a]

Compound	RCI
1α,25(OH)$_2$D$_3$	100
24-*nor*-1α,25(OH)$_2$D$_3$	46
1α,24R,25(OH)$_3$D$_3$	41
1α,24R,25(OH)$_3$D$_3$	33
3-*deoxy*-1α,25(OH)$_2$D$_3$	5.7
1α-OH-D$_3$	0.06
25-OH-D$_3$	0.06
24R,25(OH)$_2$D$_3$	0.03
24S,25(OH)$_2$D$_3$	0.03
3-*deoxy*-1α-OH-D$_3$	0.03
24-*nor*-25-OH-D$_3$	0.01
24-*homo*-25-OH-D$_3$	0.01
1αOH-*epi*-D$_3$	0.01
D$_3$	0

[a]The data are expressed on a linear scale of relative competitive index (RCI) where 1,25(OH)$_2$D$_3$ is defined as having an RCI of 100.

steroid-receptor complex. Further, it seems unlikely that the receptor would have two identical binding sites for 1,25(OH)$_2$D$_3$, one for "freezing out" each ligand.

If the foregoing postulates are valid, then we envision at least two models which may explain the observed experimental data for binding of 1,25(OH)$_2$D$_3$ to its intestinal receptor. There are several reports that the cytosol receptors for steroid hormones exist in solution as proteins, capable of two basic conformations (17). It is possible that each conformation of the receptor system can bind only one of the two chair-chair species of 1,25(OH)$_2$D$_3$. Further, if only one

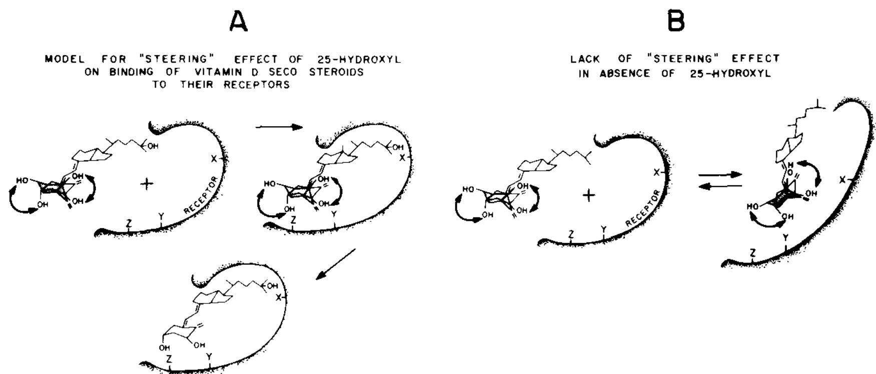

FIG. 5. Schematic representation of the postulated function of the 25-hydroxyl group and proper length side chain of $1,25(OH)_2D_3$. In (A) the 25-hydroxy group forms an initial association with a region, *X*, on the receptor. This initial association positions the A ring next to determinants, *Y* and *Z*, which will interact with A in the proper chair conformation. Without the 25-hydroxy group (B), the $1,25(OH)_2D_3$ molecule is not maneuvered into the proper position for A ring fixation.

of the steroid-receptor complexes can become activated and migrate to the nucleus, as has been proposed by Samuels and Tomkins (17), this suggests that only one of the two $1,25(OH)_2D_3$ conformers is biologically active. In fact, on the basis of a literature review, we have proposed that when the 1α-hydroxyl is oriented equatorially, there will be enhanced biological activity (20). This hypothesis is very attractive to the synthetic organic chemist. It is possible to envision the chemical synthesis of various analogs of $1,25(OH)_2D_3$ with e/a ratios for the 1α-hydroxyl ranging from 0.1 to 10, which would be more or less biologically active than $1,25(OH)_2D_3$. It follows logically from what has been said that the intracellular sequence of events must contain an irreversible step. Otherwise the "inactive" steroid conformer could be reversibly transformed back into the "active" steroid conformer, thus negating any differences due to the structural orientation of the A ring hydroxyls.

A second model describing the interaction of $1,25(OH)_2D_3$ with its intestinal receptor focuses on the key contributions of the 25-hydroxyl group on a side chain of precisely the correct length of 8 carbons. It is apparent from the data obtained from the steroid competition assays (summarized above) that $1,25(OH)_2D_3$, as it interacts with its cytosolic receptor, forms areas of "interaction" with *both* its A ring and with the 25-OH group on the end of the side chain. Thus, both ends of the seco-steroid molecule are intimately involved in the cytosol receptor system. Based on the hypothesis that the A ring of the conformationally mobile steroid will ultimately be "frozen out" in the final form of the stable steroid-receptor complex, a major problem for the receptor will be the proper "capture" of the dynamically active A ring portion of the molecule. It is in this regard that the important contribution of the 25-hydroxyl group on a side chain of the correct length may be understood. As shown in Figure 5A, the 25-OH group may function as a preliminary recognition site, which forms the first step of the interaction between the $1,25(OH)_2D_3$ molecule and the receptor. After this this interaction is effectively established, the protein receptor can ultimately capture and "freeze out" the A ring portion of the ligand. This result would be consistent with the inactivity of analogs like 1α-OH-D_3, which lack the 25-OH group, or those whose side chain is shortened, as in 24-*nor*-$1,25(OH)_2D_3$, even though they have optimal functionalities in the A ring. As shown in Figure 5B, in the absence of the 25-group, there can be no preliminary "captive process" of the side chain and, accordingly, much higher concentrations of the analog are required to permit the relatively more random "freezing out" process of the A ring.

If the model in panels A and B of Figure 5 is correct, this would be consistent teleologically with the fact that 25-hydroxylation of vitamin D precedes either 1α- or 24R-hydroxylation. The presence of the 25-OH group on the seco-steroid is required to permit further processing of this conformationally mobile molecule by the vitamin D endocrine system.

The identification and study of the molecular biology of the vitamin D endocrine system and its various interactions with the conformationally mobile seco-steroids has enormous implications for the understanding of

the plethora of disease states in man known to be related to vitamin D. These include a host of bone diseases, e.g., rickets, osteomalacia, osteoporosis, perturbations of parathyroid function, and chronic renal failure (22). It is hoped that further elucidation of details of the vitamin D endocrine system will ultimately not only prove of value to biochemical endocrinology and our conceptions of the molecular mode of action of steroid hormones, but also to the development of suitable treatment for these various disease states.

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Sterols in Yeast Subcellular Fractions

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ABSTRACT

Yeast is the most primitive organism synthesizing substantial amounts of sterols. Because of this eucaryotic organism's versatility in growth conditions, ease of culture, well-defined genetic mechanism, and characteristic subcellar architecture, it is readily applied to studies of the role of sterols in the general economy of the cell. Sterols exist in two major forms, as the free sterol, or esterified with long chain fatty acids. The importance of sterols for this organism can be demonstrated using a naturally occurring antimycotic azasterol. This agent inhibits yeast growth. Three effects are seen on sterol synthesis: inhibition of the enzymes Δ^{14}-reductase, sterol methyltransferase, and methylene reductase. Cells cultured on respiratory substrates are more sensitive to inhibition than are cells growing on glucose. We have demonstrated a relationship between respiratory competency and sterol biosynthesis in this organism. Many mutants altered in sterol synthesis are respirationally defective and must grow fermentatively. One clone has temperature conditional respiration. Experiments with purified mitochondria, prepared from this mutant and its isogenic wildtype, show that the mutant organism is able to respire at the higher temperature but lacks the ability to couple respiration to phosphorylation. No similar loss is seen in the wild-type clones. Data are given which support the proposal that, for inclusion in mitochondrial structures, yeast cells may discriminate among sterols available from the total sterol pool in favor of ergosterol.

INTRODUCTION

Yeast cells have been a source of sterols for decades. Smedley-MacLean purified ergosterol from *Saccharomyces* over three-quarters of a century ago (1). Although ergosterol is the major yeast sterol, about twenty other sterols have been isolated as intermediates in ergosterol biosynthesis or as end-products, accumulated by yeast mutants with altered sterol synthesis. Most yeast cultures produce about 0.1% of their dry weight as ergosterol, but in some strains of *Saccharomyces* 7% to 10% of their dry weight is in the form of ergosterol (2). Because of the ease with which these organisms are cultured, the ready separation of sterols from the cells by saponification, and the abundance of yeast as a product of industrial fermentation, yeast sterols have received wide attention. *Saccharomyces cerevisiae* is an excellent organism for basic studies on sterol synthesis and metabolism because a wealth of biochemical, genetic, and physiological data as well as procedures for isolating mutants defective in sterol synthesis are available.

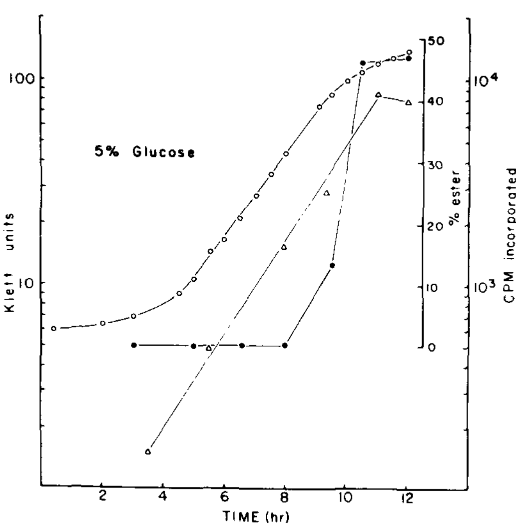


FIG. 1. Change in the level of sterol esters during fermentative growth. Growth of an aerobically shaken culture in 5% glucose was followed optically with a Klett colorimeter. Samples were taken periodically and assayed for the level of sterol esters and the incorporation of the methyl-^{14}C group of methionine into the nonsaponifiable lipid fraction. Symbols: ○, Klett units; •, percent esters; △, counts per min (cpm) per 50-ml sample incorporated into the nonsaponifiable lipids. The generation time in this experiment was 90 min. Reprinted from Ref. 6 with permission.

THE PRINCIPAL FORMS OF YEAST STEROLS

From the earliest work on the total sterol content of *S. cerevisiae*, it was observed that substantial portions of the sterol are esterified with long chain fatty acids (3,4), principally oleic and palmitoleic acids (5). We have observed considerable variation in the amount of sterol esterification, dependent upon the culture phase and growth medium of the organism (6-8). In all media, however, a rapid increase in sterol esterification occurred, preceding entry of the culture into the stationary phase of growth. Figure 1 shows one such experiment. Using an in vitro assay for sterol synthesis, we have observed a more than tenfold increase in sterol esterase activity during the later phases of culture growth (9), (Fig. 2).

Steryl ester synthesis in these organisms appears to accelerate during growth retardation. Sobus et al. (10) demonstrated that trifluperidol caused a fourfold increase in steryl esters, and others (11) have shown a sharp increase in steryl ester formation during spore formation. Thus, esterification is not necessarily stimulated by reduced levels of essential growth components from the medium.

The bulk of the steryl esters and triglycerides occurs in lipid particles (12) that can be observed microscopically. Such deposition might lead one to predict that steryl esters are simply inert storage products for the cell. However, it has been shown in our laboratory that the sterol moiety of steryl esters is exchangeable with the pool of free sterols (13).

The common fungus, *Geotrichum flavobrunneum,* produces a complex mixture of azasterols that have antifungal properties (14,15). One of these, 15-aza-24-methylene-D-homo-8,14-cholestadiene, is structurally similar to fecosterol (16) (Fig. 3). Respiring yeast cultures are much more sensitive to azasterol than are those growing fermentatively on glucose (17). At low concentrations, the azasterol inhibits the Δ^{14}-reductase, causing the accumulation of 8,14-ergosta-dien-3β-ol (18). At much higher concentrations, the Δ^{24}-sterol methyltransferase and 24(28)-methylene reductase are also inhibited (19). However, by using low but carefully determined amounts of the azasterol, it is possible to obtain cultures with both ergosterol and its 8,14 analog. We have assigned the trivial name of ignosterol to the latter compound.

A culture of yeast was grown to stationary phase in a medium containing (^{14}C-methyl)-methionine. This labels all of the C-28 sterols, but the pool contains principally ergosterol. Under these conditions, 80% of the sterol in the cell was esterified. This culture was then used as an inoculum for an unlabeled medium, containing sufficient azasterol to permit ignosterol accumulation without permitting ergosterol formation from it. As may be seen in Fig. 4, the prelabeled sterol shifted into the free form until only 15% remained esterified. Thus, mobility of the sterol components in the steryl ester pool is achieved under these conditions. In experiments we will describe elsewhere, we have found that, under a variety of cultural conditions, interconvertibility of the sterols and steryl esters may be demonstrated (Taylor and Parks, unpublished data).

Along with steryl esters and free sterols, steryl glucosides are commonly observed in higher plants. Because of the similarity of yeast sterols to those in higher plants, it might be anticipated that steryl glucosides would also be found in yeast. Using a thin layer chromatographic separation procedure, preliminary evidence for glucosides in yeast has been re-

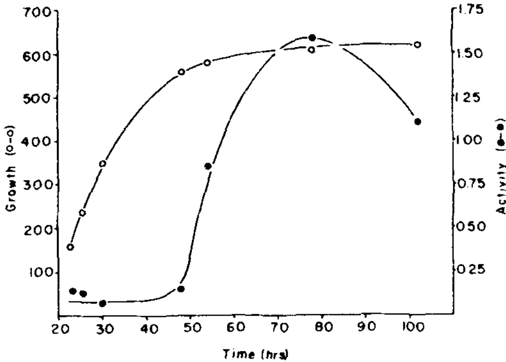


FIG. 2. Sterol esterifying activity as a function of age of the cell culture. o, Culture turbidity, as measured photometrically; •, of sterol-esterifying enzyme at the indicated points. Reprinted from Ref. 9.

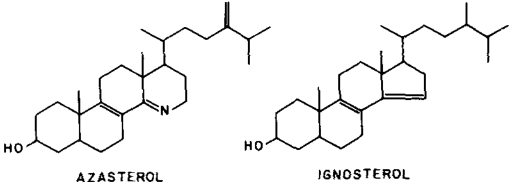


FIG. 3. Structures of the antimycotic azasterol and the ergosterol precursor, ignosterol.

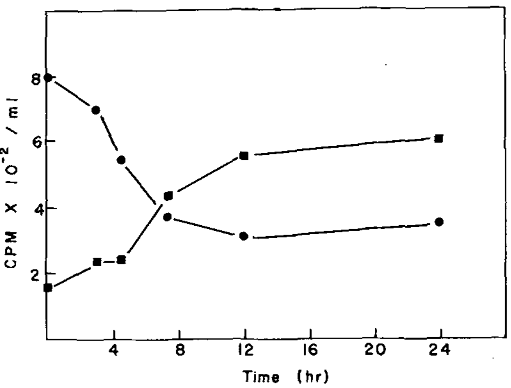


FIG. 4. A culture of yeast cells was grown to stationary phase in media containing (methyl-14C)-methionine, which is incorporated as C-28 of ergosterol. The cells were then washed three times and used as an inoculum (1:100 dilution) into unlabeled media containing 10 μg/ml azasterol. Aliquots were harvested at the indicated times during growth, and the amount of radioactivity in the free ▪ and • sterol pools was determined.

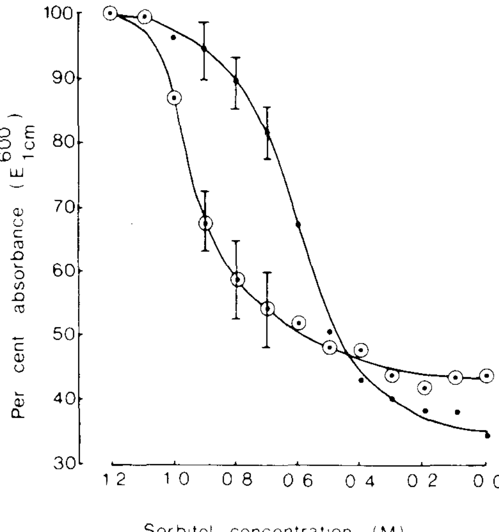


FIG. 5. Stability of spheroplasts from cells enriched in ergosterol (•) or cholesterol (○) when suspended in hypotonic solutions of buffered sorbitol. Portions (0.1 ml) of spheroplast suspension (containing 10 mg [dry weight equivalent] per ml of buffered 1.2 *M* sorbitol) were added to 2.9-ml portions of buffer, containing sorbitol in concentrations ranging from 1.2 M to zero. The contents of each tube were then shaken gently and maintained at room temperature (18 to 22 C) for 10 min. Then, the absorbance ($E^{600}_{1 cm}$) of the suspension was measured. Each value plotted is the average of at least three independent observations. The vertical bars indicate 95% confidence limits on values. Reprinted from Ref. 28 with permission.

ported (20). Purified products were not described, however. We have obtained enzymatic evidence for a glucosylation reaction in yeast, but we have not yet identified a specific steryl glucoside as the product of the reaction either. Our preliminary data indicate that the enzyme may be of broad specificity, glucosylating several products with free hydroxyl groups.

STEROLS IN THE YEAST PLASMA MEMBRANES

The yeast plasma membrane is highly enriched with free sterols (21), and over half of the total cellular lipid may be found there (22). Research from the laboratory of A.H. Rose (Bath University, Bath, United Kingdom) has given considerable insight into the relationship between sterols and the protoplast membrane. In one strain it was observed that whereas the sterol content of the whole cell was 1.89%, this membrane was enriched to 6% sterol (23). Under anaerobic contions, yeast must be provided a sterol supplement, since anaerobiosis prevents sterol formation (24). Detailed analyses of this phenomenon have been made (25,26). Incidentally, the structural requirements for sterols that satisfy anaerobic growth – a planar structure with a 3β-hydroxyl group and a long alkyl side chain – are fundamentally the same as those for sterol penetration into phospholipid liposomes (27).

By providing yeast cells growing under anaerobic conditions with a variety of sterols, Dr. Rose's group has been able to assess the effect of sterols on certain membrane properties (28). Under these conditions, plasma membranes could be enriched from 67% to 93% with the exogenously supplied sterols, but the total cellular content of sterol fractions and phospholipids were quantitatively similar. The capacity of the plasma membrane to resist stretching was measured, using cells freed of their walls by enzymic removal. The resulting spheroplasts were stabilized in sorbitol, and then their volume changes were measured as the sorbitol content was decreased. Figure 5 shows the result of one such experiment. From a variety of these analyses, one generalization could be made: spheroplasts enriched with sterols having an unsaturated side chain at C-17 were more resistant to osmotic stretching than were preparations enriched with other sterols.

STEROLS AND RESPIRATION

A substantial literature has accumulated relating ergosterol biosynthesis to respiration. Both enzymic and cytological studies have shown ergosterol to affect mitochondrial structure (29-33). We have observed that ergosterol is able to reduce the loss of respiratory competency under conditions which would otherwise induce a major decline in the maintenance of respiration (34).

Several reports show that there are effects of sterols on respiratory function and on various enzymes located in the inner and outer mitochondrial membranes. Research from the laboratory of Dr. J.M. Haslam (University of Liverpool, Liverpool, U.K.) has established that yeast mutants defective in δ-aminolevulinic acid (δ-ALA) formation are incapable of forming respiratory cytochromes, unsaturated fatty acids, and sterols. Supplementation of growth media with increasing concentrations of δ-ALA produced progressive increases in the cellular amounts of the cytochromes, unsaturated fatty acids, and sterols in the mutant (35). By manipulating the amounts of these three constituents, it was shown that there is a loss of oxidative phosphorylation in the mutant, due to the loss of the proton gradient associated

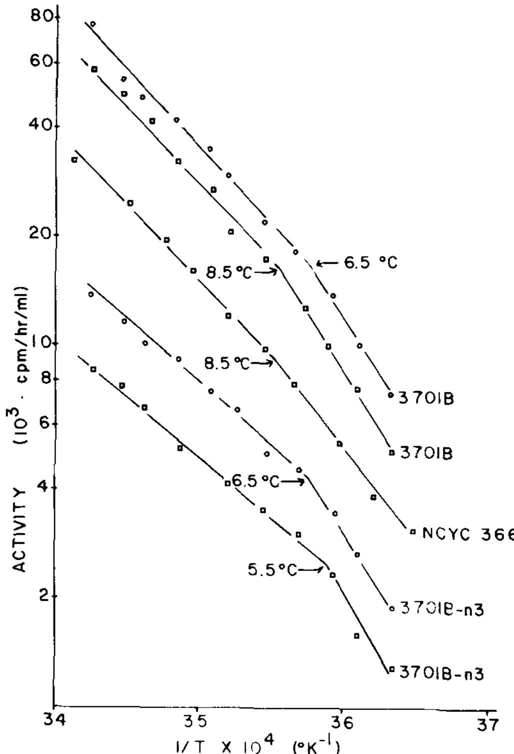
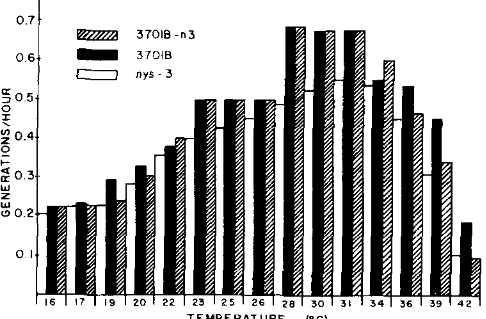


FIG. 6. Arrhenius kinetics of S-adenosylmethionine: Δ^{24}-sterol methyltransferase from wild-type (3701B) and nystatin-resistant mutants (3701B-n3, nys-3, and NCYC 366) of *S. cerevisiae.* ○, Enzyme isolated from glucose-repressed cells (promitochondria); □, enzyme isolated from respiring cells (mitochondria). Reprinted from Ref. 45 with permission.



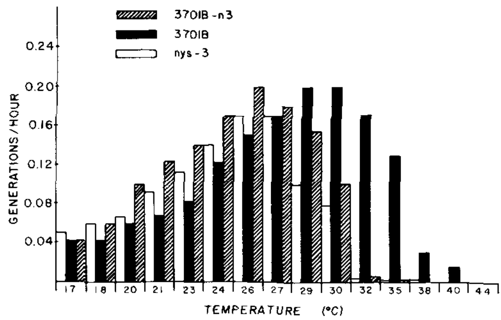


FIG. 7. Top: Temperature profile of growth rates for wild-type (3701B) and sterol mutants (3701B-n3 and nys-3) of yeast growing fermentatively. All three strains were pregrown in 5% glucose at 37 C, then transferred to the indicated temperature. Growth was measured turbidimetrically, and the maximal rate during logarithmic growth is reported. Bottom: Temperature profile of growth rates for wild-type (3701B) and sterol mutants (3701B-n3 and nys-3) of respiring yeast. All three strains were pregrown in rich broth at 27 C with ethanol as the carbon source, then transferred to the indicated temperature. Growth was measured turbidimetrially, and the maximal rate during logarithmic growth is reported. Reprinted from Ref. 45 with permission.

with the decreased sterol content (36). Further, as the sterol content decreased, the ability of the mitochondria to accumulate ATP from the cytosol was also impaired (37).

An effective technique which may be used to demonstrate the association of lipids with an enzyme is to measure the activity of the enzyme as a function of temperature. Usually, on plotting the log of activity of the enzyme as a function of the reciprocal of the absolute temperature, a straight line is obtained. Such an Arrhenius plot may be used to calculate the activation energy for that reaction. Many membrane- and lipid-associated activities reveal discontinuities in their Arrhenius plots that correspond to phase transitions in the lipid components (38). Membrane-bound enzymes from yeast mitochondria give discontinuities in the Arrhenius plots, while the soluble enzymes do not (39).

Mitochondrial ATPase with increasing amounts of sterol showed a progressive decrease in the transition temperature (40). Similar results were obtained with kynurenine hydroxylase. Monoamine oxidase showed no such temperature response even though it is also a component of the outer mitochondrial membrane (33,34,41,42).

In our laboratory, we have studied the Arrhenius kinetics of cytochrome c oxidase and sterol methyl transferase, using wild-type yeast and a mutant strain derived therefrom that is defective in sterol biosynthesis (Fig. 6). The mutant strain produces no ergosterol but produces 7,22-ergosta-dien-3β-ol and ergosta-8, 22-dien-3β-ol. While the total sterol pool of the mutant was greater than in the wild-type, the amount of mitochondrial sterol was the same. No differences were seen in the fatty acid content either (43). The transition temperatures for cytochrome c were 10 C (wild-type) and

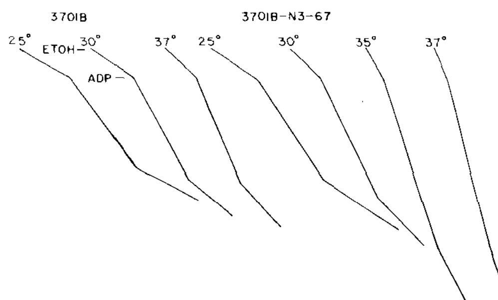


FIG. 8. Polarographic measurement of Mutant 3701B at 25, 30, and 37 C, and of Mutant 3701B-N3-67 at 25, 30, 35, and 37 C. The reaction medium contained 0.6 *M* mannitol, 10 n*M* potassium phosphate buffer (pH 6.6), 15 *mM* tris-maleate buffer (pH 6.6), 10 *mM* KCl, and 0.1 *mM* EDTA. Final protein concentration in the reaction mixture were 0.58 mg for 3701B-N3-67 and 0.55 mg for 3701B. Final concentration of ethanol as substrate was 0.5%. ADP concentration was 125 μM.

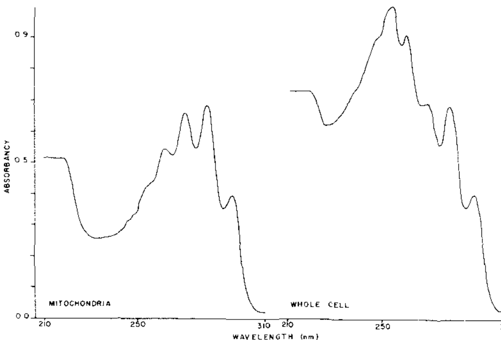


FIG. 9. The absorption spectra of the sterol fractions of whole cells and mitochondria grown in azasterol. See text for details.

4 C (mutant); whereas, the sterol methyltransferase showed transitions at 8.5 C and 5 C for the wild-type and mutant, respectively. The methyl transferase is not as tightly bound to the inner membrane as is the cytochrome c oxidase (44).

Thus, while alterations are seen in these phase transitions by clones producing different sterols, the precise localization of the enzyme will affect this result also.

This mutant also displays an interesting growth phenomenon. Optimal and permissive growth temperatures in respiring and fermenting cultures were compared using the mutant and its parent. As may be seen in Figure 7, fermentative growth was relatively unaffected by sterol composition. However, a marked decrease in both the optimal and permissive growth temperatures of cultures respiring on ethanol was observed, when ergosterol was replaced by the mutant sterols (45). The mitochondria may be more affected by sterol substitutions than the plasma membrane.

We have made polarographic measurements of oxygen consumption by intact mitochondria, prepared from the wild-type and mutant clones. Ethanol was used as the oxidizable substrate, and respiration was restricted by limited ADP concentrations. A series of comparative analyses was made over the temperature range from 25 C to 37 C. The results are shown in Figure 8. In the mutant strains, both an increase in respiration rate and a decrease in the coupling of respiration to ADP was seen in comparison with the wild-type. In addition, sodium ion prevented coupling of respiration to ADP in the mutant, but not in the wild-type. The decrease in coupling with temperature and the sensitivity to sodium ion suggest that increased cation permeability in the mutant may prevent the establishment of a proton gradient adequate to permit respiration of ethanol for growth.

SELECTIVITY IN STEROL DEPOSITION

We have found that other mutants defective in sterol synthesis also become respirationally incompetent. Similar observations have been made in other laboratories. It might be anticipated that for the maintenance of respiration there would be preferential utilization of ergosterol over other available sterols by mitochondria, and perhaps by other membranes as well. We have attempted to test this prediction, using wild-type yeast, which was altered in sterol synthesis by the specific antimycotic, azasterol. The mode of action of this compound was described earlier.

Wild-type yeast were inoculated into a complete growth medium containing 5 nm/g per ml of azasterol. The culture was allowed to grow for 36 hr and was then harvested. The mitochondria were prepared and purified by sucrose gradients. After saponification, the sterols were isolated. Whole cells were also saponified and the sterols isolated from them. Figure 9 shows absorption spectra obtained from the two preparations. Ignosterol exhibits a λ max of 250 nm, which is characteristic of a sterol containing a $\Delta^{8,14}$-heteroannular conjugated diene (46). It is clear that, although half of the total cellular pool is made up of the atypical $\Delta^{8,14}$-compound, the mitochondrial fraction contains almost exclusively ergosterol. We propose that there is selectivity in sterol deposition in the mitochondrial structure, and perhaps in other membranes as well. The basis

for this selectivity may be the sterol ester synthesizing enzyme. Under the conditions of this experiment, ignosterol exists primarily as steryl ester, whereas ergosterol is mainly uncombined. In further support of our hypothesis is the observation that anaerobic yeast cells which were supplemented with cholesterol during anaerobiosis almost completely replace that cholesterol with ergosterol in the mitochondrial membrane upon aeration (47). Thus, given the choice between cholesterol and the yeast sterol ergosterol, the cells selected the latter. The basis for this discrimination is under investigation in our laboratory.

ACKNOWLEDGMENTS

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Insect Juvenile Hormones and Pheromones of Isopentenoid Biogenesis

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ABSTRACT

In their diversity, speciation, and sheer numerical superiority, few should question that insects are the dominant life-form on earth. Their utilization of the multifunctional isopentenoids to regulate their life processes is equally diverse. To catalog or even summarize the contribution of isopentenoids in the regulatory chemistry of insect feeding, development, reproduction, diapause, and behavior is beyond the scope of this review. However, a topical treatment of the chemistry of insect juvenile hormones and pheromones provides an insight into the dependence of insects upon isopentenoids.

JUVENILE HORMONES

Metamorphosis

Since insects possess no internal skeleton, they rely upon a hard, sclerotized cuticle for support. As the insect grows, it must periodically shed its exoskeleton and form a new, looser one in order to increase in size. This process is called molting. During its immature life, the juvenile hormones (JH) are secreted from tiny glands in the head, called the corpora allata (CA). As long as JH is present, the immature insect molts successively into additional immature stages. Eventually the insect shuts off the flow of JH, and in the absence of these hormones it is able to metamorphose into the adult stage (Fig. 1). Metamorphosis, therefore, occurs in the absence of the JH. If JH is supplied to an insect in the immature stage that is about to undergo metamorphosis, the insect is forced to develop into an additional immature stage.

Reproduction

In the adult insect, the CA resume the secre-

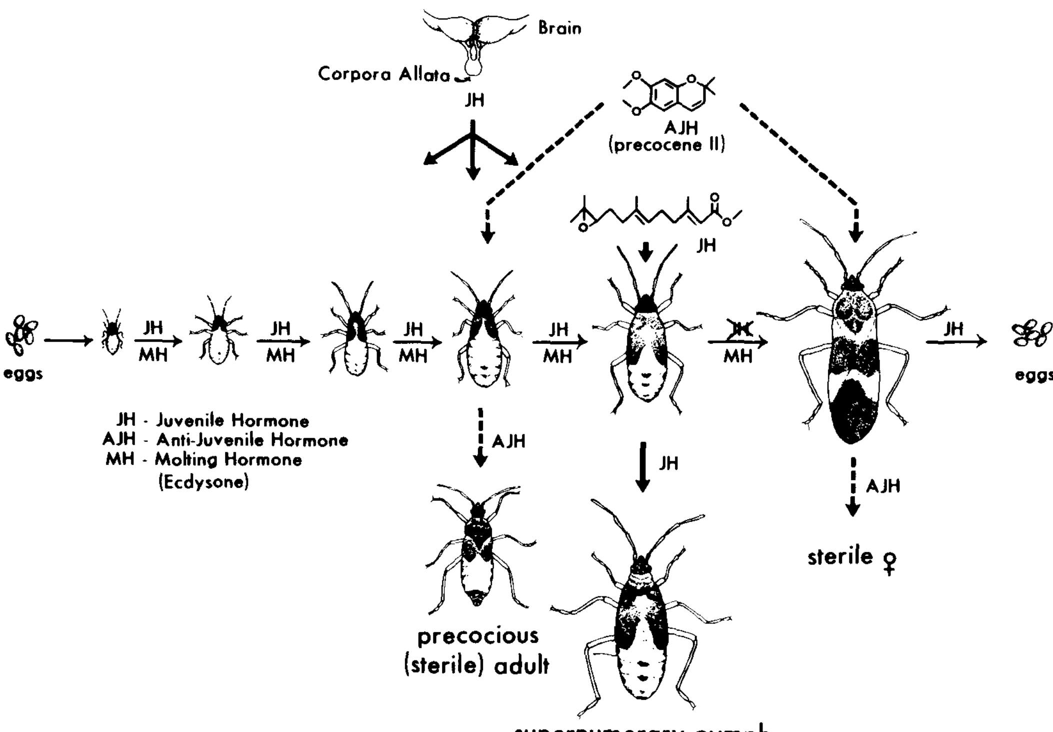

FIG. 1. Regulation of insect metamorphosis and reproduction by hormones and anti-hormones.

tion of JH, which is necessary for the development of the female ovaries. Juvenile hormones now serve as gonadotropic hormones (Fig. 1).

Diapause

During periods of climatic stress (i.e., winter, drought), many insects are able to enter a physiological resting state called diapause. During diapause, there is little or no feeding, mating, or reproduction. In insects such as the Colorado potato beetle, alfalfa weevil, and cereal leaf beetle, diapause clearly results when the flow of JH is turned off (1-4).

Sex Attractant Production

Another function of JH in adult insects is to *stimulate the production of sex pheromones*, which may serve as aggregation or distance attractants and/or promote the sexual excitement necessary for successful mating. It seems a reasonable economy for the insect to use the same hormone for ovarian maturation and for advertising its receptivity.

Chemistry of the Juvenile Hormones

The first steps toward the elucidation of the chemistry of JH began with the fortuitous discovery of the JH activity of farnesol (5). Modifications of the structure and biological activity of this ubiquitous sesquiterpenoid resulted in the synthesis of E,E-10,11-epoxy farnesenic acid methyl ester (I) (6) before it was established as a natural JH (7) (Fig. 2). Subsequently, additional JH (II, III) were identified from the lipid extract of male cecropia moths (8,9). Compounds with JH activity have been found in a wide variety or organisms from bacteria (10) to plants (11). The discovery of exceptional activity in various paper products (12) led to the identification of juvabione (11)

FIG. 2. Natural juvenile hormones.

FIG. 3. Plant products with juvenile hormone activity.

Incorporation	Product
Acetate + Mevalonate	R = CH_3
Acetate + Propionate + Mevalonate	R = CH_2CH_3

FIG. 4. Biosynthetic scheme for juvenile hormones.

Precocene I　　Precocene II

FIG. 5. Anti-juvenile hormones from *Ageratum.*

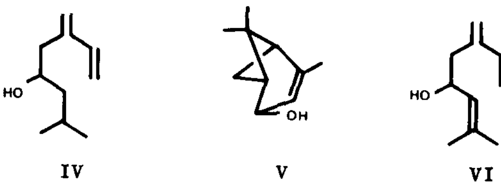

FIG. 6. Sex pheromones of the male bark beetle, *Ips paraconfusus.*

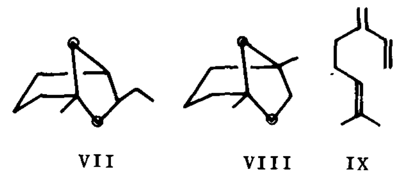

FIG. 7. Sex pheromones of the Western pine beetle, *Dendroctonus brevicomis.*

CH_2OH　H C CH_2OH　H C CHO　OHC C H

X　XI　XII　XIII

FIG. 8. Sex pheromones of the cotton boll weevil, *Anthonomus grandis.*

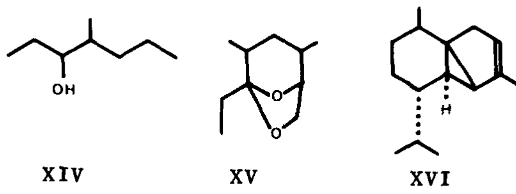

FIG. 9. Sex pheromones of the Elm bark beetle, *Scolytus multistriatus.*

and dehydrojuvabione in the balsam fir (13) (Fig. 3). Their sesquiterpenoid character and relationship to the natural JH is evident. The sesquiterpenoid character of the JH has been confirmed by biosynthetic studies (14), involving incorporation of radio-labeled intermediates (acetate, propionate, and mevalonate)

XVII

XVIII

XIX

FIG. 10. Sex pheromones of the California red scale, *Aonidiella aurantii* (XVII, XVIII) and of the yellow scale *Aonidiella citrina* XIX).

into JH II and III. Degradation studies have established a rational scheme of biosynthesis (Fig. 4).

Much of the enthusiasm over the juvenile hormones was prompted by the hope that these simple sesquiterpenoid hormones or analogs might become successful candidates for insect control. However, because the hormones are effective in deranging development only during the ultimate period of metamorphosis, they are useless for the control of most agricultural pests. However, insects that are pests in the adult stage (mosquitoes, manure breeding flies, etc.) are satisfactorily controlled by the JH analog Methoprene (15).

ANTI-JUVENILE HORMONES

When it was realized that JH are not a panacea for insect control, a few investigators sought an alternate endocrinological approach to the development of insect control agents; namely, anti-juvenile hormones (AJH). The rationale for this approach is quite clear. Insects are "full" of JH during their immature and adult life; thus, the only stage during which exogenous JH can exert a lethal morphogenetic action is during that short period when metamorphosis is programmed to proceed in its absence. However, if, JH biosynthesis, secretion, transport, or interaction at a target site could be prevented, a wide range of developmental and reproductive crises could be induced. From classical insect endocrinology, we know that extirpation of the CA from immature insects precipitates precocious metamorphosis into tiny sterile adults. Removal of the CA from adults causes female sterilization, and in certain insects it induces diapause.

(+)-bornyl acetate XX | (+)(E)-verbenyl acetate XXI | Germacrene D XXII | Periplanone B XXIII

FIG. 11. Sex pheromone mimics of the American Cockroach, *Periplaneta americana* and periplanone B, a natural pheromone component.

A secretion of the brain in *Manduca* called "allatohibin" is reported to be responsible for the inhibition of CA activity during the last larval instar of this insect (16). This discovery represents a beginning in the understanding of the natural physiological mechanism of JH regulation in insects.

Since plants have been found to contain compounds with both insect JH and molting hormone activity, it is not surprising that they were found to contain substances with AJH activity. Two compounds (Fig. 5) were isolated and identified from the bedding plant *Ageratum houstonianum,* which possessed AJH activity for a variety of hemipterous insects (17,18). They were subsequently found to be active in certain Orthoptera (19). Upon contact, these simple chromenes induce precocious metamorphosis of immature insects and sterilize the adult stages. Other AJH activities, such as the induction of diapause and inhibition of sex attractant production, have been demonstrated (17).

PHEROMONES

Insect communication by chemical agents is an exceedingly interesting and complex phenomenon, only a few elements of which have been clarified. Communication with regard to sexual receptivity, aggregation, food, alarm, and trail marking are but a few examples. The chemistry of this language is as multiform as the species and messages. The ever-plastic isopentenoids provide much of the specificity and diversity for insect communication.

Sex Pheromones

The first insect sex pheromone to be identified was (*E*)-10,(*Z*)-12-hexadecadien-1-ol (bombylcol), isolated from the commercial silkworm (20) *Bombyx mori.* Much of the subsequent work on sex pheromones was concerned with the attractants of Lepidoptera, and single components were identified in a number of species on the basis of simple laboratory bioassays. In field trials, however, most single-component pheromones performed poorly, compared to virgin females. In 1966, a multicomponent pheromone complex was reported (21) to attract both male and female bark beetles, *Ips paraconfusus* (Fig. 6). It is composed of three terpene alcohols: (-)-2-methyl-6-methylene-7-octen-4-ol (IV), (+)-(*Z*)-verbenol (V), and (+)-2-methyl-6-methylene-2,7-octadien-4-ol (VI). Another multicomponent pheromone attracts the western pine beetle, *Dendroctonus brevicomis.* It contains three compounds (Fig. 7): *exo*-7-ethyl-5-methyl-6,8-dioxabicyclo [3.2.1] octane (VII), produced by the female; 1,5-dimethyl-6,8-dioxabicyclo [3.2.1] octane produced by the male; and myrcene (IX), contributed by the pine tree itself (22-27).

One of the most complex pheromone systems described is the four-component mixture of the boll weevil, *Anthonomous grandis,* which was extracted from the fecal pellets of the male weevil (28) (Fig. 8). Three of the components are closely related: (*Z*)-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneethanol (XI), (*Z*)-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde (XII), (*E*)-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde (XIII); and the fourth component is (+)-(*Z*)-2 isopropenyl-1-methylcyclobutaneethanol (X). All four components are required for efficient attraction in the field.

The principal vector of Dutch elm disease is the smaller European elm beetle, *Scolytus multistriatus.* When a female bores into the elm tree, she produces a powerful pair of pheromones (Fig. 9): 4-methyl-3-heptanol (XIV) and 2,4-dimethyl-5-ethyl-6,8-dioxabicyclo [3.2.1] octane (V), which is attractive to both sexes (29). The host tree produces a third component, α-cubebene (XVI), which is necessary for full field attraction.

The sex attractants of the California red scale, *Aonidiella aurantii* (XVII, XVIII), and of the yellow scale, *Aonidiella citrina* (XIX) (Fig. 10) are the latest additions to the repertoire of terpenoid sex attractants (30,31).

The identification of the sex attractants of the American cockroach, *Periplaneta ameri-*

XXIV

(E)-β -Farnesene

XXV

(-)-Germacrene A

FIG. 12. Aphid alarm pheromones.

XXXI
Faranal

XXXII
Nasutene

FIG. 14. Trail pheromones of Pharaoh's ant, faranal, and of termites, nasutene.

cana, has been a long standing problem (32-34). Indeed, one previous identification (35) has been disproven (36) and retracted (37). Several mimics of the sex pheromone were discovered (38-40) and include (Fig. 11): (+)-bornyl acetate (XX), (+) (*E*)-verbenyl acetate (XXI), and germacrene D (XXII). Recently, a highly oxygenated germacrene compound, periplanone B, (XIII) has been identified as one of the authentic pheromone components (41).

Alarm pheromones.

A variety of alarm pheromones are produced by insects in response to predator or parasite attack. When attacked by predators, many aphids secret droplets of fluid containing alarm pheromones from specialized structures, called cornicles. Other aphids in the aggregation detect the alarm pheromone and run or fall away from the vicinty of the warning aphid. Two alarm substances have been identified (Fig. 12): (*E*)-β-farnesene (XXIV) is the principal alarm pheromone for aphids in the subfamilies Aphidinae and Chaitophorinae (42,43), and germacrene A (XXV) was identified as the alarm pheromone of several forage crop aphids in the subfamily Drepanosiphinae (44) including the spotted alfalfa aphid, *Therioaphis maculata*, and the sweetclover aphid, *Therioaphis riehmi.*

Social insects posses a rich variety of specialized pheromones dedicated to the survival of the socity. Quite often, defensive secretions also serve in alarm communications. Ants may simultaneously secrete a variety of terpenoid compounds from their mandibular glands (Fig. 13): 2,6-dimethyl-5-heptene-1-al, (XXVI), 2,6-dimethyl-5-heptene-1-ol, (XXVII), citronellal (XXVIII), neral (XXIX), geranial (XXX); and undecane from their Dufour's gland (45).

Trail Pheromones

Many social insects lay scent trails which assist in their orientation within the outside of their nest. Some trail pheromones are used by ants as recruitment trails for newly discovered food sources. The Pharaoh's ant, *Monomorium pharaonis,* is an urban pest which secretes a homo-sesquiterpene aldehyde trail pheromone, faranal (XXXI) (46) (Fig. 14). Similarly, termites employ a variety of trail pheromones, including nasutene (XXXII) or neocembrene-A (47). Because the diterpene nasutene is present in plants (48), there is a question whether this trail pheromone is produced by the termites or obtained from wood or ingested fungi.

It is clear that insects produce and employ an astonishing variety of compounds of isopentenoid biogenesis to regulate and direct physiological processes such as growth, development, reproduction, and diapause. Insect communication and sociality is likewise dependent upon these multiform molecules for their sexual attraction and excitement for aggregation, orientation, alarm, and defensive requirements. Although the chemistry and biology of these systems is in its infancy, a deeper understanding of these processes will undoubtedly provide the beginning for the development of safe and selective pest management.

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CHO
XXVI

CH_2OH
XXVII

CHO
XXVIII

CHO
XXIX

CHO
XXX

FIG. 13. Ant alarm pheromones.

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Insect Steroid Metabolism

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ABSTRACT

Insects are unable to biosynthesize the steroid nucleus and generally require an exogenous source of sterols. Two salient areas of insect steroid metabolism are the dealkylation and conversion of dietary C_{28} and C_{29} plant sterols to cholesterol and other C_{27} sterols, and the biosynthesis and metabolism of the steroidal insect molting hormones. Certain azasteroids and nonsteroidal amines block this conversion of 24-alkyl sterols to cholesterol and/or disrupt molting and development in insects. These inhibitors have served in charting metabolic pathways for steroids in insects and are serving as models in developing selective pesticidal chemicals and chemotherapeutic agents for use against insects and other invertebrate pests and parasites. The mode of action of some of these inhibitors on molting and development has been investigated in vivo and in vitro. Certain of these inhibitors represent a new class of insect hormonal compounds with a novel mode of action—the disruption of molting hormone metabolism. Rèsearch on sterol metabolism in insects provides important information on the comparative biochemistry and physiological functions of steroids in living systems.

INTRODUCTION

Insects in general require a dietary or exogenous source of sterol for normal growth, development, and reproduction – the only exceptions being those insects in which a sterol source may be attributed to associated symbionts or intestinal microorganisms. This dietary requirement for sterols results from a lack of the sterol biosynthetic mechanism in insects. Thus, insects, along with certain other invertebrates, differ from most plants and vertebrates which are capable of endogenous biosynthesis of sterols from simpler molecules. However, in insects as in higher animals and certain plants, sterols serve a dual role: as components of membranes of cellular and subcellular structures, and as precursors for essential bioregulators such as the steroid molting hormones (MH).

This discussion will be concerned primarily with our in depth research on steroid metabolism in insects (1-4). Research in this area of insect physiology and biochemistry is important for two reasons. First, because of the differences in steroid metabolism between insects and higher animals and most plants, it provides a target system for selective disruption of insect development. Second, because of the versatility and adaptability of insects, it provides a rich source of information on the comparative biochemistry and physiological functions of steroids in living systems.

Three aspects of our research on steroid metabolism in insects will be treated: (a) the utilization and metabolism of dietary sterols, or the mechanisms whereby insects satisfy their essential sterol requirment; (b) the utilization of sterols as precursors, and the intermediates involved in the biosynthesis of the steroid MH; (c) research on the inhibitors of steroid metabolism in insects, both in vivo and in vitro.

PHYTOSTEROL METABOLISM

Cholesterol fulfills the exogenous sterol requirement of all insect species critically studied to date– with only two known exceptions (5,6). Since cholesterol is the major sterol of higher animals, insects that feed either on vertebrates or on vertebrate products can readily satisfy their sterol requirement directly with this sterol. However, most phytophagous insects, which normally have little, if any, dietary cholesterol available, convert C_{28} and C_{29} plant sterols to cholesterol by removal of alkane or alkene substituents from C-24 on the sterol side chain (cf. Fig. 1) (1-4). This dealkylation is an important area of difference between plant-feeding insects and higher animals, since dealkylation and conversion of plant sterols to cholesterol, if it does occur to any extent in vertebrates, would be of limited physiological importance.

It was generally held for a number of years that insects could be divided into two general categories with respect to plant sterol metabolism: the zoophagous species, which are unable to dealkylate the sterol side chain were placed in one group, and the phytophagous and omnivorous species that can dealkylate the sterol side chain were placed in a second group. Recent studies have revealed some interesting biochemical differences that exist between members of this latter group, and we will consider some of these differences found in recent

Cholesterol Desmosterol Cholestanol Lathosterol

7-Dehydrocholesterol 5,22,24-Cholestatrien-3β-ol 5,7,24-Cholestatrien-3β-ol 5,7,22,24-Cholestatetraen-3β-ol

Campesterol 24-Methylenecholesterol Campestanol Δ7-Campestenol

Sitosterol Stigmasterol Fucosterol

Stigmastanol Δ7-Stigmastenol

FIG. 1. Sterols involved in plant sterol metabolism in insects.

comparative studies with several phytophagous insects.

The tobacco hornworm, *Manduca sexta* (L.), has been our primary experimental insect in these studies. It has proven to be ideal for such research because there is a satisfactory artificial diet available on which the larvae grow and develop rapidly, and because this insect efficiently converts phytosterols to cholesterol. The known intermediates and pathways involved in the metabolism of three common phytosterols in the hornworm, as elucidated in studies in which radio-labeled compounds were employed, are summarized in Figure 2 (4). All three phytosterols are converted to desmosterol, which is then reduced to cholesterol. The discovery of desmosterol as an intermediate (7) was interesting from a comparative biochemical standpoint, since this Δ^{24}-sterol serves as a terminal intermediate in the de novo biosynthesis of cholesterol in vertebrates (8,9) and since Δ^{24}-intermediates also occur in the pathways of plant sterol biosynthesis just prior to alkylation of the C-24 position (10).

In subsequent studies, it was also determined that in the hornworm fucosterol, 5,22,24-cholestatrienol, and 24-methylenecholesterol are intermediates in the conversion of sitosterol, stigmasterol, and campesterol, respectively, to cholesterol (4). These pathways are also present in other holometabolous phytophagous lepidopterans, such as the corn earworm, *Heliothis zea* (Boddie), and the fall armyworm, *Spodoptera frugiperda* (J.E. Smith), as well as in certain omnivorous hemimetabolous insects, including several species of cockroaches, and even in the primitive, ametabolous firebrat, *Thermobia domestica* (Packard) (11).

Initially, our research on sterol metabolism had been centered on insects in which the sterols are comprised primarily of Δ^5-sterols. Then we examined the sterols in the confused flour beetle, *Tribolium confusum* Jaquelin duVal, in which over 50% of the tissue sterols had been reported to be 7-dehydrocholesterol (12). This was of interest, because this

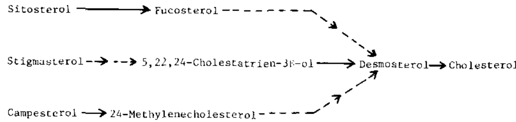

FIG. 2. Summary of phytosterol conversion to cholesterol in the tobacco hornworm.

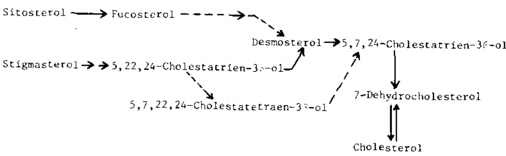

FIG. 3. Phytosterol metabolism in confused flour beetle.

$\Delta^{5,7}$-sterol usually accounts for only a few percent or less of the total sterols of most insects. We were able, through metabolic studies, to develop the scheme in Figure 3 for *Tribolium* (13). In this species, desmosterol is an intermediate in the conversion of both sitosterol and stigmasterol to cholesterol, as in the several other species previously mentioned. Interestingly, 5,7,24-cholestatrien-3β-ol was shown to be an intermediate in *Tribolium*, and 5,7,22,24-cholestatetraen-3β-ol was a metabolite of stigmasterol, indicating that this insect is capable of introducing the Δ^7-bond in a variety of sterol substrates.

Thus, there is considerable similarity between the metabolic pathways of plant sterols in *Tribolium* and in the hornworm—with fucosterol, 5,22,24-cholestatrienol, and desmosterol serving as intermediates in both species. However, the involvement of 5,7,24-cholestatrienol as an intermediate in *Tribolium* and the equilibrium between cholesterol and 7-dehydrocholesterol in *Tribolium* tissues are important differences between these two species. The reason for the presence of such high levels of 7-dehydrocholesterol in *Tribolium* remains unknown. The only known specific role for 7-dehydrocholesterol in insects is as a precursor of the MH, but only a small fraction of the total found in *Tribolium* would serve this purpose.

The Mexican bean beetle, *Epilachna varivestis* Mulsant, is an interesting insect, since it is a member of the family coccinellidae that includes the lady bug beetles, most of which are predacious. However, the subfamily to which the Mexican bean beetle belongs is composed of phytophagous species, and there are some unique aspects of plant sterol metabolism in this insect compared to other phytophagous insects previously examined.

We have analyzed the sterols of bean beetle eggs, prepupae, and adults and the dietary soybean leaves (14). There is little cholesterol present in any stage of the bean beetle, and lathosterol (Δ^7-cholestenol) was identified for the first time as one of the major sterol components of a phytophagous insect reared on a normal host plant (Table I). Large amounts of saturated sterols were present in all three stages of this insect. They consisted largely of cholestanol, campestanol, and stigmastanol. This was the first time a plant-fed insect was found to have significant levels of saturated sterols. In contrast, soybean leaves contain only very small quantities of stanols. From studies with radiolabeled sterols, it was shown that Δ^5-phytosterols are reduced to stanols and then dealkylated to produce cholestanol; the Δ^7-bond is then introduced to form lathosterol (15). Although bean beetles do dealkylate, they do

TABLE I

Relative Percentages of Sterols Identified from Various Mexican Bean Beetle Stages and Soybean Leaves

Sterol	Mexican bean beetle			Soybean leaves
	Eggs	Prepupae	Adults	
Cholesterol	2.9	4.4	4.5	0.9
Cholestanol	19.0	29.0	50.7	<0.1
Lathosterol	12.0	16.0	11.8	—
Campesterol	1.1	T[a]	T	11.3
Campestanol	7.1	10.2	6.0	0.2
Δ^7-Campestenol + Unknown	20.9	3.6	2.0	—
Stigmasterol	—	1.7	1.4	31.5
Stigmastanol	27.5	32.9	20.3	1.1
Sitosterol	3.2	2.2	2.3	55.0
Δ^7-Stigmastenol	6.3	T	1.0	—
Total saturated sterols	53.6	72.1	77.0	1.4

[a]T = detectable trace.

not convert 24-alkyl sterols to cholesterol, as might have been expected.

An equally interesting example of plant sterol utilization in a phytophagous insect was discovered in studies with the large milkweed bug, *Oncopeltus fasciatus* (Dallas). A comparison of sterols isolated from adult female milkweed bugs and eggs and those from the sunflower seeds on which they were reared is shown in Table II (16). The small amount of cholesterol in the insect may readily be accounted for by selective uptake from the diet, indicating that this phytophagous insect does not dealkylate C_{28} and C_{29} plant sterols to any measurable extent. At any rate, there is little alteration in the composition of dietary sterols that are incorporated into the insect tissues and transferred to the egg. Support for this premise has been obtained from studies in which radio-labeled campesterol and sitosterol were injected into milkweed bug nymphs and no conversion to cholesterol was detected.

These examples demonstrate that the research on sterol metabolism in phytophagous insects has revealed considerable diversification and a number of interesting biochemical variations in the ways these insects satisfy their sterol requirement. In discussion of MH biosynthesis and metabolism, it will become apparent that equally interesting comparative information is also emerging from that area of insect steroid biochemistry.

TABLE II

Content of Δ^5-Sterols of Female Adult Milkweek Bugs, Eggs, and Sunflower Seeds

	Relative percentages		
Sterol	♀ Adult	Egg	Sunflower Seeds
Cholesterol	0.4	0.5	<0.1
Campesterol	10.3	11.7	10.1
Stigmasterol	7.4	6.5	10.3
Sitosterol	81.9	81.3	79.5

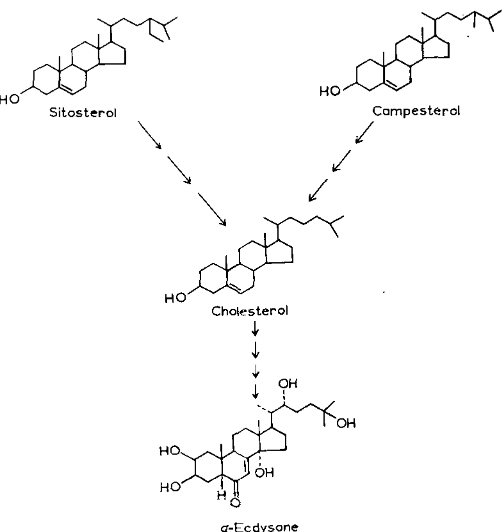

FIG. 4. Utilization of C_{28} and C_{29} phytosterols as precursors for a C_{27} insect molting hormone.

MOLTING HORMONE METABOLISM

As pointed out in earlier discussion, some phytophagous insects readily dealkylate plant sterols to form C_{27} sterols; others may directly utilize dietary cholesterol as MH precursors (Fig. 4). However, it is now becoming clear that considerable species variation also exists with respect to biosynthesis and metabolism of the MH in insects.

Molting is a characteristic feature of insect growth, development, and metamorphosis, and the invariable requisite for molting in insects is tissue stimulation by the steroid MH. To date, seven MH have been isolated and characterized from insect sources (Fig. 5). Six of these – α-ecdysone, 20-hydroxyecdysone, 20,26-dihydroxyecdysone, 26-hydroxyecdysone, 3-epi-20-hydroxyecdysone, and 2-deoxy-α-ecdysone – are C_{27} steroids. α-Ecdysone and 20-hydroxyecdysone were both first isolated from pupae of the silkworm, *Bombyx mori* (L.) (17,18), and 20-hydroxyecdysone is generally considered to be the active MH (19). Three of the C_{27} ecdysteroids, including 20,26-dihydroxyecdysone, 26-hydroxyecdysone, and 3-epi-20-hydroxyecdysone, were first characterized in our laboratory from various stages of the tobacco hornworm (20-22). These three MH, in addition to α-ecdysone and 20-hydroxyecdysone, all occur in various stages of the hornworm. Recently, 2-deoxy-α-ecdysone, the first 2-deoxy-ecdysteroid isolated from an insect, was found in ovaries and eggs of the silkworm (23).

Makisterone A, which was originally isolated from plant material (24) and a marine crustacean (25), was found to be the major ecdysteroid in the egg of the large milkweed bug (26). This is the first C_{28} molting hormone identified from an insect source, and it is of particular interest in light of the previously noted lack of dealkylation of C_{28} and C_{29} phytosterols in this insect.

Four of the C_{27} molting hormones have the same steroid nucleus as α-ecdysone and differ only in the number and position of the hydroxyl groups on the side chain. The prob-

α-Ecdysone 20-Hydroxyecdysone 20,26-Dihydroxyecdysone

26-Hydroxyecdysone 3-Epi-20-Hydroxyecdysone Makisterone A 2-Deoxy-α-ecdysone

FIG. 5. Insect molting hormones.

α-Ecdysone → 20-Hydroxyecdysone → 20,26-Dihydroxyecdysone; α-Ecdysone → 26-Hydroxyecdysone

FIG. 6. Possible interrelationships of molting hormones in tobacco hornworm.

able metabolic interrelationships of these four ecdysteroids are illustrated in Figure 6. The conversion of α-ecdysone to 20-hydroxyecdysone in insects is well documented in the literature (1-4). It is generally held that α-ecdysone functions as a prehormone that is converted to 20-hydroxyecdysone, which functions as the active hormone. 20,26-Dihydroxyecdysone is present in much lesser quantities than the other two molting hormones in the hornworm, and because of its decreased biological activity and increased polarity, it appears to be a deactivation product of 20-hydroxyecdysone (20). However, the exact roles or functions of each of these steroid hormones in insect development and/or reproduction remain to be determined. All three of these molting hormones have been found both in hornworm pupae and embryonated eggs, and they could well be intermediates in the biosynthetic-deactivation scheme for these hormones. The overall scheme in Figure 6 could represent the metabolic relationship of these hormonal compounds in the hornworm egg, 26-hydroxyecdysone being the major ecdysteroid in the developing embryo (21). On the other hand, 20-hydroxyecdysone is the major metabolite of α-ecdysone during pupal-adult development at the time of peak molting hormone titer (27), but it is only a minor metabolite in the embryonated egg of the hornworm.

A tentative composite scheme for molting hormone biosynthesis resulting from in vivo and in vitro studies with possible precursors, intermediates, and metabolites by several research groups involving a number of insect species is presented in Figure 7 (1-4). Cholesterol has been shown by a number of investigators to be converted to α-ecdysone and/or 20-hydroxyecdysone in several insect species, and in addition, the dehydrogenation of cholesterol to 7-dehydrocholesterol in insects has been well documented (1-4). Also, in certain insects there is a high percentage of 7-dehydrocholesterol in the sterols of prothoracic glands (3), and these glands have been shown unequivocally to produce α-ecdysone (28,29). The Δ^7-bond is an essential structural feature common to all molting hormones isolated from insects and, indeed, two species of insects have been shown to require a dietary source of a Δ^7-sterol (5,6).

Cholesterol

7-Dehydrocholesterol

HO OH H O

2,22,25-Trideoxy-α-ecdysone

22,25-Dideoxy-α-ecdysone

22-Deoxy-α-ecdysone

α-Ecdysone

FIG. 7. Tentative scheme for molting hormone biosynthesis.

The next possible intermediate, 2,22,25-trideoxyecdysone, was converted to 22-deoxy-α-ecdysone, α-ecdysone, and 20-hydroxyecdysone in studies with the blowfly, *Calliphora stygia* (F.) (30). In the tobacco hornworm, the labeled triol, 22,25-dideoxyecdysone, was efficiently converted to α-ecdysone and 20-hydroxyecdysone, both of which were present in the free and conjugated form, as well as to lesser quantities of 20,26-dihydroxyecdysone after injection into male diapausing pupae (31,32). The efficient conversion of 22,25-dideoxyecdysone to these three ecdysteroids strongly suggests that hydroxylation of the steroid nucleus occurs before hydroxylation of the side chain in this insect. Both in vivo and in vitro studies with hornworm prepupae showed 22,25-dideoxyecdysone to be metabolized to α-ecdysone, 20-hydroxyecdysone, and 20,26-dihydroxyecdysone through 22-deoxyecdysone in fat body and Malpighian tubules, but it is not converted beyond α-ecdysone in the ecdysial glands (33,34), the 22,25-dideoxyecdysone was also more efficiently metabolized to α-ecdysone and 20-hydroxyecdysone in prepupae of *C. stygia* (35) than the previously mentioned 2,22,25-trideoxyecdysone.

However, hornworm larvae fed 22,25-dideoxyecdysone produced mainly metabolites lacking the 22-hydroxyl group (36). The low level of conversion to α-ecdysone and 20-hydroxyecdysone in these more immature stages again emphasizes the importance of the developmental stage or period in relation to biosynthesis and metabolism of the molting hormones.

The tetraol, 22-deoxyecdysone, was converted to α-ecdysone, 20-hydroxyecdysone, and 20,26-dihydroxyecdysone in studies with hornworm prepupae (34), and it was metabolized to α-ecdysone and 20-hydroxyecdysone in *C. stygia* (35). It was an intermediate in the conversion of 22,25-dideoxyecdysone to α-ecdysone in hornworm pupae (36), but it did not appear to be a normal intermediate in this pathway in metabolic studies with grasshopper nymphs and blowfly larvae (34).

A final determination of the normal pathways of molting hormone biosynthesis must await the isolation and identification of the intermediates from insects. In the meantime, possible intermediates such as these are fulfilling the important function of helping to elucidate the sequence of side-chain hydroxylations in molting hormone biosynthesis.

The studies of the neutral sterols of the milkweed bug (16) were prompted by the discovery that makisterone A is the major ecdysteroid with MH activity in the egg of this insect, this being the first C_{28} ecdysone isolated and identified from an insect (26). The data indicate that this insect, which is incapable of converting 24-alkyl sterols to cholesterol, utilizes campesterol directly as a precursor for makisterone A. This finding provides the first evidence for the existence of biosynthetic and metabolic pathways for C_{28} and even possibly for C_{29} molting hormones in certain species of

22,25-Diazacholesterol
I

Triparanol (MER-29)
II

25-Azacholesteryl methyl ether
III

25-Azacoprostane
IV

25-Azacholestane
V

N,N,δ,7a-Tetramethyloctahydro-1-H-indene-1-butanamine
VI

N,N-Dimethyl-3,7,11-trimethyl-dodecanamine
VII

N,N-Dimethyldodecanamine
VIII

22-Iso-α-Ecdysone
IX

2β,3β-Dihydroxy-5β-cholest-7-en-6-one
X

2β,3β,5β,14α-Tetrahydroxy-5β-cholest-7-en-6-one
XI

3β,5β,14α-Trihydroxy-5β-cholest-7-en-6-one
XII

FIG. 8. Structures of inhibitors of insect steroid metabolism and certain ecdysteroids referred to in text.

insects, analogous to those known for the C_{27} MH.

It is apparent then that the matabolic pathways of MH may differ in different species of insects. Chemical and biochemical information on these steroid hormones, both during embryonic and postembryonic development, also indicates the presence of diverse metabolic pathways, as well as quantitative and qualitative variations in MH in different developmental stages of the same insect, such as the tobacco hornworm. Different ecdysteroids may function at various stages of insect development, and the qualitative nature of the molting hormones might well determine the type of molt. If so, then certain of the current concepts concerning the role of MH in the hormonal control of molting and metamorphosis in insects may need to be reassessed.

INHIBITORS OF STEROID METABOLISM IN INSECTS

The discovery that desmosterol is an intermediate in plant sterol metabolism in insects prompted us to test certain vertebrate hypocholesterolemic agents that are known to inhibit Δ^{24}-sterol reductase, which converts desmosterol to cholesterol. Upon feeding either of the two compounds, 22,25-diazacholesterol or triparanol (MER-29) (Fig. 8), in combination with dietary sitosterol to the tobacco hornworm, we noted inhibition of larval growth (37). There was also an accumulation of desmosterol and unchanged dietary sitosterol, and a decreased production of cholesterol, and these effects were most pronounced with the diazasterol.

Discovery of the inhibition of a specific enzyme system in insects – the Δ^{24}-sterol reductase – opened a whole new area of research, and the azasteroids have served as valuable tools in studies on sterol metabolism. Of particular interest are the effects on larval development, which included disruption of larval molts, formation of precocious 4th instar prepupae and inhibition of pupation, since these involve hormonally controlled processes.

In structure-activity studies with about 30

TABLE III

Effects of Combinations of N,N-Dimethyl-3,7,11-trimethyldodecanamine or 25-Azacoprostane plus 22,25-Dideoxyecdysone on Hornworm Development

Compound(s)	Concentration (ppm)	% Pupation
Control	---	80
N,N-Dimethyl-3,7,11-trimethyldodecanamine	16	67
25-Azacoprostane	1	75
22,25-Dideoxyecdysone	100	55
N,N-Dimethyl-3,7,11-trimethyldodecanamine + 22,25-dideoxyecdysone	16 100	3
25-Azacoprostane + 22,25-dideoxyecdysone	1 100	12

azasteroids, we found that not all of these compounds cause effects on molting and metamorphosis (38). However, compounds that had no effect on development could still inhibit the Δ^{24}-reductase and disrupt neutral sterol metabolism and cholesterol formation. These results indicated that steroid metabolic pathways other than those leading to cholesterol were affected by certain of these azasteroids. On the basis of this information, a number of more potent azasteroids were designed and synthesized, some of which are a thousand times more active than the original compounds tested. Certain of these inhibitors blocked development of several of the test insect species when added to the diet or medium in less than ppm quantities (39). Three of the most active of these azasteroids (25-azacholesteryl methyl ether, 25-azacholestane and 25-azacoprostane) are included in Figure 8. Thus, it was possible both to simplify the structure and to increase greatly the activity over that of the azasterols tested earlier.

The characteristic biological effects of minimal inhibitive concentrations of azasteroids that disrupt development in several of our test species (e.g., in the fourth instar of the hornworm) are related to the processes of molting and metamorphosis (39). In the yellow fever mosquito, *Aedes aegypti* (L.), first and second instar larval molts are most often blocked. *Tribolium* appears to be most susceptible at the larval to pupal molt, and the house fly, *Musca domestica* L., is most frequently affected between puparium formation and adult emergence. These effects, taken together with the fact that these inhibitors are steroidal compounds that interfere with sterol metabolism in insects, suggest that MH biosynthesis and metabolism are affected. Indeed, there is adequate cholesterol in the diets of the mosquito and house fly in these test systems, so the effects could not result from a cholesterol deficiency in these two species.

Other studies have provided further evidence that certain of these azasteroids interfere with molting hormone biosynthesis or deactivation (39). When the azasteroid, 25-azacholesteryl methyl ether, is fed to house fly larvae at 150 ppm, it has no effect. If they are fed the ecdysteroid, 22,25-dideoxyecdysone, at 75 ppm, puparium formation and adult emergence are severely affected. However, when the two compounds are fed simultaneously at these concentrations, the effect of the ecdysteroid on house fly larvae is completely reversed by the azasteroid.

Studies with *Tribolium* larvae have shown another intersting interrelationship between azasteroids and MH. *Tribolium* larvae tolerate 1,000 ppm of α-ecdysone in their diet with no adverse effects, but only 100 ppm of 25-azacholesteryl methyl ether completely blocks adult emergence. However, feeding α-ecdysone jointly with the azasteroid results in a 60% reversal of the adverse effect of the azasteroid. This reversal also occurs when 20-hydroxyecdysone is substituted for α-ecdysone.

The next step in our inhibitor research was to synthesize simpler nonsteroidal amines, such as the bicyclic amine (VI) in Figure 8 (40). This compound produces the characteristic biological and biochemical effects in several species of insects – albeit at a considerably lower level of activity. It was logical then to simplify the structure further and to synthesize and test the activities of branched and straight chain amines similar to compounds VII and VIII in Figure 8. These chain lengths and the branching of compound VII correspond to the structural features of one of the insect juvenile hormones, but these amines have no juvenile hormone effects in the tobacco hornworm or a number of other insects. Instead, these acyclic amines produce

TABLE IV

The Activity of Certain Ecdysteroids in Inducing Cuticle Formation in Cockroach Leg Regenerates when Tested in Cockroach Leg Regenerates Alone and in Leg Regenerates plus Fat Body with or without 22,25-Dideoxyecdysone

Compound[a]	Cuticle formation (%)		
	Leg regenerates	Leg regenerates + fat body	Leg regenerates + fat body + 22,25-dideoxyecdysone
20-Hydroxyecdysone	93	--	--
α-Ecdysone	82	--	--
22,25-Dideoxyecdysone	23	93	--
22-iso-α-Ecdysone	8	0	100
2β,3β-Dihydroxy-5β-Cholest-7-en-6-one	0	0	90
2β,3β,5β,14α-Tetrahydroxy-5β-Cholest-7-en-6-one	25	75	--

[a]All compounds tested at 5 μg/ml.

the same biological and biochemical effects in the hornworm and certain other species as the complex azasteroids, which were discussed earlier, and they are many times more active than the bicyclic amine (VI). Thus, it is feasible to disrupt certain essential pathways of steroid metabolism and the hormone-regulated processes of growth and development with structurally simple, readily synthesized compounds, having activities similar to those of the much more complex azasteroids we initially used in inhibitor studies.

The possible relation of some of these inhibitors with MH metabolism were examined by feeding them to hornworm larvae in combination with the triol, 22,25-dideoxyecdysone, which is converted to 22-deoxyecdysone in this stage of the hornworm. When either the branched chain amine, N, N-dimethyl-3,7,11-trimethyldodecanamine, at 16 ppm, or the ecdysteroid, 22,25-dideoxyecdysone, at 100 ppm, is fed in combination with sitosterol, there is some effect on the hornworm larvae, but feeding them together at these concentrations severely limits development to the pupal stage (Table III). Similarly, feeding 25-azacoprostane at 1 ppm with sitosterol as the dietary sterol, has little effect on development. But feeding this azasteroid and the ecdysteroid simultaneously at these concentrations has a very drastic effect. The triol by itself has no effect on converting sitosterol to cholesterol, but with the limitation of available cholesterol brought about by either of the two inhibitors, the fact that this triol may be hydroxylated to a MH-like compound that somehow interferes with the normal molting cycle may explain the combined effect. However, in vitro studies subsequently showed that some of these inhibitors may be more directly involved with blocking MH metabolism.

In depth studies of the ecdysone 20-hydroxylase enzyme system from the tobacco hornworm midgut have recently been carried out in order to define the system further. This has also made it available as a tool for further examining the effects and mode of action of some of our inhibitors. α-Ecdysone is readily converted to 20-hydroxyecdysone in a mitochondrial preparation from the midgut of the tobacco hornworm (41). This system is cytochrome P-450 mediated, as indicated by its oxygen dependence and its susceptibility to inhibition by carbon monoxide. Tricarboxylic acid cycle intermediates, such as succinate, malate and isocitrate, as well as NADPH and NADH support the reaction. Malic enzyme and nicotinamide nucleotide transhydrogenase are present in the mitochondria, and these enzymes are

TABLE V

The Effect of an Ecdysteroid, Two Azasteroids, and Two Nonsteroidal Amines in Inhibiting the Activity of 22,25-Dideoxyecdysone in Inducing Cuticle Formation in Cockroach Leg Regenerates when Tested in Leg Regenerates plus Fat Body in the Presence of 22,25-Dideoxyecdysone

Compound[a]	% Inhibition
3β,5β,14α-Trihydroxy-5β-cholest-7-en-6-one	31
25-Azacholesterol	100
25-Azacoprostane	83
	46
	46

[a]All compounds tested at 5 μg/ml.

frequently implicated in supplying NADPH to steroid hydroxylations in mitochondria. Temperature and pH optima were 30 C and 8.5, respectively (42).

In preliminary studies with a limited number of our inhibitors in this 20-hydroxylase system, an adverse effect on the hydroxylation of α-ecdysone was demonstrated. These studies have just begun, but a considerable amount of useful information may be obtained from studies with inhibitors in this in vitro system as well as in other enzyme systems involved in MH biosynthesis and metabolism.

Another very interesting technique for studying in vitro MH metabolism and effects of inhibitors utilizes a cockroach (*Leucophaea maderae* (F.)) leg regenerate system that was developed for studying effects of MH and MH analogs on deposition of new insect cuticle (43). In these experiments, a leg from a late instar cockroach nymph is removed in order to induce regeneration. After 28 days, the regenerating leg is dissected from the insect and transferred to a tissue culture system, either with or without fat body, containing an appropriate tissue culture medium. The test compounds are injected into the system, and the degree of cuticle deposition is scored 14 days later (44).

This in vitro system converts α-ecdysone to 20-hydroxyecdysone when only the leg regenerate is present in the medium (45). The C-20 hydroxylation of α-ecdysone is essential to the subsequent formation of cuticle by the leg regenerates, as in any insect cuticle formation. Both α-ecdysone and 20-hydroxyecdysone induce a high degree of cuticle formation in this system (Table IV) (44). In studies with certain other ecdysteroids, it was discovered that the triol, 22,25-dideoxyecdysone, brought about a low level of cuticle formation in the test system, but when cockroach fat body tissue was included in the preparation, cuticle formation equivalent to that realized with 20-hydroxyecdysone was obtained (46). Apparently, 22,25-dideoxyecdysone is converted to some more active compound in the presence of fat body tissue. Neither 22-iso-α-ecdysone (IX) nor 2β,3β-dihydroxy-5β-cholest-7-en-6-one (X) induces cuticle formation, nor does either compound interfere with induction of cuticle formation when 22,25-dideoxyecdysone is present. The tetrahydroxy compound, 2β,3β,5β,14α-tetrahydroxy-5β-cholest-7-en-6-one (XI), also brings about a somewhat higher percentage of cuticle formation in the presence of fat body tissue (44).

The relative inhibition of cuticle deposition induced by 22,25-dideoxyecdysone in the leg regenerate system plus fat body by an ecdysteroid, two azasteroids, and two nonsteroidal amines is presented in Table V (44). The ecdysteroid, 3β,5β,14α-trihydroxy-5β-cholest-7-en-6-one (XII), inhibits by 31%. The azasteroids, 25-azacholesterol and 25-azacoprostane, both were quite effective inhibitors (100 and 83%, respectively); the bicyclic amine (VI) and the branched chain amine (VII) were about half as inhibitory as the azacoprostane. Interestingly, the two nonsteroidal compounds are usually much less potent inhibitors than the azasteroids in in vivo tests.

Subsequently, the metabolism of radiolabeled 22,25-dideoxyecdysone in the presence of fat body without the leg regenerate was examined in this in vitro system (47). About 8 and 11% of the labeled material recovered were free tetraols and pentaols, respectively, and

TABLE VI

The Effects of an Azasteroid and Two Nonsteroidal Amines on the Metabolism of 4-^{14}C-22,25-Dideoxyecdysone in Cockroach Fat Body Cultures during an Incubation Period of 6 Days

Inhibitory compounds[a]	% Unmetabolized 22,25-Dideoxyecdysone[b]	% Metabolites[b]		
		Conjugates	Pentaol	Tetraol
Control	45.5	35.6	11.3	7.6
25-Azacoprostane	6.0	55.0	0.0	39.0
	15.9	40.8	5.6	37.8
	18.7	34.7	5.2	41.5

[a]The concentration of the inhibitory compounds and 4-^{14}C-22,25-dideoxyecdysone was 5 μg/ml.
[b]Determined by radio-chromatogram scanning of thin layer chromatography plates.

about 46% was recovered as unmetabolized substrate (Table VI). Thus, 22,25-dideoxyecdysone can be hydroxylated in one or more positions in this system. The primary sites of hydroxylation of 22,25-dideoxyecdysone are at C-25 (75%), producing 22-deoxyecdysone, and at C-26 (25%). Because the pentaols lacked the 22-hydroxyl, little, if any, α-ecdysone was formed, and the major metabolite (60%) was 22-deoxy-26-hydroxyecdysone. About one-third of the total radioactivity recovered was in the form of conjugates, and after enzymatic hydrolysis of the conjugated material with a mixture of β-glucosidase and sulfatase, tetraols, pentaols, and unhydrolyzed conjugates were found in the reaction mixture, but apparently no 22,25-dideoxyecdysone was involved.

When tested in the leg regenerate system, the tetraol and pentaol fractions both effectively induced cuticle deposition. This suggests that the 25-hydroxyl, or at least one hydroxyl group, is needed in the ecdysteroid side chain for effective activity in this system.

The cockroach fat body system was used to examine the inhibitory effects of an azasteroid (IV) and two nonsteroidal amines (VI and VII) on metabolism of 22,25-dideoxyecdysone (47, Table VI). With all three inhibitors, the amount of unmetabolized 22,25-dideoxyecdysone greatly decreased, pentaol production or accumulation decreased, and tetraol accumulation increased. A most striking effect of the azasteroid was a significant increase in quantity of conjugates formed. These results provide the first in vitro biochemical evidence that these inhibitors act by interfering with the metabolism of ecdysteroids, thus confirming our earlier in vivo studies.

Although we have obtained considerable information on the utilization and metabolism of dietary 24-alkyl sterols and although progress has been made in our understanding of molting hormone biosynthesis and metabolism, steroid metabolism in insects still remains a fertile area of investigation. Certainly, the differences that we observe with respect to steroid metabolism in insects relate to phylogeny and/or adaptation, thus indicating that this will continue to be an informative area of comparative steroid biochemistry. In addition, insects provide us with an optimal experimental system for studying steroid utilization and metabolism and the relation between steroid structure and function in organisms in which steroid composition may be controlled and is not complicated by endogenous biosynthesis.

Finally, it has been shown that certain chemicals selectively interfere with specific reactions of steroid metabolism in insects and

that some of these compounds are potent inhibitors of insect molting and metamorphosis. With increased basic knowledge in this area of insect biochemistry, it may become feasible to develop safe, selective pesticides and chemotherapeutic agents for use against insects and other invertebrate pests and parasites of agricultural and medical importance.

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Cross-Linking of Collagen in the Presence of Oxidizing Lipid

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ABSTRACT

Gelatin films containing unsaturated lipid have been exposed to ultraviolet and visible irradiation. No sign of paramagnetism could be detected in the films, although the gelatin was undergoing cross-linking reactions. The addition of nitroxyl-forming radical scavengers decreased the rate of cross-linking, as did addition of ascorbic acid to the reacting mixture. Nitroxyls could not be detected in the gels, however. The conclusion is drawn that the main reaction in the cross-linking reaction of collagen is a condensation of amino groups and extrinsic or intrinsic carbonyl groups. The extrinsic aldehydes are formed in the autoxidation of unsaturated lipid.

An object of the present study is to increase our knowledge of the causative mechanisms of the fully demonstrated premature aging of human skin which has been excessively exposed to solar radiation.

On one hand, it has been postulated by Harman (1) and others that free radicals are primary causative factors in the aging process and that these are formed in the process of lipid oxidation (2,3).

On the other hand, doubt has been raised regarding the necessity for free radicals in this reaction. The free radicals observed by Roubal (3) would seem expectable from the enzyme reactions connected with the material used.

The reaction between oxidizing fat and protein was known in the hectograph industry prior to 1915 (4). Fahrion (5,6) postulated that the reactive groups involved in the cross-linkage reaction or "tannage" with oxidizing lipids are peroxide groups formed by the action of oxygen in air on the unsaturated fatty acids of the oil (7).

The formation of acrolein and other pungent compounds in the tanning process has been known to generations of chamois tanners (8). Procter (9), suggested that acrolein was the principal tanning agent. Indeed, acrolein is an effective cross-linkage agent. Salway (10) showed that acrolein is formed by the oxidation of free unsaturated fatty acids from linseed oil. Balfe, in his review (11), stated that acrolein is the major tanning agent in oil tanning. On this basis, Kuntzel and Nungesser (12) reinvestigated the matter. Cod liver oil, exposed to air oxidation in a Mackay tester, gave acrolein yields of 0.7-1.5% of the oil being oxidized under conditions corresponding to oil tanning. When hide powder was added to the oxidizing oil, no free acrolein could be found and the hide powder became cross-linked.

Farmer (13), has shown that fatty hydroperoxides alone can effect a cross-linkage in proteins. On the other hand, the cross-linkage of proteins in proteinaceous products containing unsaturated lipids has been ascribed to effects of free radicals by Desai and Tappel (14).

Okamura and Shirai (15) studied oil tanning by incubating preoxidized cod oil in hide powders. They conclude that oil tanning follows a complex course including the combination of peroxides of cod oil with hide. No electron spin resonance (ESR) investigation was undertaken, however.

In view of the fact that both aldehydes and peroxides can induce cross-linkage and damaging reactions in proteins, it was of interest whether free radicals can be observed with ESR spectroscopy while a demonstrable accelerated protein cross-linkage by oxidizing unsaturated lipid is in progress.

EXPERIMENTAL PROCEDURES

Gelatin films containing 0, 0.17, 1, 5, and 10% of lipid, respectively, were prepared (16). As the gelatin component, pigskin gelatin (275 Bloom) was used. The lipid used was a commercially available corn oil containing 86% (w/v) unsaturated fat and free from added antioxidants. The antioxidant ascorbic acid (Pharmaceutical grade F) was dissolved in a small volume of water prior to addition. The ascorbic acid content of the gel was 0.00074%. The radical scavenger tert.-nitrosobutane was prepared as described (17), and the radical scavenger 2,4,6-tri-*tert.*-butyl nitrosobenzene was prepared by the procedure outlined (18). The scavenger was ground in a mortar together with the dry gelatin before the swelling procedure. The final concentration of the scavenger was 0.001 and 0.1%, respectively. The gels were stored before and after irradiation as previously described (16).

The gels were irradiated in a Rayonet Photochemical chamber reactor model RRR-100. The

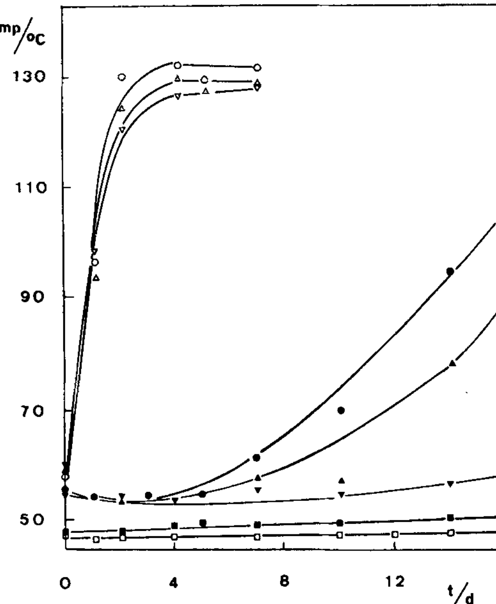

FIG. 1. Crosslinking of lipid containing collagen. Solid figures refer to samples irradiated 5 hr at 360 nm; empty figures refer to samples irradiated 60 min at 253 nm □, ■ no oil added, ▽, ▼ 1 % oil added, △, ▲ 5 % oil added, and ○, ● 10 % oil added.

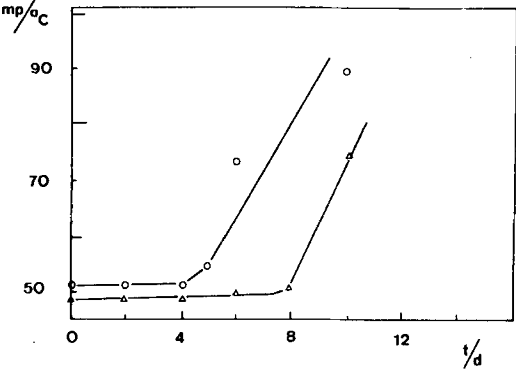

FIG. 2. Crosslinking of lipid containing collagen in presence of 7.4 x 10^{-4} % ascorbic acid. Irradiation 60 min at 253 nm., Δ 5 % oil added, ○ 10 % oil added.

gelatin films were cooled during the irradiation. The films were exposed to both daylight (360 nm) and UV-light (263 nm) in different experiments. The experimental values represent the average of at least five measurements.

The cross-linking of the collagen was followed by measuring melting points from time to time. The melting points were determined in mineral oil as has been described by Bjorksten and Collbring (16).

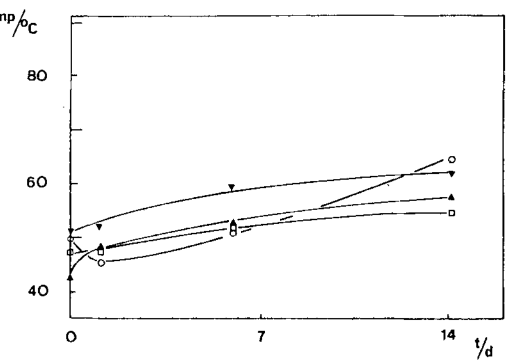

FIG. 3. Crosslinking of lipid containing collagen in presence of 7.4 x 10^{-4} % 2,4,6-tri-*tert.*-butylnitroso-benzene and 5 % oil (▼), 0.1 % 2,4,6-tri-*tert.*-butyl-nitroso-benzene and 5 % oil (▲), 0.1 % 2,4,6-tri-*tert.*-butyl-benzene (○), and no added compounds (□).

ESR measurements were performed on a Varian X-band E-3 spectrometer at temperatures ranging from +100 – -150 C with variation of all the measuring parameters. The gels were checked for paramagnetism before and after irradiation, and at time intervals equal to time between melting point measurements.

RESULTS AND DISCUSSION

The change in melting points was taken as a measure of the rate of cross-linking in the lipid-containing gels (16). The results of these determinations are collected in Figures 1, 2, and 3.

As judged by the change in melting point, there is no mechanistic difference between the reactions initiated at 263 nm and 360 nm, respectively. This finding could be explained as follows: The photochemically active chromophore (in the lipid or in the collagen) has its absorption maximum in the ultraviolet region, with some absorption probability also in the visible region, due to tailing. The difference is seen in the velocity of the aging reaction of the gels (Fig. 1). After 60 min irradiation at 263 nm, the rise in melting point is from about 50 C to about 100 C in one day, whereas 5 hr irradiation at 360 nm hardly affects the melting point in one day. By using the short wavelength light for the irradiation of the gels, we hoped to reach a higher and measurable concentration of free radicals in the gels. This was not found, however, and every effort to detect an ESR signal in the system of lipid-containing gelatin was unsuccessful.

Experiments have been reported (16) in which the effect of an added antioxidant on the melting point of the gel was tested. It was shown that tocopherol efficiently retarded the

melting point raising reaction. Accordingly, the effect of ascorbic acid was tested. The results are collected in Figure 2. The concentration of ascorbic acid was kept sufficiently low, $< 10^{-3}\%$, not to affect the pH of the gels. The addition of ascorbic acid quite evidently slows down the melting point raising reaction, after 60 min UV irradiation the melting point remains unaltered for 5 days.

The gels containing lipid and ascorbic acid were tested for paramagnetism at intervals equal to intervals between melting point determinations. No ESR signal could be detected.

Radical scavengers forming relatively stable paramagnetic products in systems where radicals are present have been developed. Among these nitroxyl-forming nitroso compounds or nitrones are commonly in use (22). The effect of the scavengers *tert.*-nitrosobutane (2-methyl-2-nitrosopropane) (17) and 2,4,6-tri-*tert.*-butylnitrosobenzene (18) on the reactions in the lipid-containing gels was tested.

In a preliminary experiment *tert.*-nitrosobutane was chosen as a scavenger. The choice was due to the high solubility of *tert.*-nitrosobutane in hydrophilic systems. The scavenger was carefully mixed in the gels after the irradiation to avoid its known photochemical degradation (19). The signal detected from the gels after this treatment was, however, shown to be due to di-*tert.*-butylnitroxyl.

Recently the use of the photochemically stable scavenger 2,4,6-tri-*tert.*-butylnitrosobenzene was reported (18) (note, however, ref. 20). The effect of this scavenger on the melting point raising reactions in the gels was similar to that of the antioxidants, tocopherol and ascorbic acid, but lasted over a longer period. The melting points of the gels remained unchanged within experimental error for 14 days. No sign of paramagnetism could be detected. The results of the measurements are collected in Figure 3.

The molecular reactions in the cross-linkage of collagen containing unsaturated lipid remain somewhat doubtful. Probably radical and condensation reactions take place in complicated sequences. It is possible that the irradiation, or an initially formed alkoxy- or alkylperoxy radical causes hydrogen abstraction in the peptide chain thereby causing intramolecular or intermolecular cross-linking between peptide chains. Reactions of these types are reported in the literature (21,22).

On the other hand, it has been pointed out that the normal sequence of events in the intramolecular cross-linkage of collagen appears to be: collagen molecules, which contain carbonyl groups, self-assemble into fibers which then become cross-linked because of the reactions that occur between the carbonyl groups and amino groups of adjoining amino acids. When aldehydes react with proteins, Schiff bases are commonly formed. Extrinsic, as well as intrinsic, aldehydes may cause this reaction (23). We, therefore, conclude that even if a free radical reaction might occur in the cross-linking reaction, the condensation between carbonyl compounds and amino groups is far more important in the cross-linking of autoxidizing lipid containing collagen. The effect of ascorbic acid or tocopherol to the lipid containing collagen as well as the radical scavengers *tert.*-nitrosobutane and 2,4,6-tri-*tert.*-butylnitrosobenzene could be the inhibition of the autoxidation of the lipid.

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Polyenoic Acid Metabolism in Cultured Human Skin Fibroblasts

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ABSTRACT

The incorporation of [1-^{14}C]linoleic acid, and [1-^{14}C]linolenic acid into cellular lipids of cultured human skin fibroblasts was studied. Cultured cells took up both labeled fatty acids at nearly the same rate and incorporated them into a variety of lipid classes. At the end of 1 hr incubation with [1-^{14}C]linoleic acid, radioactivity was found in the triacylglycerol (TG) and choline phosphoglyceride (CPG) pools preferentially. Incorporation into the TG fraction decreased rapidly, while the uptake into CPG, serine phosphoglyceride (SPG), and ethanolamine phosphoglyceride (EPG) fractions increased progressively with longer incubation times. Similar results were obtained with [1-^{14}C]linolenic acid as precursor. At the end of 24 hr, desaturation and chain elongation of 18:3 n-3 was more extensive than conversion of 18:2 n-6 to higher polyenoic acids. During pulse-chase experiments with either fatty acid precursor, the incorporated radioactivity was progressively lost from cellular lipids, particularly from the TG and CPG fractions, but continued to increase in the SPG and EPG pools. The similar labeling pattern of cellular phospholipids with linoleic or linolenic acids, and data from pulse-chase studies suggest that a direct transfer of fatty acids from CPG to EPG is a likely pathway in fibroblast cultures. Incorporation into the EPG pool during the pulse-chase experiments paralleled extensive desaturation and elongation of linoleic acid into 20:4 n-6, and 22:4 n-6; and of linolenic acid into 22:5 n-3 and 22:6 n-3.

Polyunsaturated fatty acids (PUFA) are important constituents of cell membranes and play a significant, but as yet not clearly defined, role in cellular structure and function (1-3). During the past decade, cell culture techniques have been used widely for studies of lipid metabolism. The uptake and metabolism of fatty acids, control of fatty acid biosynthesis, as well as essential fatty acid requirements for growth and normal function of several cell lines in culture, have been thoroughly investigated (4-7).

Studies from a number of laboratories on the biosynthetic pathways of polyenoic fatty acids have also been published (8,9), including previous reports from our own laboratory on cultured dissociated brain cells (10,11) and neuroblastoma cells (12). However, the metabolism of PUFA in human skin fibroblasts has not been studied as extensively as the other aspects of lipid metabolism already mentioned. Since fibroblast cultures have been shown to express the primary biochemical lesion in several hereditary disorders (13), and disturbances of PUFA metabolism have already been described in some pathological conditions (14), as well as in virally transformed cells (15), these findings prompted us to analyze polyenoic acid metabolism in human skin fibroblasts as a preliminary step to similar studies on fibroblasts from patients affected with possible genetic disorders of PUFA metabolism.

EXPERIMENTAL PROCEDURES

Fibroblast Cultures

Fibroblasts from a forearm skin biopsy of a 50-year-old normal female human donor were routinely grown for ca. 4 weeks in an atmosphere of 95% air-5% carbon dioxide at 37 C, in Eagle's minimal essential medium (MEM) with Earle's salt solution supplemented with 20% fetal calf serum and containing L-glutamine (2.0 mM), penicillin (100 units per ml), and streptomycin (100 μg per ml). Cells were subcultured at confluency (usually 1 week following inoculation) by removing the medium and washing twice with 2 ml of Hank's Ca-Mg free solution, and then treating the cells with a 0.125% trypsin solution. After 10 min at 37 C, the cells had detached from the culture flask and were harvested by centrifugation for 5 to 10 min at 300 x g. Subcultures were prepared at 1:2 splits.

Similar studies were conducted on five other fibroblast cell cultures from five donors, two of them normal subjects, three of them patients with a neurological disorder (Huntington's Disease). All yielded nearly identical results.

Reagents used for growth and maintenance of cells in culture were obtained from Grand Island Biochemical Company, Grand Island, NY. Fetal calf serum was obtained from

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Rehatuin-Reheis Chemical Company, Chicago, IL. Lipid-deficient fetal calf serum (LDS) was prepared by the method of Albutt (16), as modified by Jacobs et al. (5). Analysis of LDS by gas liquid chromatography (GLC) showed that greater than 95% of the total fatty acid had been removed. Protein recovery was in excess of 90%.

Metabolic Studies

For uptake studies, cells were plated on 60 mm petri dishes (surface area 28 cm^2) in 2 ml of MEM supplemented with 10% fetal calf serum. Inoculum densities ranged from 1.4 to 2.2 x 10^4 cells/cm^2. After 24 hr incubation at 37 C, the medium was removed, and 2 ml of the experimental medium, consisting of MEM supplemented with 10% LDS, and containing [1-^{14}C]linoleic acid (56.9 mCi/mmole, Amersham/Searle, Arlington Heights, IL) or [1-^{14}C]linolenic acid (59.6 mCi/mmole, Amersham/Searle) complexed to albumin (17) was added to the cells. Cell cultures were incubated at 37 C. At designated times, the radioactive medium was removed, and cells were washed twice with 3 ml of a solution of 0.15 M NaCl (saline) containing fatty acid-free albumin (Pentex, Miles Laboratories, Kankakee, IL, 80 mg/100 ml), and then twice with 3 ml of MEM. The cells derived from 2 to 4 petri dishes were scraped off with a rubber policeman, suspended in MEM, pooled, and harvested by centrifugation for 5-10 min at 300 x g.

Pulse-Chase Studies

For pulse-chase studies, cells were incubated for 24 hr with ^{14}C-fatty acid, the radioactive medium was removed, and cells washed as described above. Nonradioactive MEM (2 ml), supplemented with 10% LDS and antibiotics, was added, and the incubation was continued at 37 C for selected times. During the chase period, cultures were re-fed every 2 to 3 days. Results presented were derived from pooled cell extracts from 2 to 4 plates for each data point. When duplicate analyses were performed, the values were essentially identical as were the results when different cell lines were used.

Extraction of Cellular Lipids

Lipid analyses were performed on whole plus broken cells; cultures in which there was a large proportion of broken cells were discarded. The washed cell pellet of 2 to 4 combined petri dishes was homogenized with 1 ml methanol, using a mechanized Potter-Elvehjem type homogenizer with a Teflon pestle. Lipids were extracted by the addition of 2 ml chloroform-methanol (2:1, by vol) to the homogenate, which was then rehomogenized in the organic solvents. Portions were removed for the determination of protein concentration by the method of Lowry et al. (18). After allowing the homogenate to stand for 30 min at 4 C, the lipid extract was filtered through a glass-wool plug in a Pasteur pipette which had previously been washed with chloroform-methanol 2:1, (by vol). The filtrate was evaporated under nitrogen, 4 ml of chloroform-methanol (2:1), and 1 ml of water were added. After mixing, the two phases were allowed to separate, the upper aqueous phase was removed, and the organic phase evaporated to dryness under a stream of nitrogen at 20 C until further analysis.

Resolution of Cellular Lipids

Neutral lipid classes were separated on precoated 0.25 mm Silica Gel G plates (E. Merck A.G., Darmstadt, Germany) by development with a mixture of petroleum ether-diethyl ether-acetic acid (80:20:1, by vol). For the separation of polar lipids, the plates were developed with a mixture of chloroform-methanol-water (65:25:4, by vol). Areas containing the labeled lipids were identified by comparison with known standards chromatographed at the same time. After a brief exposure to iodine vapors, they were scraped off the plates and transferred to counting vials to which 3.5 ml of water and 11.5 ml Aquasol (New England Nuclear, Boston, MA) were added. Radioactivity was counted with a Beckman LS 250 liquid scintillation spectrometer. Correction for quenching was made by the external standard method. Efficiency for ^{14}C was about 65%.

CPG and EPG were isolated by thin layer chromatography (TLC) on Silica Gel G plates in the solvent system chloroform-methanol-water (65:25:4, by vol) or in chloroform-methanol-acetic acid-water (25:15:3:2, by vol) as described by Skipsky et al. (19). Recovery of radioactivity was in excess of 90%.

All solvents were ACS reagent grade, distilled before use, and contained 10 μg/ml of the antioxidant 2,6-ditert.butyl-p-cresol (BHT, butylated hydroxy-toluene, Sigma Chemical Company, St. Louis, MO). A nitrogen atmosphere was maintained throughout the whole procedure to prevent oxidation of the unsaturated fatty acids.

Quantitation of ethanolamine phosphoglyceride (EPG), choline phosphoglyceride (CPG), and total neutral lipids was performed after TLC by sulfuric acid charring according to

TABLE I

Distribution of Radioactivity among Lipid Fractions after Incubation of Cultured Fibroblasts with [1-^{14}C]Linoleic Acid[a]

	Incubation time (hr)					
	1		6		24	
Lipid fraction	A	B	A	B	A	B
Neutral lipids (NL)	36.4	61.5	26.7	322	10.7	218
DG[b]	3.5	5.9	3.0	36.1	2.6	53
FFA	2.4	4.0	1.0	12.0	1.3	26.5
TG	24.2	40.8	17.1	206	5.6	114
CE	3.4	5.7	2.5	30.1	0.9	18.3
Polar lipids (PL)	63.5	107	73.2	882	89.2	1819
SPG[c]	3.5	5.9	5.5	66.2	6.5	132
CPG	48.6	82.1	56.1	676	63.4	1293
EPG	4.6	7.7	5.4	65.0	9.6	196
CER	0.9	1.5	1.0	12.0	5.9	120
UL[d]	8.8	14.8	8.3	100.0	4.1	83.5
Ratio PL/NL	1.74		2.74		8.33	
Ratio CPG/EPG	10.56		10.38		6.60	
Total incorporation (nmol/mg protein)	1.34	169	9.54	1205	16.14	2039
Total protein (μg)	103		130		184	

[a]Human skin fibroblasts in their sixth passage were incubated with [1-^{14}C] linoleic acid (0.5-0.6 x 10^6 dpm/culture) for various periods of time. Cell lipids were extracted and separated by TLC as described in Materials and Methods. Values are given as percentages of total radioactivity incorporated into the cell lipids (A) and as dpm x 10^3/mg protein (B), and represent pooled lipid extracts of two to four cultures.

Abbreviations: DG, diacylglycerols; FFA, free fatty acids; TG, triacylglycerols; CE, cholesteryl esters; SPG, serine phosphoglycerides; EPG, ethanolamine phosphoglycerides; CPG, choline phosphoglycerides; CER, radioactivity tentatively assigned to cerebrosides.

[b]Also contains cholesterol.

[c]Also contains inositol phosphoglycerides.

[d]Unidentified lipids.

Kabara and Chen (20) using EPG, CPG, and triacylglycerol (TG) as primary standards. Total lipids were quantitated according to the method of Pande et al. (21).

Gas Liquid Chromatography (GLC)

For GLC analyses, lipids were visualized under UV light after having been sprayed with 0.001% aqueous Rhodamine 6G. Fatty acid constituents of cellular total lipids, and the CPG and EPG fractions were converted into their corresponding methyl esters by treating them with 2 ml of 0.5 N HCl in methanol in sealed, Teflon-lined, screw-cap tubes under nitrogen for 2 hr at 80 C. After transesterification was completed, 1 ml of water was added, and the methyl esters extracted three times with 2 ml of hexane. No contamination with free fatty acids could be detected by TLC on Silica Gel G plates developed with hexane-diethyl ether (90:1, by vol). The recovery of radioactivity in the form of methyl esters was greater than 90%.

Gas chromatography of the fatty acid methyl esters was performed in a Packard Model 7401 gas chromatograph equipped with a flame ionization detector, with 183 cm coiled column of 4 mm ID containing 15% polydiethylene glycol succinate on 60/80 Supelcoport (Supelco Inc., Bellefonte, PA). The column was operated at 180 C with a gas flow of 100 ml/min of helium through the column. Radioactive fatty acid methyl esters were analyzed with a Packard Model 894 gas proportional counter with an efficiency of 80% for ^{14}C and a sensitivity for counts of ca. 100 to 150 dpm.

Peaks were identified with standard mixtures of fatty acid methyl esters by rechromatography after hydrogenation for 3 hr over a platinum oxide catalyst. Peak areas were quantified by triangulation.

RESULTS

Incorporation of Linoleic and Linolenic Acids

After an incubation time of 24 hr, incorporation of [1-^{14}C]linoleic acid or [1-^{14}C]linolenic acid into cellular lipids of human skin fibroblasts ranged from 40 to 50% of the total radioactivity added to the incubation medium. During the initial phases of incubation with labeled linoleic acid, TG and CPG were labeled

TABLE II

Distribution of Radioactivity among Lipid Fractions after Incubation of Cultured Fibroblasts with [1-^{14}C] Linolenic Acid[a]

Lipid fraction	Incubation time (hr) 1 A	1 B	6 A	6 B	24 A	24 B
Neutral lipids (HL)	48.1	55.7	23.3	187	10.1	178
DG[b]	4.6	5.3	2.6	20.8	2.2	38.8
FFA	2.4	2.7	1.6	12.8	1.1	19.4
TG	31.1	36.0	10.4	83.5	4.1	72.4
CE	3.8	4.4	3.2	25.6	0.7	12.3
Polar lipids	51.8	60.0	76.6	615	89.8	1588
SPG[c]	2.5	2.9	1.7	13.6	3.1	54.8
CPG	39.9	46.2	61.5	494	61.8	1093
EPG	3.3	3.8	5.8	46.5	11.5	203
CER	0.8	0.9	1.3	10.4	1.7	30.0
UL[d]	11.5	13.3	11.8	94.7	13.7	242
Ratio PL/NL	1.07		3.28		8.89	
Ratio CPG/EPG	12.10		10.60		5.37	
Total incorporation (nmol/mg protein)	0.88	116	6.07	803	13.36	1768
Total protein (μg)	186		178		200	

[a]Human skin fibroblasts in their sixth passage were incubated with [1-^{14}C]linolenic acid (0.5-0.6 x 10^6 dpm/culture) for various periods of time. Cell lipids were extracted and separated by TLC as described in Materials and Methods. Values are given as percentages of total radioactivity incorporated into the cell lipids (A) and as dpm x 10^3/mg protein (B) and represent pooled lipid extracts of two to four cultures. Abbreviations as in Table I.

[b]Also contains cholesterol.

[c]Also contains inositol phosphoglycerides.

[d]Unidentified lipids.

preferentially (Table I). After 1 hr, ca. 72% of total radioactivity was recovered from the TG and CPG fractions. After 6 hr, the amount of radioactivity in the TG fraction decreased, reaching about 5% of total radioactivity by the end of the 24 hr incubation, while uptake into the CPG, SPG, and EPG pools increased progressively. Very little radioactivity entered monoglycerides (MG), diacylglycerols (DG), free fatty acids, or cholesteryl esters (CE) (Table I).

Significant amounts of radioactivity were found in an unidentified lipid (UL, Tables I, II). This substance migrated between CPG and EPG in all systems used.

The distribution of radioactivity after incubation of cells with [1-^{14}C]linolenic acid resembled that obtained for linoleic acid. The TG fraction contained ca. 31% of total radioactivity after 1 hr incubation, and only 4% at the end of 24 hr, while incorporation into CPG, SPG, and EPG fractions increased progressively between 1 and 24 hr incubation (Table II).

The ratio of radioactivity between the CPG and EPG fractions, using linoleic acid as the labeled substrate (Table I), was similar to the one obtained with linolenic acid (Table II), and decreased progressively in the course of incubation.

Metabolism of Labeled Linoleic and Linolenic Acids

The distribution of label among the individual fatty acids of total cellular lipids after 6 and 24 hr incubation with labeled linoleic acid is shown in Table III. After 24 hr, 86% of the radioactivity remained as 18:2 n-6, and only 9.6% of label was found in the higher PUFA (20:2 n-6, 20:3 n-6, and 20:4 n-6). The conversion of linolenic acid to its higher derivates proceeded more rapidly (Table IV). At the end of 24 hr, 58.2% of the radioactivity incorporated into cellular lipids was in the form of 18:3 n-3, while 32.7% was found in 20:3 n-3, 20:4 n-3, 20:5 n-3, and 22:6 n-3.

Quantitation of fibroblast fatty acids showed that 16:0, 18:0, 18:1, and to a much lesser extent, 20:4 were the principal components. PUFA, namely 18:2, 18:3, 20:3, 20:5, 22:4, 22:5, and 22:6, were minor components; therefore, no attempt was made to quantitate their relative specific activities.

Pulse-Chase Studies

After the initial 24 hr incubation with [1-^{14}C]linoleic acid, fibroblast cultures were maintained in a nonradioactive lipid-deficient medium for up to 12 days. As is ascertainable from Tables V, VI, and VII, the protein values

TABLE III

Distribution of Radioactivity among Fatty Acids of Total Lipids after Incubation of Cultured Fibroblasts with [1-^{14}C] Linoleic Acid[a]

	Incubation time (hr)			
	6		24	
Fatty acid	A	B	A	B
18:2 n-6	91.3	1100	86.0	1753
20:2 n-6	3.8	45.7	2.6	53.0
20:3 n-6	tr	--	1.4	28.5
20:4 n-6	3.2	38.5	5.6	114
16:0 + 16:1	0.9	10.8	2.3	46.8
18:0 + 18:1	0.7	8.4	1.8	36.7
Total lipids		1205		2036

[a]Human skin fibroblasts in their sixth passage were incubated with [1-^{14}C]linoleic acid (0.5-0.6 x 10^6 dpm/culture) for 6 or 24 hr. Cell lipids were extracted, and total lipids fatty acid methyl esters prepared and analyzed for radioactivity as described in Materials and Methods. Values are given as percentage of total radioactivity (A) and as dpm x 10^3/mg protein (B) in two pooled experiments.

TABLE IV

Distribution of Radioactivity among Fatty Acids of Total Lipids after Incubation of Cultured Fibroblasts with [1-^{14}C] Linolenic Acid[a]

	Incubation time (hr)			
	6		24	
Fatty acid	A	B	A	B
18:3 n-3	81.7	656	58.2	1029
20:3 n-3	7.4	59.4	11.2	198
20:4 n-3	tr	---	3.5	61.8
20:5 n-3	4.2	33.7	6.8	120
22:4 n-3	---	---	---	---
22:5 n-3	1.8	14.4	8.5	150
22:6 n-3	---	---	2.7	47.7
16:0 + 16:1	1.4	11.2	3.5	61.8
18:0 + 18:1	3.2	25.6	5.2	91.9
Total lipids		803		1768

[a]Human skin fibroblasts in their sixth passage were incubated with [1-^{14}C]linolenic acid (0.5-0.6 x 10^6 dpm/culture) for 6 or 24 hr. Cell lipids were extracted, and total lipids fatty acid methyl esters prepared and analyzed for radioactivity as described in Materials and Methods. Values are given as percentages of total radioactivity (A) and as dpm x 10^3/mg protein (B) in two pooled experiments.

at the termination of the experiment ranged from 21 to 94% of the values at day 0. In part, the fall in protein content was the consequence of manipulative losses.

The concentrations of EPG and CPG rose between days 0 and 12, the increase in EPG being greater; thus, there was a drop in the CPG/EPG ratio (Table V). Radioactivity in total lipids dropped slowly. The relative amounts of radioactivity in the TG and CPG fractions decreased steadily but continued to increase in the SPG and EPG pools (Table VI). After a chase period of 12 days, the ratio of radioactivity between CPG and EPG decreased to 0.75 from 6.11 at 0 days.

When these experiments were repeated in the presence of added unlabeled linoleic acid (3 μg/ml medium), the pattern of labeling and the distribution of radioactivity between the various lipid fractions were essentially identical.

Similar results were obtained in pulse-chase studies with [1-^{14}C]linolenic acid (Table VII). The recovered radioactivity in the TG and CPG pools decreased from about 56% at 0 time to 24% after the 12 day chase period, while the label in SPG and EPG increased from ca. 19% to 57%. The ratio of CPG/EPG decreased from 2.16 to 0.45 during the same period.

The distribution of radioactivity among individual fatty acids of total cellular lipids, CPG and EPG in the pulse-chase studies, after incubation with labeled linoleic acid is given in

TABLE V

Lipid Composition of Cells Grown in Lipid-Deficient Serum (LDS)[a]

Days in LDS	Total protein (mg)	Total lipids (mg)	CPG (mg/mg protein)	EPG (mg/mg protein)	Total neutral lipids (mg/mg protein)
0	0.915	0.233	0.060	0.035	0.034
12	0.861	0.230	0.074	0.064	0.039

[a]Human skin fibroblasts were incubated for 12 days in nonradioactive MEM supplemented with 10% LDS. At conclusion of incubation, the cell lipids were extracted and separated. Quantitation of total lipids, total neutral lipids, CPG, and EPG was performed as indicated.

TABLE VI

Distribution of Radioactivity among Lipid Fractions after Incubation with [1-^{14}C]Linoleic Acid in the Pulse-Chase Studies[a]

	Period of chase (days)					
	0		4		12	
Lipid fraction	A	B	A	B	A	B
Neutral lipids (NL)	18.6	84.4	13.5	42.1	7.8	15.8
DG[b]	2.1	9.5	1.8	5.6	3.9	7.9
FFA	1.2	5.4	0.8	2.4	0.9	1.8
TG	12.9	58.5	8.4	26.2	1.9	3.8
CE	0.6	2.7	0.4	1.2	0.3	0.6
Polar lipids (PL)	81.3	369	86.4	270	92.1	187
SPG[c]	2.7	12.2	4.0	12.4	13.3	26.9
CPG	57.5	261	50.6	158	28.5	57.8
EPG	9.4	42.6	14.8	46.1	37.9	76.9
CER	2.7	12.2	1.6	4.9	4.8	9.7
UL[d]	10.8	49.0	17.5	54.6	8.4	17.0
Ratio PL/NL	4.37		6.40		11.80	
Ratio CPG/EPG	6.11		3.41		0.75	
Total incorporation (nmoles/mg protein)	3.59	454	2.47	312	1.61	203
Total protein (μg)	455		419		140	

[a]Human skin fibroblasts in their eleventh passage were incubated for 24 hr (0 time) with [1-^{14}C]-linoleic acid (0.5-0.6 x 10^6 dpm/culture). After the radioactive medium was removed, the cells were further incubated with nonradioactive MEM supplemented with 10% LDS. At each specified time, two petri dishes were removed, and cell lipids extracted and separated by TLC as described in Materials and Methods. Values are given as percentages of total radioactivity incorporated into cell lipids (A) and as dpm x 10^3/mg protein (B) and represent pooled lipid extracts of two cultures. Abbreviations as in Table I.

[b]Also contains cholesterol.

[c]Also contains inositol phosphoglycerides.

[d]Unidentified lipids.

Table VIII. During the chase period, linoleic acid was readily converted to higher polyenoic fatty acids, mainly arachidonic acid (20:4 n-6) and docosatetraenoic acid (22:4 n-6).

Desturation and elongation of 18:2 n-6 to 20:4 n-6, and 22:4 n-6 was more extensive in the EPG fraction, while a significant amount of radioactivity, particularly in the CPG pool, was derived from the recycling of two carbon units, and was in the form of 16 and 18 carbon atoms fatty acids.

Results obtained in pulse-chase studies with labeled linolenic acid are given in Table IX. Labeled linolenic acid was also extensively converted into higher polyenoic fatty acids, mainly docosapentaenoic (22:4 n-3) and docosahexaenoic acids (22:6 n-3). The proportions of radioactivity in 22:5 n-3 and 22:6 n-3 were greater in the EPG fraction, while the proportion of radioactivity recycled into 16 and 18 carbon atoms fatty acids was particularly high in the CPG fractions.

DISCUSSION

Cultured human skin fibroblasts took up labeled linoleic and linolenic acids at approximately the same rate and incorporated them

TABLE VII

Distribution of Radioactivity among Lipid Fractions after Incubation with [1-^{14}C] Linolenic Acid in the Pulse-Chase Studies[a]

	Period of chase (days)					
	0		4		12	
Lipid fraction	A	B	A	B	A	B
Neutral lipids (NL)	34.9	119	25.0	60.7	6.9	9.5
DG[b]	2.2	7.4	1.8	4.3	3.1	4.2
FFA	1.5	5.1	1.2	2.9	0.9	1.2
TG	16.9	57.4	16.6	40.3	2.0	2.7
CE	2.3	7.8	1.0	2.4	4.1	5.6
Polar lipids (PL)	65.0	221	74.9	182	93.1	128
SPG[c]	1.6	5.4	2.7	6.5	7.1	9.7
CPG	39.1	133	28.8	69.9	22.9	31.6
EPG	18.1	61.5	32.7	79.4	50.8	70.1
CER	1.5	5.1	1.6	3.8	3.1	4.2
UL[d]	16.7	56.7	13.5	32.8	5.9	8.1
Ratio PL/NL	1.86		2.99		13.49	
Ratio CPG/EPG	2.16		0.88		0.45	
Total incorporation (nmoles/mg protein)	2.57	340	1.84	243	1.04	138
Total protein (μg)	369		208		79	

[a]Human skin fibroblasts in their sixteenth passage were incubated for 24 hr (0 time) with [1-^{14}C]linolenic acid (0.5-0.6 x 10^6 dpm/culture). After the radioactive medium was removed, the cells were further incubated with nonradioactive medium (MEM supplemented with 10% LDS). At each specified time, two petri dishes were removed, cell lipids extracted and separated by TLC as described in Materials and Methods. Values are given as percentages of total radioactivity incorporated into cell lipids (A), and as dpm x 10^3/mg protein (B) and represent pooled lipid extracts of two cultures. Abbreviations as in Table I.

[b]Also contains cholesterol.

[c]Also contains inositol phosphoglycerides.

[d]Unidentified lipids.

into a variety of lipid classes, mainly into TG and phospholipids. Fatty acid uptake did not appear to be influenced by the age of the donor within the limits of our experiment (ages 3 to 50 years), nor by passage number (5 to 16).

As was previously found with respect to cultured brain cells (10), there was an initial rapid incorporation of both PUFA into TG and CPG fractions. Subsequently, uptake into these fractions decelerated. Incorporation into the EPG and SPG fractions proceeded at a slower rate than did incorporation into TG and CPG, but the proportion of radioactivity in these fractions continued to increase between 24 hr and 12 days incubation. There was little difference between the labeling patterns of the various phospholipids produced by the two polyunsaturated fatty acid precursors. In this respect as well, fibroblasts behaved similarly to cultured brain cells (10) but differed from cultured neuroblastoma cells (12). In the latter cell system, CPG was the major labeled lipid when linoleic acid was used as precursor, while with linolenic acid as precursor, the EPG fraction was labeled more extensively. The similar labeling pattern of CPG and EPG by fibroblast cultures and the steady increase in the proportion of radioactivity in the EPG fraction seen in our pulse-chase studies suggest that a direct transfer of ^{14}C-labeled fatty acids between these two phospholipid moieties is a likely metabolic pathway.

In the course of being incorporated into the cellular lipids, linoleic and linolenic acids underwent desaturation and chain elongation along well-established metabolic pathways (15,22). Conversion of linoleic acid to the higher n-6 polyenoic fatty acids was slower than conversion of linolenic acid to its higher n-3 analogs. We were unable to detect any labeled 18:3 n-6 intermediate in the linoleic acid studies, nor could we verify the presence of 18:4 n-3 in the linolenic acid experiments, even though in both experiments incubation of cells was terminated after 1 hr. As suggested by Sprecher (23), the absence of these labeled intermediates could be explained by a slow rate of desaturation of 18:2 n-6 to 18:3 n-6, and of 18:3 n-3 to 18:4 n-3 followed by rapid chain elongation to the higher anaologs.

In the course of our studies, we observed that fibroblasts which had been maintained as long as 16 weeks in culture readily converted linoleic to arachidonic acid, and linolenic acid

TABLE VIII

Distribution of Radioactivity among Fatty Acids of Total Lipids, CPG and EPG Fractions after Incubation with [1-^{14}C] Linoleic Acid in the Pulse-Chase Studies[a]

	Period of chase (days)																	
	Total lipids						CPG						EPG					
	0		4		12		0		4		12		0		4		12	
Fatty acid	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
18:2 n-6	82.1	373	73.2	228	17.6	35.7	89.5	234	70.8	112	21.9	12.6	90.0	38.3	53.5	24.6	6.3	4.8
20:2 n-6	9.3	42.2	3.9	12.1	0.7	1.4	6.9	18.0	4.0	6.3	2.9	1.6	2.3	0.9	7.0	3.2	5.9	4.5
20:3 n-6	2.3	10.4	3.3	10.2	3.6	7.3	tr	---	tr	---	2.9	1.6	3.2	1.3	6.4	2.9	5.5	4.2
20:4 n-6	4.1	18.6	6.6	20.5	57.6	117	0.5	1.3	17.9	28.2	53.7	31.0	4.4	1.8	28.2	13.0	62.3	47.9
22:4 n-6	tr	---	tr	---	15.6	31.6	tr	---	tr	---	5.2	3.0	tr	---	tr	---	16.2	12.4
16:0 + 16:1	2.0	9.0	1.2	3.7	2.0	4.0	1.8	4.6	3.5	5.5	7.5	4.3	tr	---	2.9	1.3	0.3	0.2
18:0 + 18:1	tr	---	1.6	4.9	2.6	5.2	1.1	2.8	3.5	5.5	3.8	2.1	tr	---	1.7	0.7	1.0	0.7
Total		454		312		203		261		158		57.8		42.6		46.1		76.9

[a]Human skin fibroblasts in their eleventh passage were incubated for 24 hr (0 time) with [1-^{14}C]linoleic acid (0.5-0.6 x 10^6 dpm/culture). After the radioactive medium was removed, the cells were further incubated with nonradioactive MEM supplemented with 10% LDS. At each specified time, two petri dishes were removed, cell lipids extracted, the CPG and EPG fractions isolated, and their methyl ester fatty acids prepared and analyzed for radioactivity as described in Materials and Methods. Values are given as percentage of total radioactivity (A), and as dpm x 10^3/mg protein (B) of fatty acids in total lipids and in EPG and CPG fractions of pooled lipid extracts from two cultures.

TABLE IX

Distribution of Radioactivity among Fatty Acids of Total Lipids, CPG and EPG Fractions after Incubation with [1-^{14}C]Linolenic Acid in the Pulse-Chase Studies[a]

	Period of chase (days)																	
	Total lipids						CPG						EPG					
	0		4		12		0		4		12		0		4		12	
Fatty acid	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
18:3 n-3	42.1	143	9.0	21.8	5.1	7.0	70.0	93.0	9.0	6.2	7.8	2.4	14.4	8.8	8.0	6.3	1.5	1.0
20:3 n-3	10.2	34.6	6.8	16.5	2.0	2.7	tr	—	3.5	2.4	4.3	1.3	2.8	1.7	1.1	0.8	tr	—
20:4 n-3	8.2	27.8	3.6	8.7	3.6	4.9	tr	—	4.0	2.7	2.1	0.6	2.4	1.4	4.6	3.6	2.5	1.7
20:5 n-3	12.3	41.8	19.0	46.1	7.6	10.4	12.6	16.7	21.4	14.9	10.4	3.2	16.6	10.2	15.3	12.1	8.6	6.0
22:4 n-3	tr	—	7.1	17.2	9.0	12.4	tr	—	2.0	1.3	10.6	3.3	tr	—	5.3	4.2	8.1	5.6
22:5 n-3	20.1	68.3	40.8	99.1	47.6	65.6	tr	—	42.9	29.9	42.3	13.3	52.6	32.3	43.0	34.1	54.5	38.2
22:6 n-3	tr	—	6.4	15.5	14.9	20.5	tr	—	3.0	2.0	4.1	1.2	5.3	3.2	11.5	9.1	21.7	15.2
16:0 + 16:1	3.5	11.9	4.1	9.9	4.3	5.9	9.8	13.0	6.3	4.4	8.0	2.5	0.7	0.4	3.8	3.0	1.2	0.8
18:0 + 18:1	2.5	8.5	1.9	4.6	4.6	6.3	7.5	9.9	7.5	5.2	10.3	3.2	4.8	2.9	6.9	5.4	1.7	1.1
Total lipids		340		243		138		133		69.9		31.6		61.5		79.4		70.0

[a]Human skin fibroblasts in their sixteenth passage were incubated for 24 hr (0 time) with [1-^{14}C]linolenic acid (0.5-016 x 10^6 dpm/culture). After the radioactive medium was removed, the cells were further incubated with nonradioactive MEM supplemented with 10% LDS. At each specified time, two petri dishes were removed, cell lipids extracted, the CPG and EPG fractions isolated, and their fatty acid methyl esters prepared and analyzed for radioactivity as described in Materials and Methods. Values are given as percentage of total radioactivity (A), and as dpm x 10^3/mg protein (B) in total fatty acids of total lipids, EPG and CPG fractions in pooled lipid extracts of two cultures.

to docosapentaenoic and docasahexaenoic acids. By contrast, both normal brain cell cultures (12) and freshly isolated heart cell cultures (8) partially or completely lose their ability to desaturate and elongate linoleic and linolenic acids after prolonged periods in vitro.

Incorporation of labeled PUFA into the EPG fraction paralleled desaturation and chain elongation, and was associated with a decline of radioactivity in the CPG fraction. It thus would appear that both precursors were first incorporated into the CPG pool and subsequently, after desaturation and elongation to the higher polyenoic acids, were transferred into the EPG pool. The transfer of fatty acids between these two pools may occur by means of one or both of two mechanisms. The first would involve successive hydrolysis, activation to the fatty acid CoA analogs, elongation, desaturation, and finally reesterification. Desaturation may also occur when the fatty acids are esterified to the glycerol moiety of CPG. Pugh and Kates have shown the direct conversion of eicosatrienoyl lecithin to arachidonyl lecithin by a liver microsomal acyl-CoA desaturase system (24). This system, which presumably functions to provide PUFA in the absence of adequate dietary sources (24), may have become activated in our experiments by prolonged incubation in lipid-deficient medium.

The importance of these experiments on the understanding of PUFA metabolism within the central nervous system is supported by the experiments of Fewster et al. (25), who obtained similar data on the metabolism of linolenic acid by isolated oligodendroglia.

The factors that control fibroblast PUFA metabolism and the tranfer of fatty acids from one lipid moiety to another are still unknown (26,27).

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Arachidonic Acid Intestinal Absorption: Mechanism of Transport and Influence of Luminal Factors on Absorption in Vitro

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ABSTRACT

The mechanism and characteristics of intestinal absorption of arachidonic acid were studied in vitro using everted intestinal sacs of the rat. Arachidonic acid absorption was studied at concentrations of 5 μM to 8.36 mM. The plot of absorption rate vs. concentration fitted best to a rectangular hyperbola at low μM concentrations and to a straight linear relationship in the mM range of concentrations. Metabolic inhibitors and uncouplers did not change absorption in either range of concentrations. The absorption of arachidonic acid increased with thinning of the unstirred water-layer, decrease in the pH, or the substitution of sodium taurocholate by Pluronic F 68 or Tween 80. Absorption decreased following the equimolar additions of oleic, linoleic, and linolenic acids. Absorption rate did not change when the taurocholate concentration was varied from 5-15 mM or following the additions of butyric or glutamic acids, leucine, lysine, or dextrose. It was concluded that arachidonic acid is absorbed by a concentration-dependent dual mechanism of transport which is not energy dependent. At the low μM range of concentrations, facilitated diffusion is predominant, while at mM concentrations, simple diffusion is the dominant mechanism of absorption. Changes in the intestinal fluid composition, flow rate, and pH can modify the rate of absorption of arachidonic acid.

INTRODUCTION

Arachidonic acid, an essential polyunsaturated fatty acid, is a metabolic precursor of protaglandins (1-5), can be an initiator of parturition (6,7), and has been shown to inhibit gastric acid secretion in the dog (8,9). While a large body of information regarding the metabolism of arachidonic acid is available, there is no information regarding its mechanism and site of intestinal absorption. The purpose of the present series of experiments is to elucidate the mechanism of intestinal absorption of arachidonic acid and to explore some of the factors which may modify its rate of absorption.

MATERIALS AND METHODS

Materials

^{3}H-arachidonic acid (Amersham/Searle Corp., Arlington Heights, IL) with specific activity of 80 Ci/mmole was used as a tracer compound. The radiochemical purity of the compound was ascertained by thin layer chromatography (TLC) on silica gel developed in petroleum ether-ether-acetic acid (80:50:1) and was found to be greater than 98%. Nonradioactive arachidonic acid (Sigma Chemical Co., St. Louis, MO) had less than 1% impurities. ^{14}C-Carboxylic inulin (Amersham/Searle Corp.) with specific activity of 7.7 mCi/mmole was used as a marker for adsorption (10). Purified sodium taurocholate (K & K Laboratories, Plain View, NY) was found to have less than 2% impurities by TLC (11). Pluronic F 68 (BASF Wyandotte Corp., Wyandotte, MI) and Tween 80 (Fisher Scientific Co., Fairlawn, NJ) were used as nonionic surfactants for solubilizing arachidonic acid in some of the experiments.

Butyric, oleic, linoleic, and linolenic acids with purity of 99% were obtained from Sigma Chemical Co. L-Leucine, L-lysine, and glutamic acid (Aldrich Chemical Co., Inc., Milwaukee, WI) were of 99% purity when purchased. Dextrose (99% purity) was used as obtained (Fisher Scientific Co.).

A micellar solution of the surfactant or bile salt in the standard phosphate buffer solution was prepared by ultrasound irradiation for 5 min at 70 watts of power with a sonicator (Artek Corporation, Farmingdale, NY). A.R. grade sodium dihydrogen phosphate and disodium hydrogen phosphate (J.T. Baker Chemical Co., Phillipsburg, NJ) were used as buffer components. The pH of the solutions was varied by changing the relative concentrations of the sodium salts of phosphate. The final incubation solution contained the following compounds at given concentrations: arachidonic acid (5 μM to 8.35 mM), sodium dihydrogen phosphate (22.82 mM), disodium hydrogen phosphate (87.45 mM), and tracer amounts of ^{3}H-arachidonic acid and ^{14}C-inulin. Inulin, which is a nonabsorbable sugar, was used throughout this study as a marker compound for correction of the apparent gross absorption of arachidonic acid for adsorption (10). On

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separate occasions, either sodium taurocholate (5-20 mM), or Pluronic F 68, or Tween 80 (5 mM) were added to the buffer solution to act as ionic or nonionic surfactants for solubilization of arachidonic acid.

Everted Sac Preparation

Male Sprague-Dawley rats (Spartan Research Animals, Inc., Haslett, MI) weighing 180-220 gm had free access to water and Purina Rat Chow (Ralston Purina Co., St. Louis, MO). The rats were not fasted prior to experimentation and were sacrificed by stunning and cervical dislocation. The small intestine was exposed and rinsed in situ with chilled normal saline. The entire small intestine was then removed and everted. A 15 cm segment distal to the ligament of Treitz was designated as proximal jejunum, and the distal 15 cm of the small bowel immediately proximal to the ileocecal junction was designated as distal ileum. Each segment was subdivided with sutures into 1.5 cm sacs which were identified with tags indicating their exact origin. Intestinal segments which contained Peyer's patches were not used. No fluid was placed in the serosal compartment since no transmural transport of lipid compounds has been found under similar in vitro conditions (12-15).

Incubation Methods

The sac was immediately immersed in 50 ml of the micellar incubation solution which had been equilibrated to 37 C prior to experimentation and which was contained in a plexiglass incubation chamber with internal dimensions of 2 x 6 x 30 cm. The chamber was placed in a metabolic water bath (Precision Scientific Co., Chicago, IL) and was agitated at 80 oscillations/min. Preincubation samples were withdrawn in triplicate and were used for calculation of the initial specific activity of arachidonic acid and inulin. Proximal and distal intestinal sacs were removed from the chamber every 2 min for a total period of 8 min. The sacs were immediately immersed for 15 sec in a beaker which contained 200 ml of either 1 mM sodium taurocholate, or 0.1 mM pluronic F 68, or 1 mM Tween 80 solution which was sitrred by a magnetic stirrer at a constant rate. The rinse was designed to remove some of the incubation solution that had remained adherent to the sacs. All sacs were then gently blotted on a paper towel and dried in a vacuum oven at 50 C under 20 in. of mercury vacuum for 24 hr. The sutured ends of each sac were removed, and the sacs were weighed in the dry state. All calculations pertaining to tissue weight refer to the dry weight of the tissue. All experimental work was performed under subdued lighting and an aluminum foil cover in order to prevent decomposition of arachidonic acid by ultraviolet light irradiation.

Radioactivity Determinations

The radioactivity of the absorbed arachidonic acid and the adsorbed inulin was separated by total combustion of the intestinal sacs by a sample oxidizer (Tri-Carb Model 306, Packard Instrument Co., Downers Grove, IL). The ^{3}H-arachidonic acid and ^{14}C-inulin were thus converted to tritiated water and $^{14}CO_2$ gas, respectively. Monophase-40® (Packard Instrument Co.) was used as a scintillator for tritiated water. Carbosorb® and permafluor V® (Packard Instrument Co.) were used for $^{14}CO_2$ scintillation counting. All radioactivity measurements were carried to a counting error of ± 1% by using a liquid scintillation counter (Beckman LS 250, Fullerton, CA) with automatic quench calibration at ambient temperature.

Statistical Analysis

The data were plotted by using least squares regression analysis (16). The absorption of arachidonic acid under various experimental conditions was compared to baseline data by using analysis of variance (ANOVA) and Student's t-test (17).

RESULTS

Viability and Stability of Experimental Preparation

At first, the incubation solution concentration of arachidonic acid was 2.1 mM, its pH was 7.4, and its incubation temperature was 37 C. The incubation solution also contained 10 mM sodium taurocholate, 22.82 mM sodium dihydrogen phosphate, 87.45 mM disodium hydrogen phosphate, and tracer amounts of ^{3}H-arachidonic acid and ^{14}C-inulin. The relationship between arachidonic acid absorption and time was found to be linear ($r > 0.95$, $p < 0.01$) for the entire 8 min of incubation. The absorption of arachidonic acid by the proximal jejunum was not different ($p > 0.05$) from that by the distal ileum (Table I).

Absorption of Arachidonic Acid at Varied Incubation Fluid Concentrations

In order to examine the influence of arachidonic acid concentration on its absorption rate, the concentration of arachidonic acid in the mucosal solution was varied from 0.21 to 8.36 mM while the other components of the solution and conditions of the experiments were kept

TABLE I

Influence of Metabolic Inhibitors and Uncouplers on 2.1 mM Arachidonic Acid Absorption

Region and incubation time (min)	Absorption[a] (nmol/100 mg dry wt sac)			
	Arachidonic acid (2.1 mM)	+ DNP (0.1 mM)	+ KCN (1.0 mM)	+ NaN_3 (1.0 mM)
Proximal				
2	172.2 ± 2.7	163.35 ± 9.14[b]	172.56 ± 13.37[b]	195.08 ± 32.95[b]
4	262.92 ± 7.39	236.17 ± 14.31[b]	243.11 ± 12.41[b]	270.28 ± 8.92[b]
6	319.40 ± 6.92	322.81 ± 17.55[b]	317.94 ± 16.20[b]	299.31 ± 13.86[b]
8	353.35 ± 8.73	334.68 ± 10.19[b]	401.81 ± 18.95[b]	411.45 ± 19.30[b]
Distal				
2	157.17 ± 6.58	181.25 ± 10.72[b]	177.18 ± 17.82[b]	172.91 ± 7.95[b]
4	242.53 ± 6.81	240.26 ± 19.87[b]	268.21 ± 42.66[b]	241.84 ± 25.19[b]
6	280.04 ± 9.92	297.16 ± 30.58[b]	350.52 ± 16.55[b]	291.35 ± 24.51[b]
8	322.74 ± 7.46	367.22 ± 11.49[b]	358.39 ± 15.92[b]	324.32 ± 40.55[b]

[a]Values represent mean ± standard error for four different rat experiments.

[b]$p > 0.05$; statistical comparison between groups at given time was performed by using Student's t-test.

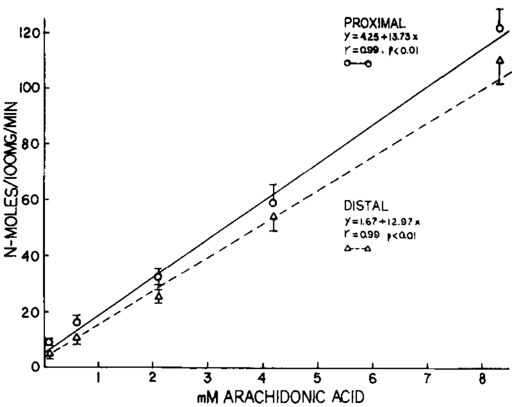

FIG. 1. Absorption rate of arachidonic acid at high concentrations. Each point represents mean ± SE absorption rate. At least four animals were used to define each point. Statistical line was obtained by least squares regression analysis.

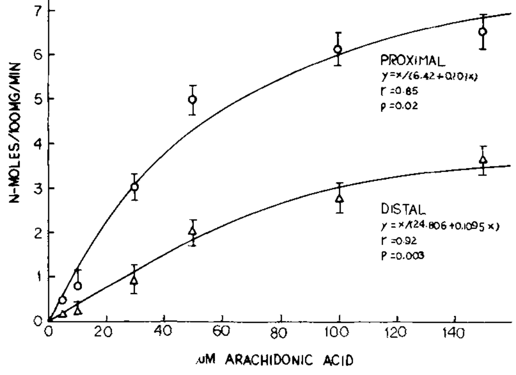

FIG. 2. Absorption rate of arachidonic acid at low concentrations. Each point represents mean ± SE. At least four animals were used to define each point. The rectangular hyperbolic equations were obtained by NLIN computer program.

constant. The absorption remained linear with time in all experiments ($r > 0.95$, $p < 0.01$). The rate of arachidonic acid's absorption by the proximal and distal segments increased with increases in its concentration. The differences between the absorption of neighboring concentrations were significant ($p < 0.05$), but the difference between the proximal and distal segments' absorption rates was not significant. The relationship between the absorption rate of arachidonic acid and its concentration remained linear throughout this range of concentrations (Fig. 1). In the next series of studies, we investigated the absorption of arachidonic acid at a much lower range of concentrations (5-150 μM). The experimental conditions and the composition of the incubation solution were kept

TABLE II

Influence of Metabolic Inhibitors and Uncouplers on 100 μM Arachidonic Acid Absorption

Inhibitor (mM)	No. of rats	Absorption rate (nmol/min per 100 mg)			
		Proximal	p[a]	Distal	p[a]
None	4	6.14 ± 0.28	---	2.39 ± 0.10	---
DNP (0.1)	4	5.23 ± 0.47	> 0.05	2.10 ± 0.09	> 0.05
KCN (1.0)	4	5.35 ± 0.39	> 0.05	2.42 ± 0.13	> 0.05
NaN_3 (1.0)	4	6.05 ± 0.34	> 0.05	2.28 ± 0.16	> 0.05

[a]Statistical analysis of the data was made by comparing the absorption rate without inhibitors to the absorption rate following the additions of DNP, KCN, and NaN_3.

TABLE III

Influence of pH on the Absorption of 100 μM Arachidonic Acid

Incubation pH	No. of rats	Absorption rate (nmol/min per 100 mg)			
		Proximal	p[a]	Distal	p[a]
7.4	4	6.14 ± 0.28	---	2.39 ± 0.10	---
6.4	5	7.68 ± 0.46	< 0.05	4.05 ± 0.41	< 0.05
5.4	5	9.26 ± 0.67	< 0.05	6.40 ± 0.49	< 0.05

[a]ANOVA was used for statistical comparison of the absorption rate at pH 7.4 to absorption rate at lower pH values.

constant. The relationship of absorption vs. time remained linear in individual experiments ($r > 0.95$, $p < 0.01$). However, the rate-concentration plot of the data fitted best to a rectangular hyperbola (Fig. 2) and demonstrated saturation of the absorption process at concentrations above 100 μM.

Influences of Other Fatty Acids on Arachidonic Acid Absorption

Jejunal and ileal absorption of 100 μM arachidonic acid was studied in a micellar solution of 10 mM sodium taurocholate at pH 7.4. The fatty acids were added at 100 μM concentrations. In all the experiments, the relationship between arachidonic acid uptake and time was linear ($r > 0.95$, $p < 0.01$) during the 8 min of incubation. Butyric acid (4:0) addition did not change the rate of arachidonic acid absorption (Fig. 3). In contrast, the addition of oleic (18:1), linoleic (18:2), and linolenic (18:3) acids resulted in a significant and progressive decrease ($p < 0.05$) in the absorption rate of arachidonic acid by the jejunum and ileum (Fig. 3).

Influence of Metabolic Inhibitors and Uncouplers on Arachidonic Acid Absorption

Absorption of 100 μM and 2.1 mM arachidonic acid was investigated in the presence of a variety of metabolic inhibitors and uncouplers. The basal absorption rate was compared with absorption experiments in which 0.1 mM 2,4-dinitrophenol (DNP), 1 mM potassium cyanide (KCN), or 1 mM sodium azide (NaN_3) were added separately to the incubation medium containing 100 μM or 2.1 mM arachidonic acid, respectively. The addition of these metabolic inhibitors and uncouplers (Tables I, II) did not change the rate of arachidonic acid absorption when compared to absorption under basal conditions at either low or high concentrations of arachidonic acid ($p > 0.05$).

Influence of pH on Arachidonic Acid Absorption

Since the intestinal luminal pH can vary, its influence on the absorption rate of arachidonic acid was studied by changing the ratio of the monobasic and dibasic sodium salts of phosphate in the buffer solution. The incubation fluid contained 100 μM arachidonic acid and 10 mM sodium taurocholate. The absorption of arachidonic acid increased with the decrease in incubation fluid pH (Table III).

Influences of Surfactants on Arachidonic Acid Absorption

We investigated the absorption of arachidonic acid in the presence of 5, 10, 15, or 20 mM sodium taurocholate in the standard phosphate buffer solution. At taurocholate concentrations of 5-15 mM, the absorption rate was not significantly ($p > 0.05$) different but at 20

mM sodium taurocholate concentration, the absorption rate decreased when compared to baseline experiments at 10 mM taurocholate concentration (Table IV).

Since sodium taurocholate is an anionic surfactant, we studied the influence of two nonionic surfactants on arachidonic acid absorption. Sodium taurocholate was replaced by Pluronic F 68 or Tween 80 in separate series of experiments. Pluronic F 68 is a nonionic surfactant composed of a mixture of polyoxyethylene-polyoxypropylene ethers of an average molecular weight of 8350. Tween 80, which has an average molecular weight of 1308, is a nonionic surfactant composed of a mixture of polyoxyethylene ethers with partial oleic acid esters of sorbitol anhydrides. Pluronic F 68 and Tween 80 were used separately at an equimolar concentration of 5 mM. The absorption rate of arachidonic acid in the presence of either Pluronic F 68 or Tween 80 was higher than absorption of the fatty acid solubilized in a 5 mM sodium taurocholate solution (Table V).

Influence of Shaking on Arachidonic Acid Absorption

We evaluated the influence of the unstirred water-layer thickness on arachidonic acid absorption by varying the oscillation speed of the incubation chamber from 0-120 oscillations/min. The absorption rate of arachidonic acid increased when the chamber was oscillated at speeds higher than 40/min (Table VI).

Influence of Dextrose and Amino Acids on Arachidonic Acid Absorption

Since sugars and amino acids are usually present in the intestinal fluids post-prandially, the effects of 2.1 mM dextrose, glutamic acid (acidic), lysine (basic), or leucine (neutral) on the absorption of 2.1 mM arachidonic acid was studied in the next series of experiments. When compared to control values, the addition of these nutrients caused no change in the absorption rate of arachidonic acid by the proximal or distal intestinal sacs (Table VII).

DISCUSSION

The mechanism and characteristics of intestinal absorption of arachidonic acid were studied in vitro by using everted sacs of the rat's proximal and distal small intestine. In order to delineate the mechanism of absorption of arachidonic acid, the relationship between its concentration and its absorption rate was investigated over a wide range of concentrations (5 μM to 8.36 mM). At low concentrations (5 to 150 μM), the absorption of arachidonic acid delineated apparent saturation kinetics (Fig. 2) indicating that the mechanism of absorption has the features of carrier-mediated transport, which could be energy requiring or passive. At higher concentrations of arachidonic acid (up to 8.36 mM), the relationship between the concentration and the absorption rate was linear (Fig. 1) indicating that, in this range of concentrations, absorption of arachidonic acid is taking place by simple diffusion. In order to clarify whether absorption of arachidonic acid in the saturable range of concentrations is an energy-requiring process or a facilitated diffusion process, metabolic inhibitors and uncouplers such as 2,4-dinitrophenol, potassium cyanide, and sodium azide were added to the incubation medium in separate experiments. The addition of these inhibitors and uncouplers to the incubation medium did not change the absorption rate of arachidonic acid in either range of concentrations (Tables I, II). These observations, when coupled with the apparent saturation kinetics (Fig. 2), indicate that in the micromolar range of concentrations the mechanism of arachidonic acid absorption is a facilitated diffusion process. At higher concentrations of arachidonic acid, which are more in the pharmacological range of concentrations, both the linearity of the relationship between the concentration and the absorption rate (Fig. 1) and the lack of change in the absorption rate following the addition of metabolic inhibitors and uncouplers (Table I) indicate that the absorption mechanism is simple diffusion. Thus, simple diffusion, which is the predominant mechanism of arachidonic acid absorption in the pharmacological range of concentrations, obscures the co-existing facilitated diffusion process that was dominant at physiological concentrations of the compound. A similar concentration-dependent dual mechanism of transport has been described for other micronutrients such as thiamine (18,19), cyanocobalamine (20), and retinol (15). Absorption at the physiological range of concentrations (Fig. 2) could be mediated by the fatty acid binding protein (FABP) which is thought to be present in the cytosol of intestinal cells (21,22) and is known to have a high affinity for long chain fatty acids (21-23). This binding protein is thought to participate in the intracellular transport of fatty acids from the lipid cell membrane through the aqueous cytosol to the intracellular organelles (22,23). It is reasonable to assume that FABP is the carrier involved in arachidonic acid absorption; however, a more direct proof will be needed to validate this assumption.

The influence of the composition and physical conditions of the solution on the absorption

of arachidonic acid was investigated by measuring its absorption rate under a wide variety of experimental conditions. The pH of the incubation fluid was changed from 7.4 to 5.4 by varying the relative concentrations of the monobasic and dibasic phosphate buffer components. An increase in the hydrogen ion concentration of the incubation fluid caused a parallel increase in the absorption rate of arachidonic acid by the proximal and distal small intestinal segments (Table III). Two distinct mechanisms may account for this observation. The luminal cell membrane and the micellar particles are known to be negatively charged (24-26). As the negatively charged micellar particles approach the absorptive cell membrane, the resistance to their diffusion toward the negatively charged absorptive cell membrane increases (27). As the hydrogen ion concentration in the incubation solution was increased, the negative surface charge of the cell membrane was *partially* neutralized and the resistance to diffusion of the micellar particles toward the cell membrane would be reduced resulting in the observed increase in the rate of absorption of arachidonic acid (Table III). The other mechanism which could account for these observations would be changes in the degree of ionization of arachidonic acid which has a pKa of 6.5 in sodium taurocholate micelles (28). As the pH of the incubation fluid is lowered below 6.5, a larger proportion of arachidonic acid would exist in a protonated form thereby decreasing the negative surface charge of the micelles which contain arachidonic acid. The decrease in the negative surface charge would decrease the diffusional resistance of the micelles toward the negatively charged cell membrane enhancing the absorption of arachidonic acid. The sodium taurocholate component of the micelles which has a pKa of 1.85 (26) would not be expected to change the micellar surface charge under the present range of incubation solution hydrogen ion concentrations. The relative acidity of the intraluminal contents of the proximal small bowel when compared to the distal small bowel would, therefore, be advantageous for enhanced absorptin of arachidonic acid in vivo.

Foods containing arachidonic acid would contain other fatty acids of different degrees of saturation or different chain lengths. The most common unsaturated fatty acids present in the diet are oleic (18:1), linoleic (18:2), and some linolenic (18:3). We investigated the influence of equimolar addition of these fatty acids on the absorption of arachidonic acid. We also investigated the influence of a short chain fatty acid, butyric (4:0), on the absorption rate of arachidonic acid. The addition of butyric acid

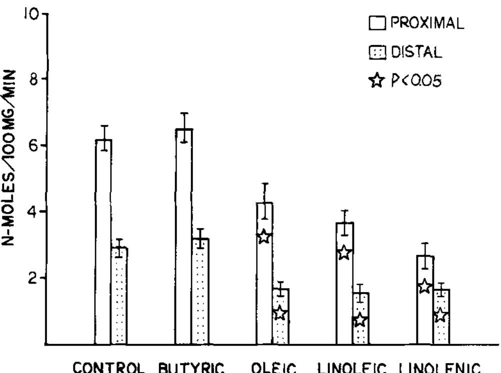

FIG. 3. The effect of other fatty acid additions on the absorption rate of 100 μM arachidonic acid. The fatty acids were added at equimolar concentration (100 μM). Each bar represents the mean absorption rate obtained from at least four rat experiments. Statistical comparison between groups was performed by ANOVA. The absorption of arachidonic acid in the absence of other fatty acids was used as a control value. Starred (☆) bars are significantly ($p < 0.05$) different from control values.

to the incubation medium did not change the absorption rate of arachidonic acid (Fig. 3). The lack of interaction between butyric and arachidonic acids is likely to be due to the vastly differing modes of absorption and transport pathways of the two fatty acids. Butyric acid is known to be absorbed rapidly by an active transport mechanism primarily into the portal vein rather than the lymphatic system (29,30). It is also known to be water soluble and not to require micellar solubilization for its absorption. In contrast, the additions of the long chain unsaturated fatty acids, oleic, linoleic, and linolenic, resulted in marked and significant decrease in the absorption rate of the arachidonic acid (Fig. 3). These long chain fatty acids are known to require micellar solubilization for absorption. Their inclusion in the micellar particles would cause an enlargement of the size of the micelles which would diminish the rate of arachidonic acid absorption by slowing its diffusion rate in the micellar phase. The long chain fatty acids may also hinder arachidonic acid absorption by their known binding affinity to the FABP (21-23). The FABP has been shown to have greater binding affinity for fatty acids with longer chain lengths and greater numbers of unsaturated sites (22,23). Therefore, oleic, linoleic, and linolenic acids would possess progressively greater binding affinity for the FABP and and could progressively interfere with arachidonic acid's own binding to FABP during its absorptive pathway. This known competitive

TABLE IV

Influence of Bile Acid Concentration
on 2.1 mM Arachidonic Acid Absorption

Taurocholate concentration (mM)	No. of rats	Absorption rate (nmol/min per 100 mg)			
		Proximal	p[a]	Distal	p[a]
5	4	26.5 ± 2.7	> 0.05	26.4 ± 0.8	> 0.05
10	4	30.0 ± 6.3	---	26.7 ± 0.9	---
15	4	28.3 ± 0.9	> 0.05	23.1 ± 0.8	> 0.05
20	4	20.4 ± 0.5	< 0.05	17.1 ± 0.4	< 0.05

[a]Analysis of the data was performed by comparing the absorption rate at 10 mM taurocholate concentration to absorption rates at 5, 15, and 20 mM taurocholate concentrations.

TABLE V

Influence of Nonionic Surfactants
on 2.1 mM Arachidonic Acid Absorption

Surfactant (5 mM)	No. of rats	Absorption rate (nmol/min per 100 mg)			
		Proximal	p[a]	Distal	p[a]
Sodium taurocholate	4	26.5 ± 2.7	---	26.4 + 0.8	---
Pluronic F 68	6	42.3 ± 4.5	< 0.01	38.2 3.4	< 0.05
Tween 80	7	34.3 ± 2.9	< 0.05	31.4 2.1	< 0.05

[a]Analysis of the data was performed by comparing the absorption rate in the presence of sodium taurocholate to the absorption rate in the presence of nonionic surfactants, Pluronic F 68, and Tween 80.

binding could explain the observed gradual decrease in arachidonic acid absorption seen in the present set of experiments (Fig. 3).

The bile salt concentration in the lumen of the small intestine is variable under normal physiological conditions and can be markedly reduced in individuals with liver disease or diseases affecting the absorptive capacity of the ileum. We, therefore, investigated the influence of the bile salt concentration in the incubation solution on the absorption rate of arachidonic acid using 10 mM taurocholate concentration as a baseline value (Table IV). The absorption rate of arachidonic acid did not change at taurocholate concentrations of either 5 or 15 mM but did decrease at 20 mM (Table IV). The decrease in arachidonic acid absorption at 20 mM sodium taurocholate concentrations may be due to interference with water absorption and changes in the permeability characteristics of the small intestine (31) rather than specific interaction between the two classes of compounds. When sodium taurocholate was substituted with the nonionic surfactants, Pluronic F 68 and Tween 80, an increase in the rate of absorption of arachidonic acid was found (Table V). The increase may be due to two separate mechanisms which are probably additive. The nonionic surfactants, Pluronic F 68 and Tween 80, do not impart a negative surface charge to the micellar particles in contrast to the negative surface charge of the sodium taurocholate micelles. The lack of surface charge would allow a more rapid diffusion of the micelles toward the absorptive cell membrane. A separate mechanism which could account for the increased absorption of arachidonic acid out of the nonionic surfactant micelles would be the partitioning of arachidonic acid between the micellar and monomeric species. Arachidonic acid has to escape the micellar particles and partition into the monomeric phase prior to its entry into the lipid cell membrane of the intestinal cells. If the Pluronic and Tween micelles possessed a lower affinity for arachidonic acid than do the sodium taurocholate micelles, then the rate of transfer of arachidonic acid from the micellar particles to the monomeric phase could be higher and would allow a more rapid uptake of arachidonic acid into the absorptive cell membrane. It is likely that both the above mechanisms play a role in the observed increase in the absorption rate of arachidonic acid out of the nonionic surfactant solutions (Table V).

The unstirred water-layer is a known barrier to lipid absorption (32,33). Its importance in arachidonic acid absorption was investigated by decreasing its thickness by varying the rate of oscillation of the incubation chamber. At higher oscillation rates, the unstirred water-layer is thinner due to increased flow rate of the incu-

TABLE VI

Influence of Incubation Chamber
Oscillation Rate on 2.1 mM Arachidonic Acid Absorption

Oscillations per minute	No. of rats	Absorption rate (nmoles/min per 100 mg) Proximal	p[a]	Distal	p[a]
0	7	19.1 ± 1.2	---	17.3 ± 0.8	---
40	4	27.4 ± 1.5	< 0.05	24.2 ± 0.9	< 0.05
80	4	30.0 ± 1.3	< 0.05	26.7 ± 0.9	< 0.05
120	7	42.4 ± 3.8	< 0.01	34.1 ± 2.4	< 0.05

[a]Absorption rate at no oscillations was compared statistically to absorption rate at 40, 80, and 120 oscillations/min.

TABLE VII

Influence of Amino Acids and Dextrose
on 2.1 mM Arachidonic Acid Absorption

Excipients (2.1 mM)	No. of rats	Absorption rate (nmoles/min per 100 mg) Proximal	p[a]	Distal	p[a]
None	4	30.0 ± 1.3	---	26.7 ± 0.9	---
Glutamic acid	4	29.3 ± 2.4	> 0.05	26.1 ± 1.3	> 0.05
Lysine	4	29.5 ± 1.9	> 0.05	25.8 ± 2.5	> 0.05
Leucine	4	31.7 ± 3.0	> 0.05	27.2 ± 2.7	> 0.05
Dextrose	4	31.6 ± 2.4	> 0.05	24.7 ± 1.8	> 0.05

[a]No significant difference in the absorption rate of arachidonic acid in the absence or presence of equimolar concentrations of glutamic acid, lysine, leucine, or dextrose.

bation solution around the intestinal surface. As the oscillation rate of the incubation chamber was increased, the absorption rate of arachidonic acid increased both by the proximal and distal small intestinal segments (Table VI). Thus, the unstirred water-layer is an important rate-limiting step in the pathway of arachidonic acid absorption by the small intestine.

The influence of the addition of three amino acids, glutamic acid, lysine, and leucine, and the sugar dextrose on the absorption rate of arachidonic acid was studied to simulate post-prandial conditions in the intestinal fluid. The addition of these nutrients caused no change in the absorption rate of arachidonic acid (Table VII). Since these nutrients are water soluble and are known to be absorbed by transport pathways (34,35) that are quite different from those of arachidonic acid, this finding by itself is not surprising. The findings suggest that the presence of multiple nutrients of different chemical and solubility characteristics does not interfere with each other's absorption.

Because of the immense importance of arachidonic acid as a precursor of prostaglandins, information regarding its absorptive mechanisms is crucial. Its concentration-dependent dual mechanism of transport, its pH, surfactant, and unstirred water-layer dependence could be helpful in controlling its absorption for therapeutic or nutritional purposes.

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Fatty Acid and Sterol Specificity of Cholesterol Esterifying Enzyme in Developing Rat Brain

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ABSTRACT

The properties and fatty acid and sterol specificity of cholesterol-esterifying enzyme (EC 3.1.1.13) in rat brain were studied. The enzyme utilized free fatty acid for esterification, and activity was maximal at pH 5.6. Exogenous ATP and CoA did not stimulate the incorporation of free fatty acids into sterol esters. Substrates dispersed in Tween 20 or Triton X-100 were just as effective as the substrates dissolved in acetone solution, while dispersion in propylene glycol or sodium taurocholate was not as effective. Snake venom phospholipase A_2 (EC 3.1.1.4) increased the esterification of cholesterol in the absence of added fatty acid. The fatty acid specificity data indicated that oleic and palmitic acids were the preferred fatty acids. Little or no esterification occurred in the presence of long chain fatty acids (C_{20}-C_{24}). Esterification of cholesterol with palmitate or stearate was not affected by the presence of oleic acid in the mixture. Thus, the nonrequirement of the brain-esterifying enzyme for a bile acid or for an amphiphile such as an unsaturated fatty acid suggests that micellar solubilization of the substrate is not essential for activity. Although the brain enzyme catalyzed the esterification of desmosterol, cholesterol was the preferred substrate. Neither lanosterol (C_{29} sterols) nor Δ7-dehydrocholesterol was esterified to any significant extent. The presence of low concentrations of desmosterol increased cholesterol esterification slightly, while there was a concentration-dependent inhibition of desmosterol esterification by cholesterol. These data on fatty acid and sterol specificity of the esterifying enzyme correlate well with the composition of sterol esters present in developing rat brain.

INTRODUCTION

The presence of a cholesterol-esterifying enzyme in rat (1) and human (2) brain has been reported, and it has been shown that the enzyme in brain utilizes free fatty acids for esterification. In a recent study, Ramsey and Davison (3) observed that the addition of snake venom phospholipase A increased cholesteryl ester formation by rat brain homogenate. However, the fatty acid pattern of the free fatty acids released differed from the fatty acid pattern of the cholesteryl esters synthesized. This suggests that the esterifying enzyme in rat brain utilizes specific fatty acids for esterification with cholesterol. In the present study, we therefore examined the fatty acid specificity of cholesterol-esterifying enzyme in rat brain. Since the sterol ester fraction in developing rat brain also contains sterol precursors of cholesterol (4), we also examined the sterol specificity of the esterifying enzyme in brain.

MATERIALS AND METHODS

Chemicals

[4-^{14}C]Cholesterol (sp. act. 50 mC/mmole), [26-^{14}C]desmosterol (sp. act. 50 mC/mmole), [1-^{14}C]nervonic acid (sp. act. 55.2 mC/mmole), [1-^{14}C]oleic acid (sp. act. 6.86 mC/mmole), [1-^{14}C]stearyl CoA (sp. act. 57.6 mC/mmole), and [1-^{14}C]oleyl CoA (sp. act. 5.45 mC/mmole) were purchased from New England Nuclear Corp. (Boston, MA). [1-^{14}C]lauric acid (sp. act. 28.8 mC/mmole), [1-^{14}C]palmitic acid (sp. act. 6.73 mC/mmole), and [1-^{14}C]stearic acid (sp. act. 6.45 mC/mmole) were purchased from Amersham Searle, Inc. (Arlington Heights, IL). [^{14}C]Lanosterol was synthesized according to the procedure described by Tchen (5). The radiopurity of all labeled compounds was established prior to use. Unlabeled commercial grade cholesterol (Sigma Chemical Co., St. Louis, MO) was purified by crystallization in ethanol. Unlabeled desmosterol, lanosterol, fatty acids, and cholesterol oleate were purchased from Applied Sciences Labs (State College, PA) and checked for purity. Tween 20, Triton X-100, sodium taurocholate, and snake venom phospholipase A_2 were products of Sigma Chemical Co. (St. Louis, MO).

Preparation of 800 g Supernatant Fraction

Sprague-Dawley rats bred in our colony (ca. 3 weeks old) were killed by decapitation, and the brains were removed and washed with saline. The tissues were homogenized in 5 vol of ice cold 0.32M sucrose, pH 7.2, and centrifuged at 800 g for 10 min. The 800 g supernatant fraction thus obtained was employed as enzyme

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TABLE I

Effect of Detergents, Cofactors, and Phospholipase A_2 on Cholesterol Esterification by 800 g Supernatant from Brain[a]

Additions	Cholesteryl oleate formed (nmoles/mg/hr)
None (substrates in acetone)	1.41
None (substrates in acetone sonicated)	1.34
Propylene glycol (0.05 ml)	0.66
Tween 20 (0.1%)	1.40
Triton X-100 (0.1%)	1.23
Taurocholate (0.1%)	0.70
ATP (10μmole) + CoA (2.0 μmole)	1.10
Oleyl CoA[b]	0.22
Minus oleic acid	0.13
Phospholipase A_2[c] 0.06 unit	0.67
Phospholipase A_2[d]	1.28

[a]Incubation mixture contained: [4-^{14}C]cholesterol, 100,000 cpm + unlabeled cholesterol, (0.60 μmole) and oleic acid, (1.2 μmole) dissolved either in 0.05 ml of acetone or in appropriate detergent or bile acids; 0.90 ml of 0.15 M citrate-phosphate buffer, pH 5.6; 0.10 ml of 800 g supernatant fraction. The values are averages of duplicate incubations with variations of less than 5%.

[b][1-^{14}C]-oleyl CoA, 100,000 cpm, (1.2 μmoles) replaced oleic acid and [4-^{14}C]-cholesterol.

[c]Added phospholipase A_2, no added oleic acid.

[d]Phospholipase A_2 treated 800 g supernatant used as enzyme source, no added oleic acid.

source. Protein determinations were carried out using the biuret reaction (6).

Assay Procedure

Except in experiments examining the effect of various detergents and bile acids, the labeled substrate, 100,000 cpm, and unlabeled substrates (0.60 μmole of sterol and 1.2 μmole of fatty acid) were dissolved in 2 ml of 0.05% Tween in absolute ethanol, and the solvent was evaporated under nitrogen. The residue was resuspended in 0.9 ml of 0.15M citrate phosphate buffer, pH 5.6 by vigorous mixing. In some experiments, the substrates were dissolved in acetone (0.05 ml), and then 0.9 ml of 0.15 M citrate-phosphate buffer, pH 5.6, was added, and, in some experiments, the incubation mixture containing acetone solution of the substrates was sonicated. The tubes containing the mixture were immersed in ice bath during sonication. The samples were sonicated for 30 sec using 60 watts of acoustic energy. The esterification reaction was started by adding 0.1 ml (2 mg protein) of 800 g supernatant from brain. The tubes were flushed with nitrogen, stoppered, and incubated for 3 hr at 37 C in a shaking water bath. Control incubations contained all components except brain 800 g supernatant. The reaction was stopped by adding 5 ml of chloroform-methanol (2:1, v/v) and 100 μg of unlabeled cholesteryl oleate to each incubation tube. The lipids were extracted from the mixture according to Folch-Pi et al. (7), and cholesteryl esters were separated from either cholesterol or free fatty acids on silicic acid columns according to Creech and Sewell (8), or by thin layer chromatography (TLC) on Silica Gel G with hexane-benzene (1:1, v/v) as the developing solvent. In the latter system, sterol esters migrate to Rf 0.5 while free sterols and free fatty acids remain near the origin. The lipid areas on the TLC plates were visualized under UV light after spraying with Rhodamine 6G, and the bands migrating with authentic cholesteryl oleate, cholesterol, or oleic acid, were scraped into counting vials, and radioactivity determined.

Preparation of Phospholipase A_2 Treated 800 g Supernatant

In the presence of phospholipase A_2 (1.2 units) 2.0 ml (40 mg protein) of 800 g supernatant fraction was pre-incubated for 15 hr at pH 6.5. The incubation mixture was subsequently adjusted to pH 5.6 with 0.1 N HCl, and aliquots equivalent to 2.0 mg protein were used as an enzyme source for assaying cholesterol-esterifying activity.

RESULTS

As reported by Eto and Suzuki (1), sterol-esterifying activity in brain utilized free fatty acids and did not require ATP or CoA as cofactor (Table I). Moreover, the results in Table I also show that the dispersing agents taurocholate (0.1%) and propylene glycol (5%) inhibited

TABLE II

Fatty Acid Specificity of Cholesterol-Esterifying Enzyme in Rat Brain[a]

[^{14}C]labeled substrate	Unlabeled substrate	Cholesteryl esters formed (nmoles/mg/hr)
Cholesterol	Laurate (12:0)	0.08
Cholesterol	Myristate (14:0)	0.50
Cholesterol	Palmitate (16:0)	1.48
Cholesterol	Stearate (18:0)	0.61
Cholesterol	Arachidate (20:0)	0.10
Cholesterol	Lignocerate (24:0)	0.06
Cholesterol	Palmitoleate (16:1)	0.50
Cholesterol	Oleate (18:1, *Cis*)	1.64
Cholesterol	Elaidate (18:1, *Trans*)	0.17
Cholesterol	Linoleate (18:2)	0.29
Cholesterol	Linolenate (18:3)	0.04
Cholesterol	Eicosaenoate (20:1)	0.28
Cholesterol	Arachidonate (20:4)	0.05
Cholesterol	Nervonate (24:1)	0.05
Laurate (12:0)	Cholesterol	0.10
Palmitate (16:0)	Cholesterol	1.59
Palmityl CoA (16:0)	Cholesterol	0.10
Stearate (18:0)	Cholesterol	0.58
Stearyl CoA (18:0)	Cholesterol	0.07
Oleate (18:1)	Cholesterol	1.88
Oleyl CoA (18;1)	Cholesterol	0.23
Nervonate (24:1)	Cholesterol	0.16

[a]Labeled substrate, 100,000 cpm, plus unlabeled cholesterol (0.6 μmole) and fatty acid (1.2 μmole) were dissolved in 2.0 ml 0.05% Tween 20 and the solvent was evaporated under nitrogen. 0.9 ml of 0.15 M citrate-phosphate buffer, pH 5.6, plus 0.1 ml (2.0 mg protein) 800 g supernatant were added. Incubations were carried out for 3 hr. Assay mixtures were then extracted and the cholesteryl esters were separated from free cholesterol or free fatty acid as described in the text. Values are the averages of duplicate incubations with variations of less than 5%.

enzyme activity by 50% while Triton X-100 (0.1%) and Tween 20 (0.1%) had no effect on cholesterol esterification. There was little or no esterification of cholesterol in the absence of added fatty acid (0.13 nmoles, Table I). However, the addition of phospholipase A_2 during the incubation (in the absence of added oleic acid) enhanced cholesterol esterification significantly (0.67 nmoles). Preincubation of the 800 g supernatant fraction with phospholipase A_2 for 15 hr further enhanced cholesterol esterification (1.28 nmoles).

Other experiments carried out to ascertain the optimal conditions for cholesterol-esterifying enzyme in brain showed that: (a) the activity was optimal at pH 5.6, and (b) the increase in esterification was linear up to 4 hr and up to 6 mg of protein per ml of incubation mixture. The amount of cholesterol esterified increased proportionately with the amount of cholesterol and oleic acid present in the reaction mixture up to the concentrations of 0.5 mM and 1.1 mM, respectively. In studies to determine the sterol and fatty acid specificities of cholesterol-esterifying enzyme in brain, protein concentrations of 2.0 mg/ml, sterol and fatty acid concentrations of 0.6 mM and 1.2 mM, respectively, and an incubation interval of 3 hr were used.

The data in Table II compare the amounts of [^{14}C]cholesterol esterified in the presence of various fatty acids. The amount esterified was highest (1.59 nmoles) when oleic acid was the added fatty acid and was slightly lower (1.46 nmoles) in the presence of palmitic acid. While cholesterol esterification in the presence of myristic acid (0.5 nmoles), stearic acid (0.61 nmoles), and palmitoleic acid (0.50 nmoles) was about half of that observed in the presence of palmitic acid, there was little or no esterification in the presence of elaidic acid, linoleic acid, lauric acid, arachidonic acid, linolenic acid, or nervonic acid. Similar results were obtained when the assays were carried out using [^{14}C]labeled fatty acids. In order to test whether palmitic and oleic acid compete with each other for esterification, or whether the esterification of cholesterol with saturated acids enhanced in the presence of unsaturated fatty acids, we examined the esterification of cholesterol with both oleic and palmitic acid or oleic and stearic acids present in the incubation mixture. The results in Table III show that the amount of oleic acid, palmitic acid, and stearic acid esterified were not affected by the presence of the other fatty acid, and suggests that

TABLE III

Effect of Added Oleic Acid on Cholesterol Esterification with Palmitic or Stearic Acids by Rat Brain 800 g Supernatant[a]

[^{14}C]labeled substrate	Unlabeled substrates	Cholesteryl esters formed (nmoles/mg/hr)
Palmitate	Palmitate (0.6 μmole)	1.49
	Palmitate (1.25 μmole)	2.42
	Palmitate + Oleate (0.6 μmole) (0.6 μmole)	1.62
Stearate	Stearate (0.6 μmole)	0.59
	Stearate (1.25 μmole)	1.23
	Stearate + Oleate (0.6 μmole) (0.6 μmole)	0.65
Oleate	Oleate (0.6 μmole)	1.21
	Oleate (1.25 μmole)	2.40
	Oleate + Palmitate (0.6 μmole) (0.6 μmole)	1.30
Cholesterol	Palmitate (0.6 μmole)	1.08
	Palmitate (1.25 μmole)	1.93
	Stearate (0.6 μmole)	0.39
	Stearate (1.25 μmole)	0.76
	Oleate (0.6 μmole)	0.98
	Oleate (1.25 μmole)	1.80
	Palmitate + Oleate (0.6 μmole) (0.6 μmole)	1.87
	Stearate + Oleate (0.6 μmole) (0.6 μmole)	1.08

[a]Each incubation mixture contained: 800 g supernatant fraction (2.0 mg protein), 0.1 ml; 0.15 M citrate-phosphate buffer, pH 5.6, 0.9 ml; 100,000 cpm of labeled substrate; the designated amounts of unlabeled fatty acid; and 0.6 μmole of unlabeled cholesterol. Values are the averages of duplicate determinations with variation of less than 5%.

none of the fatty acids tested inhibits or enhances the esterification of cholesterol with any other fatty acids.

Since the sterol ester fraction in developing brain contains other sterols in addition to cholesterol, namely methyl sterols and desmosterol (4), we tested the sterol specificity of the esterifying enzyme in rat brain. As indicated in Table IV, esterification of desmosterol (0.90 nmoles) was about half that observed for cholesterol, while the esterification of Δ7 dehydrocholesterol and of lanosterol was about 0.35 nmoles. The presence of cholesteryl esters in developing brain when the levels of desmosterol are also high (4,9) indicates that desmosterol may play a role in cholesterol esterification. We, therefore, examined cholesterol esterification in the presence of desmosterol and vice versa. The results of a single experiment presented in Table V show that the presence of up to 0.1 μmole of desmosterol enhanced slightly the esterification of cholesterol. On the other hand, there was a concentration-dependent inhibition of desmosterol esterification by cholesterol. The data obtained from several other experiments showed that the esterification of cholesterol in the presence of 0.1 μmole of desmosterol was 122.0 ± 10.7% of control, and in the presence of 0.3 μmole of desmosterol, it was 77.2 ± 8.55% of control. Both values were significantly different from control.

DISCUSSION

Results of our studies indicated that sterol-esterifying enzyme in rat brain utilizes free fatty acid for sterol esterification, has a pH optimum of 5.6, and is inhibited in the presence of propylene glycol and taurocholate and are consistent with the findings of Eto and Suzuki (1). On the other hand, our observations that the enzyme activity was unaffected by low levels of Tween 20 or Triton X-100 differ from those of these investigators. This may perhaps be due to significantly higher (10- to 20-fold) concentrations of these detergents employed in their studies.

The sterol-esterifying enzyme in rat brain resembles the cholesterol-esterifying enzyme in pancreas, intestinal mucosa, and aorta, in that all utilize free fatty acid for the esterification reaction (10-12). On the other hand, the brain-esterifying enzyme differs from that in these other tissues with respect to the requirement for taurocholate and the pH at which optimal

TABLE IV

Sterol Specificity of the Esterifying Enzyme in Rat Brain[a]

[^{14}C]labeled substrate	Unlabeled substarte	Sterol esters formed (nmoles/mg/hr)
Cholesterol	Oleate	1.73
Desmosterol	Oleate	0.73
Lanosterol	Oleate	0.31
Oleate	Cholesterol	2.10
Oleate	Δ7-Dehydrocholesterol	0.30
Oleate	Desmosterol	0.90
Oleate	Lanosterol	0.39

[a]Labeled substrate, 100,000 cpm, plus 0.6 μmole unlabeled sterol and 1.2 μmole unlabeled fatty acid were added in Tween 20 suspension. See text for other details of the experiment. Values are the averages of duplicate determinations with variation of less than 5%.

TABLE V

Effect of Sterol Precursors on Cholesterol Esterification in Rat Brain[a]

[^{14}C] Labeled substrate	Unlabeled substrates	Sterol esters formed (nmole/mg/hr)
Cholesterol	Cholesterol 0.30 μmole	0.67
	Cholesterol 0.60 μmole	1.42
	Cholesterol 0.30 μmole plus Desmosterol 0.05 μmole	0.71
	Cholesterol 0.30 μmole plus Desmosterol 0.10 μmole	0.80
	Cholesterol 0.30 μmole plus Desmosterol 0.20 μmole	0.62
	Cholesterol 0.30 μmole plus Desmosterol 0.30 μmole	0.54
	Cholesterol 0.30 μmole plus Lanosterol 0.30 μmole	0.69
	Cholesterol 0.30 μmole plus Δ7-Dehydrocholesterol 0.30 μmole	0.65
Desmosterol	Desmosterol 0.30 μmole	0.32
	Desmosterol 0.60 μmole	0.58
	Desmosterol 0.30 μmole plus Cholesterol 0.05 μmole	0.22
	Desmosterol 0.30 μmole plus Cholesterol 0.10 μmole	0.16
	Desmosterol 0.30 μmole plus Cholesterol 0.20 μmole	0.12
	Desmosterol 0.30 μmole plus Cholesterol 0.30 μmole	0.09

[a]All incubations contained labeled sterol, 100,000 cpm, plus designated amounts of unlabeled sterol and 1.2 μmole oleic acid dissolved in 0.05 ml of acetone, 0.9 ml of 0.15 M citrate-phosphate buffer, pH 5.6, and 0.1 ml of 800 g supernatant fraction. Each value is an average of duplicate incubations with variation of less than 5%.

activity is observed (12-14). The esterifying enzyme in these tissues requires taurocholate and has an optimal pH of 6.2. The fatty acid specificity of the brain enzyme is also different from that of the enzyme in pancreas and intestine (10), which shows increased esterification with increasing unsaturation and decreasing chain length of the fatty acid. On the other hand, for the enzyme in brain, there was no correlation between the extent of esterification and the degree of unsaturation or of chain length of fatty acid.

The in vitro fatty acid specificity of the brain enzyme observed in the present study agrees fairly well with the fatty acid composition of sterol esters synthesized in vivo in developing rat brain (15), but differs significantly from the fatty acid composition of the sterol esters present in demyelinating brain. Relatively high proportions of long chain fatty acids are present in the cholesteryl ester fraction (16-18), while we observed very little, if any, esterification of cholesterol with long chain fatty acids. Sterol specificity studies for the brain enzyme indicate that cholesterol is the preferred sterol for the esterification reaction. Since the sterol specificity of the enzyme in other tissues is not known, we cannot at present conclude that the brain sterol-esterifying enzyme's preference for cholesterol is peculiar to this tissue. The relative specificity of the brain enzyme for cholesterol is consistent with the sterol composition of the sterol ester fraction in developing rat brain. Ramsey et al. (4) have shown that cholesterol constitutes over 50% of the sterols in the sterol ester fraction in

young rat brain. The slight increase in the in vitro esterification of cholesterol observed in the presence of small amounts of desmosterol in our study is also consistent with the recent findings of Volpe et al. (19) that the presence of desmosterol in culture media causes an increase in amount of cholesterol esterified by C_6 glial cells. These investigators also reported that the increase in the cholesteryl ester fraction observed in the presence of desmosterol was accompanied by a reduction in the synthesis of cholesterol from acetate, and suggested that both cholesteryl esters and desmosterol have a role in regulating cerebral cholesterol synthesis.

The physical state of the substrates required for esterification by the brain enzyme seems to differ from that for the enzyme from pancreatic juice and intestinal mucosa. Micellar solubilization of the substrate is essential for in vitro cholesterol esterification by the enzyme in these tissues. Bile acids, plus an amphiphile such as an unsaturated monoglyceride or unsaturated fatty acid, are required for micellar solubilization of substrates (20). Since brain cholesterol-esterifying enzyme demonstrated neither a bile acid requirement nor a requirement of oleic acid (an unsaturated fatty acid) for esterification with palmitic or stearic acids (saturated fatty acids), it is reasonable to suggest that micellar solubilization of substrates is not essential for in vitro cholesterol esterification by the brain enzyme. This suggestion is also supported by the fact that sonication of the substrate dispersion was not essential for esterification.

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Isolation and Identification of the Metabolites of 22,25-Dideoxyecdysone from Cockroach Fat Body Cultures

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ABSTRACT

Hydroxylation and conjugation were the principal pathways of metabolism of 22,25-dideoxyecdysone in cockroach fat body cultures. The major metabolite isolated and identified was the tetrahydroxy steroid 22-deoxyecdysone; other exdysteroids isolated, in order of decreasing quantities, were 22-deoxy-26-hydroxyecdysone, 22,25-dideoxy-26-hydroxyecdysone, and 22-deoxy-20-hydroxyecdysone. Cockroach fat body from late-instar nymphs appears to lack the mechanism for hydroxylating at C-22. Radioanalyses of the material obtained from enzymic hydrolysis of the conjugate fraction showed 65, 15 and 20% of tetraols, pentaols, and unhydrolyzed conjugates respectively, and no 22,25-dideoxyecdysone. An azasteroid and two nonsteroidal amines that effectively inhibit the activity of 22,25-dideoxyecdysone in the cockroach leg regenerate-fat body culture system enhanced the metabolism of 22,25-dideoxyecdysone, decreased the quantity of the pentaol fraction present, and caused an increase or accumulation of the tetraol and conjugate fractions in the fat body culture system.

INTRODUCTION

The effectiveness of the insect molting hormone 20-hydroxyecdysone in initiating cuticle deposition in the cockroach leg regenerate tissue from *Leucophaea maderae* (F.) is dependent on both the concentration of and the length of time of exposure to this hormone. α-Ecdysone, which is converted to 20-hydroxyecdysone by cockroach leg regenerates (1), differs in that its effectiveness in initiating cuticle deposition is dependent primarily on the length of time that tissue is exposed to the hormone rather than on the amount of hormone present. However, the molting hormone effect of the synthetic ecdysteroid 22,25-dideoxyecdysone (I) in inducing cuticle deposition in leg regenerates is quite low unless the leg regenerates are cocultured with cockroach fat body tissue. This suggests that the fat body tissue converts this ecdysteroid to a steroid(s) with molting hormone activity in the system, and it was proposed that this cockroach in vitro system could be used to evaluate compounds that inhibit molting hormone metabolism (2). Recently it was demonstrated that a number of azasteroids and nonsteroidal amines that inhibit molting and metamorphosis and/or steroid metabolism in insects did indeed inhibit the molting hormone activity of 22,25-dideoxyecdysone in the leg regenerate-fat body organ culture system (3). This paper reports on the isolation and characterization of the metabolites of 22,25-dideoxyecdysone in the cockroach fat body tissue culture system without leg regenerates and certain effects of an azasteroid and two nonsteroidal amines on the metabolism of this ecdysteroid.

EXPERIMENTAL PROCEDURES

Instrumentation

Ultraviolet spectra were taken in methanol with a Bausch and Lomb Spectronic 505 spectrophotometer. Measurements of radioactivity were made with a Packard Tri-Carb Liquid Scintillation Spectrometer Model 3003, and radio thin layer chromatographic (TLC) analyses were obtained with a Packard Radiochromatogram Scanner Model 7201. The mass spectra were obtained by using an LKB Model 9000 gas chromatograph mass spectrometer (LKB Produkter AB, Stockholm, Sweden) equipped with a Varian Spectro System 100 MS data system; the samples were introduced directly into the ionization chamber, and the ionization energy was 70 ev. Nuclear magnetic resonance (NMR) spectra were recorded at 60

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MHz with a JEOL FX60-Q Fourier Transform NMR equipped with a 1.7 mm capillary probe. Deuterated pyridine and chloroform were used as the solvents for the ecdysteroids and their acetate derivatives, respectively, and TMS was the internal NMR standard. HP-TLC plates 10 x 10 cm Silica Gel 60 F-254 (E. Merck, Darmstadt, Germany) for Nano TLC were used in TLC analyses, and Analab Anasil H, 5 x 20 cm TLC plates were used for radiochromatogram scans. The solvent systems were chloroform-ethanol (4:1) for the ecdysteroids and benzene-ethyl acetate (1:3) for their acetates.

Labeled Compound

The 4-^{14}C-22,25-dideoxyecdysone (I) with a specific activity of 1.4 x 10^6 dpm/mg was prepared from 4-^{14}C-cholesterol according to procedures used for the syntheses of the unlabeled compound (4). Its radiochemical purity was greater than 98% as determined by column and thin layer chromatography. For these studies, the 4-^{14}C-22,25-dideoxyecdysone was diluted with the unlabeled compund to give a final observable specific activity of 5 x 10^5 dpm/mg (500 dpm/μg). The purity of the unlabeled compound used for dilution was equivalent to that of the labeled ecdysteroid as determined by column chromatography, TLC and spectral analyses (UV, IR, NMR, mass spectrometry).

Fat Body Cultures

Each culture was prepared with 20 to 30 cu mm of cockroach *L. maderae* (F). abdominal fat body in 1 ml of M20S culture medium (with 7% fetal calf serum). Either 10 or 30 μg of 4-^{14}C-22,25-dideoxyecdysone in 1 μl of freshly distilled dimethyl sulfoxide (DMSO) with or without a 10 μg quantity of an amine inhibitor were injected into the medium on the day following explantation. In cultures in which the antibiotic gentamicin (Schering Corp., Kenilworth, NJ) was used, it was added to the medium before use at the level of 5 μg/ml of medium. The cultures were incubated at 26 C, and incubations were terminated and material preserved by adding 2 ml of methanol 3, 6 or 14 days after dosing. Upon receipt in Beltsville, the methanolic solutions were stored at -20 C until analyzed. The procedures were similar for cultures in which we used nonradioactive 22,25-dideoxyecdysone.

Leg Regenerate Bioassay

Late-instar nymphs of the cockroach *L. maderae* were isolated from the laboratory colony while still white. Twenty-four hours later the mesothoracic legs were removed at the coxotrochanteral joint. After 28 days the coxa was removed, and the regenerating leg was dissected. Two leg regenerates were placed about 3 mm apart on the lower coverslip of a Rose multipurpose tissue chamber (5) and covered with dialysis strip soaked in M20 culture medium (6). The chamber was then assembled and filled to a volume of 2 ml with M20S culture medium (with 7% fetal calf serum.) Ten μg of the test compound (10 μg/μl of DMSO) was injected under the dialysis strip peripheral to the leg regenerates. The treated chambers were incubated at 26 C and at the end of 14 days were examined with the aid of phase contrast optics and scored for the presence of cuticle (7). Five chambers were scored for each treatment, and the significance of the results was determined by using 2 x 2 contingency tables based on Fisher's exact test.

Extraction and Isolation of Labeled Compounds

The incubation mixture of medium, fat body and labeled compound I with or without an inhibitory amine was transferred in methanol to a Tenbroeck tissue grinder and homogenized. The mixture was filtered through a fritted disc glass funnel, and the filtrate was concentrated to dryness under vacuum. The residue was partitioned between 6 ml each of preequilibrated butanol and water through four 13-ml centrifuge tubes, and 5 transfers of the upper phase were made. The butanol phases collected at the 4th tube were combined and concentrated to dryness under vacuum. The residue was then partitioned between 6 ml each of hexane and 70% methanol as in the case of the butanol-water partition system, and the 70% methanol phase was concentrated to dryness. The residue was dissolved into 1 to 3 ml of methanol, and aliquots were taken out for radioassay by TLC and by liquid scintillation spectrometery.

Separation of 4-^{14}C-Ecdysteroids from the 4-^{14}C-Ecdysteroidal Conjugates

The residue that contained both ecdysteroids and very polar conjugates was partitioned in 13-ml centrifuge tubes between a solvent system of cyclohexane-butanol-water, 5:5:10, 6 ml each of upper and lower phase. Nine transfers of the upper phase were made over 4 tubes, and the upper phases were collected, combined and concentrated to dryness. The lower phases of tubes 1 and 2, and 3 and 4 were separately combined and concentrated to dryness under vacuum. The residues from the respective fractions were dissolved into a measured volume of methanol, and aliquots were taken for radioassay by liquid scintillation spectrometry and by TLC.

Isolation of 4-^{14}C-Ecdysteroidal Moieties of Conjugates

The ecdysteroid conjugates from incubation of fat body, 4-^{14}C-22,25-dideoxyecdysone and M20S culture medium for 6 and 14 days were each incubated with a mixture of sulfatase and β-glucosidase (0.6 mg each in 0.3 ml of 0.2% sodium chloride solution) in 2 ml of 0.2 M sodium acetate-acetic acid buffer solution (pH 5) for 18 hr at 30 C. Each mixture was then diluted with 4 ml of the aqueous phase from a 1:1 mixture of butanol-water and extracted 4 times with 6 ml of the upper phase. The upper phases were combined and concentrated to dryness under vacuum, the residue was dissolved into 3 ml of methanol, and aliquots were assayed by liquid scintillation spectrometry and by radiochromatogram scanning.

Extraction, Isolation and Identification of Unlabeled Metabolites of 22,25-Dideoxyecdysone From Fat Body Cultures

The ecdysteroids and their conjugates were isolated, as described above for the labeled compounds, from the pooled contents of 115 culture flasks of which each flask contained fat body, M20S culture medium and 30 μg of 22,25-dideoxyecdysone and incubated for 6 days. From the resulting residue (46 mg), the conjugates were separated from the ecdysteroids as described for the labeled compounds by partitioning between the solvent system of cyclohexane-butanol-water (5:5:10). The upper phases were combined and concentrated to dryness under vacuum to give 30 mg of residue that contained the unmetabolized 22, 25-dideoxyecdysone (I), and its metabolites. The lower phases were combined and concentrated to dryness under vacuum to give 25 mg of residue that contained the conjugates. Enzymic hydrolysis of this residue as before with a mixture of β-glucosidase and sulfatase at 30 C for 48 hr followed by partitioning between butanol and water gave 10.4 mg of residue in the combined butanol phases after concentrating to dryness under vacuum. The residue was partitioned between the upper and lower phases of the solvent system of cyclohexane-butanol-water, 5:5:10. The upper phases were combined and concentrated to dryness under vacuum to give 2 mg of residue that contained the ecdysteroidal moieties of the conjugates.

The 30 mg of residue which contained the unmetabolized I and its metabolites was chromatographed over 4 g of Unisil, and the following 40 ml fractions were collected: 1 chloroform, 2-3 chloroform-5% ethanol, 4-5 chloroform-10% ethanol, 6 chloroform-15% ethanol, 7 chloroform-20% ethanol. The fractions monitored and quantitated by UV analyses showed that fractions 2 and 3 contained ca. 2.4 mg of unmetabolized I, and fractions 4 and 5 contained 320 μg and 120 μg of tetraols and pentaols, respectively.

The ecdysteroids from the enzymic hydrolysis of the conjugates chromatographed similarly showed that fraction 2 contained only a trace of compound I, and fractions 4 and 5 contained 215 μg and 75 μg of tetraols and pentaols, respectively.

Since the tetraols and pentaols obtained from hydrolysis of the conjugates gave R_f values identical with those of the unconjugated tetraols and pentaols, the respective tetraol and pentaol fractions were combined and further purified by rechromatography over Unisil. From the rechromatography, we obtained 400 μg of tetraols, R_f 0.38, NMR, δ 0.74 (18-methyl), 1.09 (19-methyl), 1.04 and 0.98 (21-methyl) and 1.41 (26- and 27-methyl). The mass spectrum, $C_{27}H_{44}O_5$, exhibited a M^+ at m/e 448 and a fragmentation pattern similar with that of 22-deoxyecdysone (8).

The rechromatography of the pentaol fraction yielded 175 μg of the pentaols, R_f 0.23, NMR, δ 0.70 (18-methyl), 1.08 (19-methyl), 1.04 and 0.98 (21-methyl), and 1.35 (26- and 27-methyls), 1.48 (27-methyl of a compound with hydroxyl groups at C-25 and C-26), 1.16 (18-methyl of a compound with a hydroxyl group at C-20); MS, $C_{27}H_{44}O_6$ (m/e, rel. intensity) 464 (M^+, 0.5), 446 (M^+-H_2O, 3), 428 (M^+-$2H_2O$, 14), 410 (M^+-$3H_2O$, 18), 395 (M^+-$3H_2O$-CH_3, 8), 392 (M^+-$4H_2O$, 8), 327(15), 301(17), 300(14), 283(14), 278(21), 267(14), 256(16), 250(15), 239(18), 231(25), 211(38), 199(21), 185(26), 171(20), 161(22), 145(23), 135(60), 127(53), 109(78), 81(37), 55(74), 43(100).

Analyses of Acetylated Tetraol Fraction

The acetates (pyridine-acetic anhydride (3:1), 24 hr at room temperature) of the tetraols were prepared. Analyses by TLC of the acetates showed the presence of two compounds with R_fs of 0.58 and 0.31. The two compounds were readily separated by column chromatography over 4 g of dichloromethane-chloroform (1:1) washed Unisil. Chloroform eluted 62 μg of the triacetate (Va), R_f 0.58, NMR δ 0.68 (18-methyl), 0.97 and 0.88 (21-methyl and 27-methyl), 1.03 (19-methyl), 2.11, 2.01 (2- and 3- acetoxy), 2.06 (26-acetoxy); MS, $C_{33}H_{50}O_8$, (m/e, rel. intensity) 574(M^+, 1.3), 556(M^+- H_2O, 32), 546(M^+- CO, 19), 514(M^+- CH_3COOH, 3), 496(M^+-CH_3COOH - H_2O, 19), 481(M^+- CH_3COOH - H_2O - CH_3, 9), 454(M^+- $2CH_3COOH$, 7),

I R=R′=R″=R‴=H
II R=R′=R″=H;R‴=OH
IIa R=R′=Ac;R″=H;R‴=OH
III R=R′=H;R″=R‴=OH
IIIa R=R′=Ac;R″=R‴=OH

IV R=R′=R‴=H;R″=OH
IVa R=R′=R‴=Ac;R″=OH
V R=R′=R″=R‴=H
Va R=R′=R‴=Ac;R″=H

VI

VII

VIII

Scheme I

411(9), 385(M^+- C_8H_{15} - CH_3COOH - H_2O, 20), 361(7), 333(52), 283(14), 265(13), 231(19), 213(20), 173(14), 171(10), 157(11), 145(13), 121(12), 81(19), 69(24), 43(100).

Chloroform-5% ethanol eluted 185 μg of (IIa), R_f 0.31, NMR δ 0.69 (18-methyl), 1.00 and 0.89 (21-methyl), 1.04 (19-methyl) and 1.23 (26- and 27-methyl); MS, $C_{31}H_{48}O_7$, (m/e, rel. intensity) 532(M^+, 0.2), 514(M^+- H_2O, 9), 496(M^+- $2H_2O$, 26), 486(37), 454(M^+- CH_3COOH - H_2O, 7) 426(M^+- C_8H_{15} - $2H_2O$, 19), 412(M^+- $2CH_3COOH$, 7), 411(M^+- C_8H_{15} - $2H_2O$ - CH_3, 12), 385(25), 384(37), 366(12), 334(23), 333(55), 313(11), 291(11), 283(13), 230(35), 213(25), 199(13), 161(20), 147(16), 131(15), 121(20), 91(18), 81(28), 43(100).

Analysis of Acetylated Pentaol Fraction

Acetylation of the pentaol fraction with pyridine-acetic anhydride (3:1) for 24 hr at room temperature gave a mixture of acetates NMR, δ 0.68 (18-methyl), 0.96 and 0.87 (21-methyl), 1.03 (19-methyl), 1.21 (26-and 27-methyl), 1.23 (27-methyl of a compound with a hydroxyl group at C-25 and acetoxy group at C-26), 0.85 (18-methyl of a compound with a hydroxyl group at C-20), 2.01 (3-acetoxy), 2.11 (2- and 26-acetoxy).

Analyses by TLC showed a major compound with an R_f of 0.30 and another compound with an R_f of 0.12. Both compounds were scraped from plate and analysed by mass spectrometry; the major compound (R_f 0.30) is the triacetate IVa; MS, $C_{33}H_{50}O_9$, (m/e, rel. intensity) 590(M^+, 1), 572(M^+- H_2O, 3), 530(M^+- CH_3COOH, 3), 512(M^+- CH_3COOH - H_2O, 16), 502(M^+- CH_3COOH - CO, 15), 486(6), 396(5), 385(M^+- $C_{10}H_{19}O_3$ - H_2O, 22), 368(46), 340(5), 333(44), 283(11), 280(10), 264(14), 257(20), 255(25), 250(12), 236(46), 229(17), 213(21), 194(14), 181(14), 173(16), 159(21), 147(21), 123(33), 111(44), 97(97), 83(100), 55(95).

The MS of the minor component (R_f 0,12) indicates a $C_{31}H_{48}O_8$, the diacetate IIIa (m/e, rel. intensity) 548(M^+, 2), 530(M^+- H_2O, 2), 512(M^+- $2H_2O$, 16), 497(M^+- $2H_2O$ - CH_3, 24), 498(M^+- CH_3COOH, 6), 494(M^+- $3H_2O$, 46), 479(M^+- $3H_2O$ - CH_3, 20), 447(13), 429(45), 411(13), 404(M^+- $C_8H_{15}O_2$, 48), 395(11), 386(32), 385(32), 371(12), 368(57), 334(70), 327(57), 302(20), 283(23), 269(26), 259(23), 239(23), 232(49), 213(26), 191(36), 145(44), 127(100), 109(97).

RESULTS AND DISCUSSION

The analyses of fat body cultures were conducted on pooled material from 3 or more cultures that contained 10 or 30 μg of 4-^{14}C-22,25-dideoxyecdysone (I) per culture. Approximately 63 to 85% of the initial radioactivity was recovered by extraction of the medium with butanol. All of the radioactivity was found in the 70% methanol phase after partitioning of the extractives between 70% methanol and hexane, which suggests that no

less polar metabolites were formed by the elimination of hydroxyl groups during the incubation. At this stage the material was of sufficient purity for TLC and radiochromatogram analyses.

Radiochromatogram scans of TLCs of the material that had been developed in the solvent system of chloroform-ethanol (4:1) indicated that a large portion of the material remained at the origin (conjugates). The scan also indicated the presence of unmetabolized compound I and two more polar metabolites. The R_f of the less polar metabolite was identical to the R_f of the tetraol 22-deoxyecdysone (II), and the more polar compound exhibited an R_f that was between the R_fs of α-ecdysone and 20-hydroxyecdysone. This suggestsed that the latter compound was a pentaol that contained a more polar or a differently positioned or oriented hydroxyl group than α-ecdysone.

In our initial experiments, it was observed that fat body cultures incubated for 14 days in the presence of labeled I and gentamicin contained a lesser quantity of conjugates than those without gentamicin. For this reason experiments were conducted on the metabolism of labeled I in fat body cultures with and without gentamicin. The results in Table I indicate that gentamicin interferes with conjugation and does affect the overall metabolism of I, since enzymic hydrolysis of the conjugates obtained from either 6 or 14 days of incubation of I with fat body cultures yields 65 and 15% of tetraols and pentaols, respectively. Interestingly, no labeled I could be detected.

In order to accumulate a sufficient quantity of material for the identification of the tetraols and pentaols, we chose to carry out the incubation of unlabeled I in fat body cultures for 6 days without gentamicin. From the 115 incubated cultures of compound I and fat body, we obtained 400 µg of the tetraol fraction that gave an R_f and NMR and mass spectral data that were identical with the NMR and mass spectral data of 22-deoxyecdysone (8), and this established the identity of the major tetraol as 22-deoxyecdysone (II).

The molecular ion at m/e 464 indicates that the ecdysteroid with an R_f of 0.23 is indeed a pentahydroxy compound(s). Its greater polarity than α-ecdysone (R_f 0.28) suggests that one of the two additional hydroxyl groups was at C-26. The presence of a methyl resonance peak in the NMR spectrum as a singlet and of equal intensity as the 18- or 19-methyl group at δ 1.48 is in the typical region for the C-27 methyl (1.47) of 20,26-dihydroxyecdysone and 26-hydroxyecdysone (9,10). This places the two additional hydroxyl

TABLE I

Metabolism of 4-^{14}C-22,25-Dideoxyecdysone in Cockroach Fat Body Cultures with and without Gentamicin during Incubation Periods of 6 and 14 days

22,25-Dideoxyecdysone (µg/ml of Medium)	Gentamicin (µg/ml of Medium)	% Unmetabolized 22,25-Dideoxyecdysone[a]	% Metabolites[a]		
			Conjugates	Pentaols	Tetraols
6 Days					
10	--	36.0	44.9	17.2	1.8
10	5	43.0	31.6	13.4	12.0
30	5	65.1	13.0	5.5	16.4
14 Days					
10	--	38.1	44.8	9.0	8.1
10	5	25.8	36.4	22.7	15.2
30	--	24.8	51.7	12.8	10.7
30	5	52.1	30.9	6.4	10.6

[a]Determined by radio TLC analyses.

groups in the major pentaol at C-25 and C-26 and indicates that it is 22-deoxy-26-hydroxyecdysone (IV). However, the additional methyl resonance peak at δ 1.16 (18-methyl) suggests the presence of an additional pentaol with a hydroxyl group at C-20.

The metabolism of 22,25-dideoxyecdysone (I) usually proceeds via hydroxylation and conjugation. The normal sequence of side chain hydroxylation of I to the insect ecdysones does not occur in the metabolism of this ecdysteroid in fat body cultures. Also, an orderly sequence of hydroxylation does not seem to occur, even though the major sites of hydroxylation appear to be first at C-25 then at C-26. We have observed in prior in vivo metabolic studies (8,11) that the metabolism of I does not always proceed in an orderly sequence. Since certain ecdysteroids containing an identical number of hydroxyl groups at different positions are not readily separable by TLC, though their acetates often are, the acetates of the tetraol and pentaol fractions were prepared. The results indicate that the tetraol fraction does indeed contain a mixture of at least two ecdysteroid acetates. The NMR and mass spectral data further indicate that the major component (75%), which has an R_f of 0.31, is 22-deoxyecdysone 2,3-diacetate (IIa), and the other compound (R_f 0.58) is 22,25-dideoxy-26-hydroxyecdysone 2,3,26-triacetate (Va).

The NMR spectrum of the pentaol fraction and its acetate indicated the presence of more than a single compound. Analyses of the acetylated pentaols by TLC also showed two ecdysteroid acetates. Although we did not analyze the individual acetates by NMR spectroscopy because of the limited quantity of material, the acetates were separated and analysed by mass spectrometry. The mass spectrum of the acetate (R_f 0.30) that represented about 60% of the mixture showed this compound to contain three acetoxy groups. Its fragmentation pattern was similar to that of 22-deoxy-26-hydroxyecdysone 2,3,26-triacetate obtained from acetylation of pentaols isolated from frass of larvae of the tobacco hornworm, *Manduca sexta* (L.) (8). These data taken together with the other results indicate that the major pentaol acetate is 22-deoxy-26-hydroxyecdyson 2,3,26-triacetate (IVa). However, in view of the identification of Va in the acetylated tetraol fraciton, this fraction could contain some 22,25-dideoxy-20,26-dihydroxyecdysone.

The molecular ion of 548 in the mass spectrum of the pentaol acetate (R_f 0.12) that was more polar than 20-hydroxyecdysone triacetate (R_f 0.14) indicated that the compound contained two acetoxy and three unacetylated hydroxyl groups. Since the hydroxylation most likely occurs in the side chain, this places the two additional teriary hydroxyl groups at C-20 and C-25. The peak at m/e 404 in the mass spectrum supports the cleavage of a side chain containing two hydroxyl groups. Furthermore, the fragmentation pattern of this ecdysteroid acetate is similar to that of 22-deoxy-20-hydroxyecdysone 2,3-diacetate isolated from the acetylated pentaols of frass of tobacco hornworn larvae (8). The 22-deoxy-20-hydroxyecdysone 2,3-diacetate is also more polar by TLC than 20-hydroxyecdysone 2,3,22-triacetate. Thus, the second pentaol acetate has the structure of 22-deoxy-20-hydroxyecdysone 2,3-diacetate (IIIa).

As in previous in vivo studies with other insect species (8,11,12), the principal pathway of metabolism of 22,25-dideoxyecdysone in the cockroach fat body cultures are hydroxylation and conjugation. The major metabolite was the tetrahydroxy steroid 22-deoxyecdysone (II), indication that hydroxylation at C-25 precedes that at the 20, 22, and 26 positions. The other metabolites in order of decreasing amounts were 22-deoxy-2-hydroxyecdysone (IV), 22,25-dideoxy-26-hydroxyecdysone (V), and 22-deoxy-20-hydroxyecdysone (III). Compounds II, III, and IV have been previously isolated from tobacco hornworm larvae and the frass of larvae when the larvae were fed compound I in their diets (8). The isolation of these ecdysteroids indicated that in vitro cultures of cockroach fat body are similar to the tobacco hornworm larvae in that they appear to lack the mechanism for hydroxylating at C-22. It further indicates that hydroxylation does not proceed in a completely orderly sequence and that there are enzymes in the cockroach *L. maderae* abdominal fat body that hydroxylate at a number of positions. However, since the fat body was obtained from late instar nymphs, this tissue from a different developmental stage of *L. maderae* most likely could contain qualitatively different hydroxylating enzymes.

Whe tested in the leg regenerate test system, the tetraol fraction that consisted of 75% 22-deoxyecdysone (II) and 25% 22,25-dideoxy-26-hydroxyecdysone (V), induced cuticle formation in 62% of the leg regenerates in 10.8 days, while 22,25-dideoxyecdysone induced only 27% of the leg regenerates to deposit cuticle. With the pentaol fraction that consisted of ca. 60% 22-deoxy-26-hydroxyecdysone (IV) and 40% 22-deoxy-20-hydroxyecdysone (III), 80% of the leg regenerates produced cuticle in 11.25 days. The results suggest that the

TABLE II

The Effects of an Azasteroid and Two Nonsteroidal Amines on the Metabolism of 4-^{14}C-22,25-Dideoxyecdysone in Cockroach Fat Body Cultures During an Incubation Period of 6 Days

Inhibitory compounds[a]	% Unmetabolized 22,25-Dideoxyecdysone[b]	% Metabolites[b]		
		Conjugates	Pentaols	Tetraols
Control	45.5	35.6	11.3	7.6
VI	6.0	55.0	0.0	39.0
VII	15.9	40.8	5.6	37.8
VIII	18.7	34.7	5.2	41.5

[a]The concentration of the inhibitory compounds and 4-^{14}C-22,25-dideoxyecdysone were 10 μg/each per culture flask.
[b]Determined by radio TLC analyses.

25-hydroxyl group, or at least one hydroxyl group, is needed in the side chain for effective activity in the leg regenerate system, and that the leg regenerates alone do not appear to be capable of hydroxylating at C-25. However, there may be other specific structural requirements, since 26-hydroxyecdysone, the major molting hormone of developing eggs of the tobacco hornworm (10), was completely ineffective in the leg regenerate system.

Clearly, an interesting aspect of this study has been the results obtained from the enzymatic hydrolysis of the conjugates that showed 65, 15 and 20% of tetraol, pentaol, and unhydrolyzed conjugate fractions, respectively, and none of the original 22,25-dideoxyecdysone. Whether this resulted from the hydrolysis of the conjugates from 6 or 14 days of incubation, the findings were similar. This suggest that hydroxylation in this system precedes conjugation and that these hydroxylated metabolites serve as substrate for the conjugating enzymes while the ecdysteroid I does not. Although the position(s) conjugated or the nature of the conjugates formed have not been determined, this finding does strongly suggest that the site of conjugation is most likely at one of the newly hydroxylated positions in the side chain.

The effects of the azasteroid VI and the two nonsteroidal amines VII and VIII on the metabolism of labeled compound I is summarized in Table II. These amines, which are effective inhibitors of molting, metamorphosis and steroid metabolism in insects (13,14), and also inhibit the activity of I in the leg regnerate-fat body culture system (3), enhanced the metabolism of I, decreased the quantity of pentaols present, and caused an accumulation of the tetraols and conjugates in the fat body culture test system. The azasteroid VI that was the most active in inhibiting the acitivty of I in inducing cuticle deposition of cockroach leg regenerates-fat body system (3) completely prevented the production or accumulation of the pentaols. Since both the tetraol and pentaol fractions isolated from the tissue culture effectively induced cuticle deposition in the leg regenerate system without the addition of fat body, the results in Table II do not readily or fully explain the action of these compounds on I in inhibiting the induction of cuticle deposition in the cockroach leg regenerate system in the presence of fat body (3). Further work is required to determine whether the tetraols and pentaols that accumulated in the presence of the inhibitory amines are identical with those isolated in this study and whether, in addition to the observed effects, the inhibitory amines may also be affecting other steps in the metabolism of 22,25-dideoxyecdysone in the fat body-leg regenerate system.

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The Behavior of Rat Bile Phospholipids in the Intestine and in Incubation Media Containing Pancreatic Juice

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ABSTRACT

Samples of radioactive bile were collected from rats after intravenous injection of potassium soaps ([9-10 $^3H_2$] or [1^{14}C]oleate, [1^{14}C] linoleate or [9-10 $^3H_2$] palmitate). These radioactive acids were chosen because it is well established that, in natural phosphatidyl cholines, palmitic acid is located chiefly at the 1 position and linoleic and oleic acids at the 2 position. After incubation of bile with pancreatic juice, the labeling of unchanged biliary phospholipids was higher when native bile was labeled with oleic acid than with palmitic or linoleic acids. These data suggest that monounsaturated molecular species of biliary phospholipids are more resistant than the diunsaturated ones to in vitro hydrolysis by phospholipase A_2. Ninety min after introduction of the radioactive bile into the upper part of the rat duodenum, high labeling of luminal phospholipids was observed regardless of the bile sample used, although labeling of free fatty acids was always low. The passage of intact biliary phospholipids through the intestinal epithelium is discussed.

INTRODUCTION

In previous in vitro (1) and in vivo (2) experiments using rat bile labeled with [9-10 $^3H_2$] oleic acid, we demonstrated that, unlike isolated phosphatidyl cholines (PC) or diacylphosphatides (PL), biliary PL were poorly hydrolyzed by pancreatic phospholipase A_2. Recently, we observed high proportions of radioactivity in 1-acyl-lyso-phosphatidylcholines (LL) or free fatty acids (FFA) after incubations with rat pancreatic juice of rat bile labeled with palmitic or linoleic acids. Because these latter results conflicted with the former, we decided to reinvestigate this problem.

In the present investigation, we have studied the behavior of rat bile PL labeled with oleic, palmitic or linoleic acids in incubation media containing rat pancreatic juice and in the intestinal lumen and mucosa. We have chosen these labeled fatty acids because natural PL contain predominantly unsaturated fatty acids (FA) in the 2 position and saturated fatty acids in the 1 position, and because pancreatic phospholipase A_2 catalyses hydrolysis of the fatty ester bond in the 2 position of PL, giving as a product a 1-acyl lysophosphatide.

MATERIALS AND PROCEDURES

[9-10 $^3H_2$] and [1-^{14}C] oleic (35 mCi/mM and 53 mCi/mM) and [9-10$^3H_2$] palmitic (40 mCi/mM) acids were purchased from CEA-Saclay, France; [1-^{14}C]linoleic acid (60 mCi/mM) was supplied by the Radiochemical Centre, Amersham, U.K. Examination of these fatty acids by thin layer chromatography (TLC) and gas liquid chromatography (GLC) indicated a radiopurity greater than 98%.

Wistar male rats weighing 220-250 g were maintained on a commercial diet containing 5% lipid and 17% protein (Villemoisson/Orge, France).

Under ether anesthesia a cannula was inserted into the bile duct proximal to the pancreas. All the operated animals were placed in restraining cages with free access to water containing 0.6% NaCl and 0.3% KCl. To obtain radioactive bile, the donor animals bearing a bile fistula were injected (during 15 sec) with labeled K soaps (2 mg FA; 1 mCi oleate or palmitate; 0.5 mCi linoleate) bound to 90 mg serum albumin dissolved in 1 ml of 0.9% NaCl solution into the portal vein instead of the jugular wein as was done previously. Immediately after the operation, bile was collected for 6 hr at 0 C. Nonradioactive bile, collected in a similar way, was added to labeled bile to obtain the desired specific activity of biliary lipids, 25,000 dpm/mg and 950,000 dpm/mg for the in vitro and in vivo experiments, respectively, regardless of the nature of the radioactive FA. The bile samples were stored at 4 C and used within 3 days.

Pancreatic juice was collected at 0 C for 48 hr by cannulation of the pancreatic duct of rats provided with an external bile fistula. The samples of pancreatic juice were immediately used or stored at -20 C. Contrary to previous experiments (1), in the present studies, pancreatic juice was not heated before use. In fact, pancreatic lipase would not hydrolyze the FA in the 1 position of PL (3), and we confirmed that very low proportions of labeled FA were released after incubation of isolated PC labeled with palmitic acid (more than 90% in the 1 position).

TABLE I

Distribution (in %) of Radioactivity in Bile Lipids
after Administration of Labeled Fatty Acids to Rats

<table>
<tr><th>Bile samples</th><th>Labeled fatty acids</th><th>Lysophosphatidylcholines</th><th>Other phospholipids</th><th>Free fatty acids</th><th>Neutral[a] glycerides</th></tr>
<tr><td>a</td><td>^{3}H 18:1</td><td colspan="2">92</td><td>2</td><td>6</td></tr>
<tr><td>b</td><td>^{14}C 18:1</td><td colspan="2">91</td><td>4</td><td>5</td></tr>
<tr><td>c</td><td>^{3}H 16:0</td><td>3</td><td>91</td><td>3</td><td>3</td></tr>
<tr><td>d</td><td>^{14}C 18:2</td><td colspan="2">89</td><td>7</td><td>4</td></tr>
<tr><td></td><td>^{3}H 18:1</td><td colspan="2">91</td><td>1</td><td>8</td></tr>
<tr><td>e</td><td>^{14}C 18:2</td><td colspan="2">96</td><td>1</td><td>3</td></tr>
<tr><td></td><td>^{3}H 18:1</td><td colspan="2">93</td><td>2</td><td>5</td></tr>
<tr><td>f</td><td>^{14}C 18:2</td><td colspan="2">85</td><td>4</td><td>11</td></tr>
<tr><td>g</td><td>^{3}H 18:1</td><td colspan="2">88</td><td>4</td><td>8</td></tr>
</table>

[a]Mainly as triglycerides.

TABLE II

Proportions of Radioactivity Present as Diacylphosphatides
after Incubation of Native Bile or Various Mixtures with Pancreatic Juice[a]
(Constant shaking at 37 C for 30 or 60 min)

Labeled fatty acids	Bile samples	Pancreatic juice samples	Incubation time (min)	Number of experiments	% of labeling present as phospholipids[b]	
					^{3}H	^{14}C
^{3}H 18:1	a	A	60	6	73.5 ± 2.8	
^{14}C 18:1	b	B	60	3		66.2 ± 2.5
^{3}H 16:0	c	B	60	8	19.7 ± 2.4	
^{14}C 18:2	d	B	60	3		28.7 ± 8.1
^{3}H 18:1	e	C	30	4	87.3 ± 1.7	80.2 ± 0.3
^{14}C 18:2			60	4	70.5 ± 0.9	28.7 ± 3.1
id.	f	D	60	4	68.6 ± 1.9	34.2 ± 1
^{3}H 18:1 + ^{14}C 18:1	a + TL of ^{14}C Bile b	A	60	3	76.9 ± 2.2	25.1 ± 1.6
^{3}H 18:1	Synthetic mixed micelles	A	60	3	43.4 ± 0.4	

[a]Various mixtures are either bile "a" (labeled with ^{3}H oleic acid) added to total lipids (TL) extracted from 1 ml of bile "b" (labeled with ^{14}C oleic acid), or synthetic mixed micelles containing isolated PC. Composition of the incubation media: bile (1ml); trypsin (FLUKA) (0.5 mg); CaC12 (5 mg); pancreatic juice (1 ml); borate buffer 0.05 M pH 7.6 (Up to a total volume of 4.5 ml). ((one ml of bile contained about 2.9 mg of PL.)

[b]The radioactivity of PL in controls (incubations without pancreatic juice) set at 100 (100 ± 0.9 for 8 analyses). The values represent the mean ± SE.

Synthetic mixed micelles with a composition similar to that found in native rat bile (7 mg Na taurocholate, 0.5 mg cholesterol, 2 mg isolated biliary PC labeled with ^{3}H oleic acid) were prepared according to Nalbone et al. (4). The incubation media contained, in addition to 1 ml of labeled bile, 1 ml of mixed micelles or lipids extracted from 1 ml of labeled bile, 0.5 mg of trypsin, 5 mg of $CaCl_2$, 1 ml of pancreatic juice and borate buffer 0.05 M, pH 7.6 up to a total volume of 4.5 ml. The incubation assays were performed under constant shaking at 37 C for 1 hr. To determine the position of labeled FA in the biliary PL, incubation mixtures containing these isolated PL and snake venom (Näïa Näïa) were prepared according to the method of Tattrie (5).

In animal experimentation each rat received rapidly 0.5 ml of labeled bile through a polyethylene catheter inserted in the upper duodenum. The animals were sacrificed 90 min later. The total small intestine was removed. The lumen was washed with 0.9% NaCl solution containing Na taurocholate (Schuchardt) (3

g/l). The lipid extracts were carried out according to Folch et al. (6) and analyzed (TLC-radioactivity) as described previously (1).

RESULTS

Distribution of the Radioactivity between the Lipid Classes of Bile (Table I)

Similar distributions were observed in the different samples of bile. Eighty five to 96% of the radioactivity was present as PL (mainly as PC) regardless of labeled FA. The degradation of isolated biliary PC by snake venom (5) indicated that about 88% of ^{3}H or ^{14}C oleic acid and over than 90% ^{14}C linoleic acid was in the 2 position and that over 90% of ^{3}H palmitic acid was in the 1 position.

In Vitro Experiments (Table II)

After incubation of bile, regardless of the samples of pancreatic juice used, the highest labeling of intact PL was in the bile samples labeled with ^{3}H or ^{14}C oleic acid. This fact was most apparent when double-labeled bile was employed. After incubation of bile "a" (^{3}H oleic acid) mixed with total ^{14}C lipids extracted from 1 ml of bile "b" (^{14}C oleic acid), the ^{3}H labeling of intact PL was high and similar to that found when bile "a" was the only substrate, whereas the ^{14}C labeling of intact PL was lower. After incubations of synthetic mixed micelles, extensive hydrolysis of ^{3}H PC occurred by comparison with that observed when native bile "a" (labeled with ^{3}H oleic acid) was the substrate.

In Vivo Experiments (Table III)

The proportions of the lipid radioactivity (% of the initial dose) recovered were 30 to 50% in the lumen and 30 to 40% in the mucosa, whatever the bile samples used. In the intestinal lumen, the predominant labeling was always found in PL amounting to about 83 to 90% of total radioactivity. In the mucosa these components were still highly labeled (51 to 72% of total radioactivity), but higher proportions of labeled triglycerides appeared.

DISCUSSION

In the present experiments, when the labeled K soaps were introduced into the portal vein instead of the jugular vein as previously described (1,2), 85% of total lipid radioactivity was present in bile as PL, compared to 65-70% in our previous investigations (1). It is obvious that the different bile samples collected have a similar lipid composition because they were

TABLE III

Distribution of the Radioactivity in Luminal and Mucosal Lipids, 90 min after Introduction of Different Radioactive Bile Samples (0.5 ml) into the Duodenum of Rats[a]

Experiments	Nature of the fatty acids labeling bile	Bile sample	Lumen: Lyso-phosphatidylcholines	Lumen: Other Phospholipids	Lumen: Free fatty acids	Lumen: Glycerides[b]	Mucosa: Lyso-phosphatidylcholines	Mucosa: Other phospholipids	Mucosa: Free fatty acids	Mucosa: Glycerides[b]
I (4)[c]	^{3}H 18:1	g	89.2 ± 3.2		7.7 ± 2.3	3.1 ± 1.3	51.4 ± 6.9		9.3 ± 1.8	39.3 ± 5
II (5)	^{3}H 16:0	c	9 ± 1.8	88.6 ± 4.6	1.8 ± 0.4	0.6 ± 0.2	9.8 ± 2	57.5 ± 6	32.7 ± 3.1	
	^{3}H 18:1		90.5 ± 3.9		4 ± 0.9	5.5 ± 1	not determined			
III (3)	^{14}C 18:2	f	83.5 ± 4		5.5 ± 1	11 ± 2.1	not determined			
	^{3}H 18:1		80.2 ± 4.8		5.8 ± 1	14 ± 1.5	72.7 ± 4.6		1.7 ± 0.4	25.6 ± 3.8
IV (2)	^{14}C 18:2	f	86.5 ± 2.9		9 ± 1.2	4.5 ± 0.8	57.3 ± 3.8		13.1 ± 1	29.6 ± 4.4

[a]The values represent the mean ± SE.

[b]Mainly as triglycerides.

[c]Number of Rats.

prepared under identical conditions using a tracer dose of labeled fatty acid. In the incubation media containing various samples of bile, the amounts of total unchanged PL, FFA released or LL formed were also similar regardless of the radioactive FA.

The in vitro and in vivo results presented here concerning bile labeled with oleic acid corresponded closely to previous findings (1,2). In the previous experiments, we proposed that a direct correlation existed between the mass and the radioactivity of the FA released from native bile PL in the intestinal lumen or in the incubation media. Because their radioactivity was always low, we concluded that the biliary PL were poorly hydrolyzed. Moreover, when isolated radioactive PL were added to bile, we demonstrated that the biliary PL were hydrolyzed more slowly than isolated PL. However, in the present studies, that conclusion is shown to be no longer valid because when bile labeled with ^{3}H palmitic or ^{14}C linoleic acid was incubated with pancreatic juice, higher proportions of radioactivity appeared in LL and FFA fractions. In addition the data show that bile PL labeled with palmitic acid are more vulnerable to attack by pancreatic juice. We suggest that the various molecular species of biliary PC cannot be hydrolyzed to the same extents. 1 Palmitoyl-2 linoleyl PC, which is the major species of PC in bile (about 60%), may be more readily attacked by phospholipase A_2 than the monounsaturated molecular species (1 palmitoyl-2 oleyl PC, 20% of total PC). These in vitro results could be due to a specificity of the bile PC or to a specific action of the phospholipase A_2. Because no information exists on the relative affinities of this enzyme towards individual FA located in the 2 position of PL, we carried out in vitro experiments, which were described under methods. Instead of bile the substrates were liver PC labeled with ^{3}H oleic acid or ^{14}C linoleic acid (1 part) added to nonradioactive PC (9 parts) isolated from only one liver. The molecular species of PC were similar, and consequently so were their physical properties. After incubation identical proportions of radioactivity were found as FFA regardless of the labeled FA. These findings demonstrate that phospholipase A_2 releases fatty acids located in the 2 position at similar rates regardless of degree of unsaturation. Because the results obtained with isolated PC or bile were different, we conclude that the monounsaturated molecular species of biliary PC are in vitro, hydrolyzed more slowly than the diunsaturated molecular species, possibly because they might not be too easily accessible to the enzyme. This view is consistent with the findings of some workers (7-9), assuming that biliary PL is associated with proteins. Our own data obtained with synthetic micelles and the mixture containing lipids extracted from ^{14}C bile and native ^{3}H bile are compatible with this possibility. In fact, isolated total biliary PC labeled with FA of any kind were hydrolyzed to the same extent. Even when these biliary PC were incorporated into synthetic micelles, they were hydrolyzed more easily than native bile PC.

The hydrolysis pattern of native bile PC might be similar in the incubation media and in the intestinal lumen. However, in the latter most of the radioactivity was always found in PL whatever the labeled acid. In particular, no greater amounts of labeled FFA were observed with bile labeled with linoleic acid than we would expect from in vitro results. Either the luminal hydrolysis of biliary PC is reduced, or the released FA and lysocompounds formed are absorbed very rapidly. After ingestion of triglycerides, high proportions of hydrolyzed products (FFA and monoglycerides) are always present in the lumen contents. It appears unlikely that the process of FA absorption is different when they are released from biliary PC. However, we must consider that the concentration of phospholipase A_2 was markedly lower than that of lipase in the pancreatic juice (10) and lumen (3). Under these conditions, the amounts of FA released from PC would be low and rapidly absorbed. However, a recent report by Nalbone et al. (4) demonstrated that native bile PC, unlike mixed synthetic PC, was absorbed in the absence of pancreatic secretion. These findings are consistent with the idea that a major part of the biliary PC is absorbed intact in the rat, chiefly the monounsaturated molecular species.

In addition, the high labeling of PL in mucosa (over 50%) supports this explanation. On the contrary, in man, according to Borgström (11) and Arnesjö et al. (3), biliary and dietary PC undergo an intense luminal hydrolysis.

Whatever the degree of luminal hydrolysis of native bile PC, considerable degradation occurred either prior to their passage through the intestinal epithelium (brush border) or intracellularly, since we observed a wide distribution of radioactivity among the different mucosal lipids. In particular, an active synthesis of triglycerides occurred via the Kennedy pathway (12).

The present findings require us to verify our previous conclusions (13) because they were obtained in experiments performed with bile labeled with oleic acid. For this purpose,

studies performed with double-labeled bile (glycerol-linoleic acid, glycerol-palmitic acid, oleic acid-choline) are in progress.

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In Vitro and In Vivo Effects of Exogenous Lipids on the Enzymatic Hydrolysis of Rat Bile Phospholipids

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ABSTRACT

The addition of total phospholipids, phosphatidylcholines, triglycerides, cholesterol or glycerol to incubation media containing rat pancreatic juice and bile labeled with [9,10$^3H_2$] oleic acid (90% of the radioactivity present as phospholipids) had no effect on the hydrolysis of bile endogenous phospholipids. The introduction of 2 or 10 mg of phosphatidylcholines and 0.5 ml of bile ($\sim$ 1.5 mg of phospholipids)into the rat upper duodenum decreased the rate of absorption of native bile phospholipids. It was not followed by an increase of free fatty acids released from biliary phospholipids in the intestinal lumen. The introduction of bile (0.5 ml) and small amounts of triolein (1.4 – 3.5 mg) into the duodenum had little effect on the rate of hydrolysis and absorption of native bile phospholipids, but caused a reduced absorption of the free fatty acids released or those coming from initial nonphosphorus biliary lipids. The introduction of bile (0.5 ml) and large amounts of triolein (30 mg) into the duodenum increased the rates of hydrolysis and absorption of endogenous bile phospholipids. These observations suggest that luminal lipid components can modify the organization of luminal micelles and, consequently, the action of the pancreatic phospholipase A_2 and the absorption of bile lipids.

INTRODUCTION

In recent studies (1) we demonstrated that the rate of in vitro hydrolysis of rat endogenous bile diacylphosphatides (PL) by pancreatic juice varied with their fatty acid composition. The monounsaturated PL hydrolyzed slowly, whereas the diunsaturated (1 palmitoyl-2 linoleoyl PC) class underwent a more rapid hydrolysis. Moreover, higher proportions of radioactivity were found in free fatty acids (FFA) fractions when bile labeled with oleic acid was replaced by phosphatidylcholines (PC) isolated from this bile in the incubation media. These findings seemed to be compatible with the existence of a lipoprotein complex in bile (2-4) in which the monounsaturated molecular species of PC are protected against the action of phospholipase A_2, whereas the other molecular species are accessible to the enzyme.

While appreciable amounts of radioactive FFA appeared in the incubation media, by contrast, we observed small proportions of radioactivity as FFA in the intestinal lumen after introduction of labeled native bile into the duodenum of rats. These results were consistent with our previous findings (5) and could stem either from a reduced hydrolysis of native bile in the lumen, or unchanged biliary PC absorbed by the intestinal mucosa, or hydrolyzed products absorbed very rapidly.

It is possible that endogenous lipids such as triglycerides and PL may have modified the physical organization of the micelles and consequently facilitated or decreased the contact between bile PL and pancreatic phosphoipase A_2. These modifications could have affected the rates of hydrolysis and absorption of biliary PC. Moreover, some experiments performed in man (6,7) showed that dietary and biliary PL underwent similar intestinal metabolism, indicating the appearance of large amounts of acyllysophosphatidylcholines (LL) in the intestinal lumen.

In the present report, we studied the effect of different amounts of isolated PC or triglycerides on the hydrolysis of rat native bile PL, in vitro, by pancreatic juice and in the intestinal lumen of the rat. Bile labeled with [9,10-$^3H_2$] oleic acid was employed because this acid permits, following the behavior of the molecular species of biliary PC, most resistance to the in vitro action of pancreatic phospholipase A_2 as demonstrated previously (1). Moreover, we confirmed that 88% of the ^3H oleic acid was located in the 2 position of the biliary PC (1), since phospholipase A_2 releases specifically the FA located in this position. Under these conditions LL formed were unlabeled. Isolated PC were added to bile instead of isolated PL, because PC represent more than 92% of the total rat biliary PL.

MATERIALS AND PROCEDURES

Male Wistar rats weighing 220-250 g were maintained on a commercial diet (UAR, Villemoisson/Orge, France) containing 5% lipid and 17% protein. Details of the collection of pure pancreatic juice and labeled native bile, the preparation of incubation media, the removal of tissues and the extraction and analysis

TABLE I

Percent of Radioactivity Recovered as Intact PL[a] after Incubation of Bile Labeled with [9,10 $^3H_2$] Oleic Acid Added to Various Substrates

Additions	mg	Samples of bile	% intact. PL
none		A	73.5 ± 2.8
TO	0.8	B	78.4 ± 2.3
	1.4	B	79.5 ± 3.8
	6	B	74.3 ± 1.2
	10	C	76.4 ± 0.8
	17	C	76.6 ± 3.2
Isolated liver PL	7	A	76.1 ± 0.6
Isolated liver PC	7	A	72.5 ± 4.9
Glycerol	1	B	75.6 ± 0.2
Cholesterol	1	C	78.5 ± 1.8
	3	C	76.8 ± 0.4

[a]The radioactivity present as PL in control (incubations without pancreatic juice) was set at 100 (100 ± 0.4 for 8 analyses). The values represent the mean ± SE obtained from 4 analyses except in the first case (no substrate added) in which 8 analyses were performed. Each assay contained: 1 ml radioactive bile (no. 3 mg PL), 0.5 mg trypsin (FLUKA), 5 mg $CaCl_2$, 1 ml nonheated rat pancreatic juice, borate buffer pH 7.6, 0.05 M up to a total vol of 4.5 ml. This mixture was incubated under constant shaking at 37 C for 1 hr. Pure pancreatic juice used here was collected from many rats; the samples were pooled, then divided in some flasks, and stored at -20 C until used.

of lipids have been described previously (1). The exogenous lipids added to labeled bile were as follows: nonradioactive triolein (TO) was purchased from NU–CHEK PREP, (Elysian, MN), and labeled TO was prepared according to the method of MATTSON et al. (8) with [1-^{14}C] oleic acid (CEA-Saclay, France). The specific activity was 700,000 dpm/mg. Isolated radioactive PC were prepared from lipid extracts of rat liver after introduction of K[1-^{14}C] oleate (0.2mCi) into the mesenteric vein (specific activity, 16,000 to 840,000 dpm/mg). Nonradioactive PL or PC were also isolated from rat liver. Examination of ^3H or ^{14}C oleic acid by thin layer chromatogrpahy (TLC) and gas liquid chromatography (GLC) indicated a radiopurity greater than 97%. The purity of radioactive and nonradioactive TO was greater than 98%. The exogenous substrates added to the incubation media were not labeled. They were dissolved in chloroform-methanol (1:1, v/v) and added to each incubation vial. The solvents were evaporated to dryness and bile was added. The vials were shaken at 30 C for a short time to obtain the mixture. In some experiments other components (cholesterol, glycerol) were added to the bile (for composition, see Table I).

Animal Experimentation

The recipient animals received rapidly (30 sec), through a polyethylene catheter inserted in the upper duodenum, either pure radioactive bile followed by 0.5 ml of physiological saline or pure radioactive bile followed by 0.5 ml of physiological saline containing labeled PC or TO, after sonication. Drinking water was withheld. The animals were killed by decapitation 45 or 90 min later.

RESULTS

In all samples of bile used, about 90% of the radioactivity was present as PL. No significant variations in rates of the enzymatic hydrolysis of the endogenous bile PL occurred when various substrates were added to the incubation media. TO was hydrolyzed to a large extent and high amounts of FFA and partial glycerides (PG) appeared.

Luminal Lipids

The absorption of biliary lipids as measured by transfer of ^3H lipids from lumen to mucosa was always significant. However, the disappearance of ^3H lipids from the lumen was slower when 0.05 ml of bile was introduced into the duodenum than with 0.5 or 2 ml of bile (Table II). The absorption of biliary lipids decreased when 2 or 10 mg of isolated PC were added, whereas it was not significantly modified after the addition of 1.4 or 3.5 mg of ^{14}C TO. When 17 or 30 mg of ^{14}C TO were present, the disappearance of biliary lipids was more rapid. In experiments using 0.05 ml of ^3H bile added to 30 mg of ^{14}C TO, the ^3H and ^{14}C radioactivity recovered indicated a linear rate of lipid

TABLE II

Distribution and Amounts of Radioactivity in Luminal and Mucosal Lipids (L and M) after Duodenal Administration of Labeled Bile and Lipids to Rats

Exp.		Samples of native bile ml[a]		Lipids added mg		Time Min		^3H TL[b]	^3H PL	^3H FFA	^3H TG	^3H PG	^{14}C TL[b]	^{14}C PL	^{14}C FFA	^{14}C TG	^{14}C PG
I	(3)[c]	0.05	D	0		90	L	48.8	60	6	3.9	- - -					
							M	29.1	28.4	2.7	13.7	- - -					
II	(3)	2.	D	0		90	L	30.7	1,410	119	116	133					
							M	31.	1,140	142	141	383					
III	(4)	0.5	D	0		90	L	23.7	294	23	10	- - -					
							M	24.6	135	30	160	- - -					
IV	(4)	0.5	D	2	PC	90	L	33.5	435	25	25	- - -	39	724	32	24	
							M	24	174	42	130	- - -	29	344	37	194	
V	(4)	0.5	D	10	PC	90	L	44.5	625	11	10	- - -	49	4,780	76	0	
							M	24	213	41	90	- - -	25	1,745	258	555	
VI	(4)	0.5	E	1.4	TO	90	L	28	215	46	62	79	5	11	25	19	15
							M	31	158	72	149	72	13	47	27	92	17
VII	(4)	0.5	E	3.5	TO	90	L	27	244	68	30	50	2	7	25	13	20
							M	40	160	79	272	64	12	28	51	54	248
VIII	(4)	0.5	E	17.	TO	45	L	33	335	72	33	32	22	318	1,462	976	890
							M	35	77	81	283	46	30	131	748	3,300	714
IX	(3)	0.05	D	30.	TO	45	L	43	42	9	3	6	21	380	2,826	600	2,325
							M	27	7	7	19	4	42	450	2,290	8,520	1,110
X	(4)	0.05	D	30.	TO	90	L	23.7	19	6	4	4	11	264	1,560	530	920
							M	30	9	4	22	4	41	1,146	1,185	9,000	900

[a]1 ml of bile = 2.9 mg PL. Bile (samples D and E) were labeled with '[9,10 $^3H_2$]oleic acid; exogenous lipids (PL, PC, TO) were labeled with [1-^{14}C]oleic acid.
[b]Per cent of initial dose. The other values are expressed as μg of labeled components (calculated from initial specific activity of FA of bile or PC or TO). The values represent means. For the sake of clarity, SE have been omitted from the table, but they revealed differences equal to ± 12%
[c]( ) number of rats.

absorption. The ^{14}C lipids disappeared quickly from the lumen even when as much as 30 mg ^{14}C TO was used.

In all the experiments, the ^{3}H labeling of luminal PL was high by comparison with that of other lipids. However, the ^{3}H labeling of FFA, TG and PG was higher when ^{14}C TO was added to bile (Table II). The ^{14}C labeling of TL was always high (39 and 49%) and, in proportion, similar to that of ^{3}H labeling of PL (30 and 43%) when the ^{14}C PL were added to ^{3}H bile. When ^{14}C TO was added to bile, the highest ^{14}C labeling was usually found as FFA.

Mucosal Lipids

Considerable hydrolysis of the injected labeled material (biliary PL, isolated PC or TO) occurred, leading to the appearance of various radioactive ^{3}H and ^{14}C lipids regardless of the experiments. However, it should be noted that the proportion of the ^{14}C labeling of PL was higher when ^{14}C PC was added to bile instead of ^{14}C TO.

DISCUSSION

The purpose of these experiments was to change the nature and the size of the mixed micelles and, consequently, the enzyme substrate contact by the addition of various components normally present in the intestinal lumen during fat digestion. The amounts of exogenous material added to bile were probably similar to those coming from endogenous sources or food.

The volumes of exogenous bile (0.05 to 0.5 ml) introduced into the duodenum did not represent abnormal values. Indeed, total endogenous biliary PL determined in the total small intestinal lumen was about 1.5 mg, while the radioactive bile samples introduced into the duodenum were 0.15 to 1.5 mg of PL. In any case no accumulation of biliary lipids occurred in the lumen.

As might be expected for the in vitro and in vivo experiments using bile labeled with [9,10 $^{3}H_2$] oleic acid (without addition), the present results were in agreement with those found previously (1,5,9). The addition of various substrates to bile did not seem to modify the rate of in vitro and in vivo hydrolysis, except in the latter case when large amounts of TO were added. According to Borgström (10), the action of phospholipase A_2 on PL occurs chiefly in the micellar phase, and it is probable under our experimental in vitro conditions that the hydrolyzed products (FFA, lysocompounds) were accumulated, and in fact, that the enzyme-biliary PC contact was no better than in the experiments without additions.

In the animal experimentation, the uptake of fats by the intestinal mucosa was always high, although the addition of isolated PC or TO had some effect on the behavior of the biliary PC. It is well known that PC produce larger micelles, which would be absorbed more slowly. This fact might explain the decrease in the absorption of biliary and isolated PC when the amounts of the latter increased from 2 to 10 mg. However, even when this decreased absorption occurred, no accumulation of hydrolysis products in the lumen was observed; on the contrary, intact PL always formed the major labeled luminal lipid components. When large amounts of ^{14}C TO (17-30 mg) were added to bile, an enhancement of the rates of hydrolysis (ca. 66%) and disappearance (ca. 50%) of biliary PC from the lumen occurred. The active degradation of ^{14}C TO and absorption of its hydrolysis products must provide large amounts of micelles in which biliary PC are present, and consequently these last components would be absorbed more rapidly. In these cases (exp. VI, VII and VIII), the higher ^{3}H labeling of luminal FFA and glycerides could be explained by some exchange of ^{3}H and ^{14}C oleic acid between these components, as was demonstrated by Ahrens and Borgström (11). The problem of luminal hydrolysis of native bile PC has been discussed elsewhere (1), but it was of particular interest to reexamine some of its aspects. In fact, in the lumen, the ^{3}H labeling of FFA (and other lipids) coming from bile was always low, which could mean either that PC hydrolysis was very small (and bile PC could be absorbed unchanged as some workers have demonstrated) (12), or else it was higher with rapid absorption of the hydrolysis products. However, this last explanation is questionable, because in the experiments in which larger amounts (30 mg) of ^{14}C TO were used, higher amounts of free ^{14}C oleic acid were found in the lumen. It is unlikely that uptake of ^{3}H or ^{14}C oleic acid by the mucosa would not be identical unless we accept different solubility of FFA in various luminal lipid components (PG, PC, LL, etc.). Thus, the investigations of Rodgers and O'Connor (13) indicated that addition of PC to oleic acid produced significant inhibition of uptake of FFA by the mucosa. In this respect our observations do not support this view.

The behavior of isolated ^{14}C PC was similar to that of biliary PC in the lumen and mucosa. It is well established that the enzymatic degradation of PC is slower than the degradation of PL or phosphatidylethanolamines, and our in vivo results are consistent with these findings. Parthasarathy et al. (14) and Arnesjö et al. (7)

reported a considerable intraluminal hydrolysis of isolated PC in rat and in man, although the former authors found, as we did, a low rate of FFA and lysocompounds in the lumen. In this respect the amounts of luminal 1-acyl lysophosphatides (nonradioactive in our experiments) that we determined were low (1:20 of total PC). The hydrolysis of biliary and isolated PC may be considerable either in the brush border or inside the enterocytes, as the label was distributed between the different cell lipids, and a synthesis of triglycerides occurred according to the current concepts. The labeling of mucosal PL was always higher when the ^{3}H or ^{14}C labeling of PL was high in the lumen, suggesting an uptake of intact PL by the mucosa in agreement with the conclusions of some authors (15,16).

In conclusion, several variables, particularly lipid components, may modify the organization of luminal micelles and consequently the lipid absorption process.

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The Incorporation of Orally Administered Radiolabeled Dihomo γ-Linolenic Acid (20:3ω6) into Rat Tissue Lipids and Its Conversion to Arachidonic Acid

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ABSTRACT

Radioactivity from orally administered radiolabeled dihomo-γ-linolenic acid (20:3ω6) was recovered from the liver, plasma and brain lipid fractions. After administration the fatty acid was metabolized to arachidonic acid, the 22 carbon chain length fatty acid, and was also β-oxidized. However, 22 hr after administration of [1-^{14}C] 20:3 between one-third and one-half of the recovered radioactivity was still associated with dihomo-γ-linolenic acid in the liver and plasma lipid fractions. Orally administered dihomo-γ-linolenic acid is incorporated into lipid fractions and is, therefore, available in the metabolic pool for PGE_1 synthesis.

INTRODUCTION

The metabolism of linoleic acid (18:2ω6) – an essential fatty acid that must be supplied in the diets of vertebrates – to its longer chain polyunsaturated metabolites involves a Δ6 desaturation to γ-linolenic acid (18:3ω6) and followed by a chain elongation to dihomo-γ-linolenic (20:3ω6), the latter is then desaturated (Δ5 desaturase) to arachidonic acid (20:4ω6) (1). Previous studies in vitro (2) and in vivo (3,4) have demonstrated that the Δ6 desaturase is the key enzyme limiting the metabolism of linoleic acid to its metabolites.

Dihomo-γ-linolenic acid (20:3ω6) and arachidonic acid (20:4ω6), besides being principal components of cell structural lipids, are also the direct precursors of the physiologically active prostaglandins. Dihomo-γ-linolenic acid gives rise to PGE_1 – one of the most potent known inhibitors of platelet aggregation – and has other antithrombotic properties, while arachidonic acid gives rise to PGE_2, which potentiates platelet aggregation (5,6). The balance between these two series of prostaglandins and/or their precursors are thought to regulate platelet functions, and it has been suggested that dietary dihomo-γ-linolenic acid supplementation may be used as an antithrombotic agent (7).

It is thought that these prostaglandin precursor fatty acids are derived from the acyl phosphoglycerides. It has been shown that oral administration of dihomo-γ-linolenic acid results in the incorporation of this fatty acid into the tissue lipids (8,9). However, no detailed information is available on the distribution of the orally administered dihomo-γ-linolenic acid into the lipid classes or the extent to which it is desaturated to arachidonic acid.

TABLE I

The Incorporation of Radioactivity into the Liver, Brain and Plasma Lipids 22 hr after Oral Administration of [-1^{14}C]20:3[a]

	% administered dose
Liver	6.65 ± 0.95
Brain	1.02 ± 0.29
Plasma	0.27 ± 0.06

[a]Figures are mean of 4 animals ± SEM.

TABLE II

Percentage Distribution of Radioactivity after 22 hr. from Orally Administered [1^{14}C]20:3[a] in the Lipid Fractions of the Liver Brain and Plasma[a]

Lipid fractions	Liver	Brain	Plasma
Phospholipids	78.2 ± 1.64	88.7 ± 0.25	54.4 ± 1.84
Cholesterol	1.0 ± 0.07	8.0 ± 0.42	1.6 ± 0.91
Free fatty acids	0.4 ± 0.09	1.6 ± 0.10	1.3 ± 0.24
Triglycerides	19.4 ± 1.61	1.6 ± 0.28	18.7 ± 1.6
Cholesteryl esters	1.0 ± 0.19	0.2 ±0.10	24.1 ± 0.78

[a]Figures are mean of 4 animals ± SEM.

TABLE III

Percentage Distribution of Radioactivity in the Fatty Acids of the Liver, Brain and Plasma Lipid Fractions

Fatty acid fractions	Liver[a]		Plasma[b]		Brain[a]
	Phospholipids	Triglycerides	Phospholipids	Triglycerides	Phospholipids
16:0+16:1ω7+ 18:0+18:1ω9	1.5 ± 0.19	1.9 ± 0.36	3.1	3.8	22.1 ± 1.6
18:2ω6+ 18:3ω6+ 20:2ω6	0.5 ± 0.08	1.8 ± 0.20	1.4	3.5	2.1 ± 0.29
20:3ω6	26.4 ± 2.0	47.0 ± 1.6	46.8	51.1	15.2 ± 1.7
20:4ω6	67.1 ± 2.6	38.2 ± 3.4	42.5	28.8	51.5 ± 4.2
22:4ω6+ 22:5ω6	3.3 ± 0.74	10.0 ± 1.9	5.6	11.8	8.2 ± 0.66

[a]Mean of 4 animals ± SEM.
[b]Mean of 2 determinations from a pooled sample of 4 animals.

TABLE IV

Decarboxylation Studies on the Liver TG, PL and Brain PL Fatty Acids Collected by Preparative Gas Liquid Chromatography

Fatty acid fraction collected by preparative GLC	Relative Carboxyl activity (RCA) $\left(\frac{^{14}C \text{ in the -COOH group}}{^{14}C \text{ in total fatty acids}}\right)$	Activity in -COOH groups as a % of RCA in the original [1-^{14}C]20:3[a]
Liver — Liver TG		
20:3ω6	0.81[b]	100
20:4ω6	0.79	98
22:4 + 22:5ω6	0.57	70
Liver PL		
20:3ω6	0.81	100
20:4ω6	0.82	101
22:4 + 22:5ω6	0.47	58
Brain PL		
16:0 + 16:1ω7	0.14	17
18:0 + 18:1ω9	0.07	9
20:3ω6	0.76	94
20:4ω6	0.78	96
22:4 + 22:5ω6	0.51	63

[a]The original [1-^{14}C]20:3 had a relative carboxyl activity of 0.81 ± 0.66 (mean ± standard error of six determinations).
[b]Mean of two determinations of pooled samples.

MATERIALS AND METHODS

Suckling rat pups bred from females (Wistar strain) raised in this laboratory on a semisynthetic diet were used. Four pups aged between 15 and 16 days were dosed orally with [1-^{14}C]8,11,14-20:3 (57 mCi/mmole, radiopurity 99%, New England Nuclear Chemicals, Boston MA). About 6 μCi of isotope were administered to each pup. The pups were killed 22 hr later, and blood, liver and brain were collected for lipid analysis.

Lipids were extracted, and the distribution of isotope into the lipid fractions, after separation on thin layer chromatography, was determined by means of techniques described previously (3,10). The distribution of the radioactivity in the fatty acid fractions of lipids was determined by the separation and collection of fatty acid methyl esters on a preparative gas liquid chromatograph and followed by measurement of the radioactivity in a liquid scintillation spectrometer (3,10). Fatty acid fractions were also decarboxylated by the Schmidt procedure, as described by Goldfine and Bloch (11), after separation and collection on a preparative gas liquid chromatograph.

RESULTS AND DISCUSSION

After an oral dose of [1-^{14}C]20:3, about

8% of the administered dose was recovered from the liver, plasma and brain lipids (Table I), which is less than recoveries found for arachidonic acid, but is much greater than those found for linoleic acid (10). In the liver lipids, the radioactivity was mainly in the phosphoglyceride (PL) and triglyceride (TG) fractions (Table II). The brain lipids contain very little triglycerides, and the radioactivity was mainly recovered from the PL fraction. In the plasma lipids, besides PL and TG fractions, radioactivity was also recovered from the cholesteryl ester (CE) fraction. The phosphoglyceride fractions accounted for more than 50% of the recovered activity.

The distribution of the radioactivity in the fatty acids of these lipid fractions is presented in Table III. In the liver PL fraction, two-thirds of the radioacitivity was in the arachidonic acid (20:4ω6), while 26% of the activity was still as dihomo-γ-linolenic acid (20:3ω6). In the liver TG, the radioactivity was evenly distributed between dihomo-γ-linolenic acid (47%) and its metabolites, namely arachidonic (20:4ω6), docosatetraenoic (22:4ω6) and docosapentaenoic (22:5ω6) acids, which in total accounted for 48% of the recovered activity. In the plasma PL and TG fractions, the recovered radioactivity was again more or less evenly distributed between dihomo-γ-linolenic acid and its longer chain metabolites. However, in the brain PL fraction, half of the recovered radioactivity was arachidonic acid, 8% in the 22 carbon chain length metabolites and 15% as 20:3ω6. Significant amounts of radioactivity (22%) were also present as 16 and 18 carbon chain length saturated and monounsaturated fatty acids in the brain PL fraction.

The presence of radioactivity in the brain phosphoglyceride 16:0, 18:0, 16:1ω7 and 18:1ω9 fatty acids and the brain cholesterol fraction suggests β-oxidation of the orally-administered [1-^{14}C]20:3, and this was confirmed by decarboxylation of these 16 and 18 carbon fatty acids (Table IV). The β-oxidation and the reincorporation of the isotope into structural lipids by *de novo* synthesis of fatty acids has also been shown to take place for both linoleic (18:2ω6) and γ-linolenic (18:3ω6) acids, both of which are precursors of dihomo-γ-linolenic acid (20:3ω6) (3,12). Comparison of these earlier studies with the present study indicates that dietary dihomo-γ-linolenic acid undergoes β-oxidation to an extent less than that shown by linoleic or γ-linolenic acids.

In conclusion, up to one-third or even one-half of the radioactivity recovered from the liver and plasma lipid fraction, 22 hr after oral administration of [1-^{14}C]20:3, is still present as dihomo-γ-linolenic acid. Therefore, orally administered 20:3ω6, besides being metabolized to arachidonic acid and the 22 carbon chain length derivatives, is also available for incorporation as 20:3ω6 into the tissue lipids, the Δ5 desaturase regulating the conversion of dihomo-γ-linolenic acid to arachidonic acid. Such a regulation would result in the availability of dihomo-γ-linolenic acid for PGE_1 synthesis. This is important for the normal homeostatic balance in which the prostaglandins of the E_1 series inhibit platelet aggregation, while the prostaglandins of the E_2 series have the opposite effect.

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Effects of Feeding Ethyl-dihomo-γ-Linolenate on Rabbit Renomedullary Lipid Composition and Prostaglandin Production In Vitro

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ABSTRACT

Feeding the ethyl ester of dihomo-γ-linolenic acid for 25 days to rabbits resulted in increased PGE_1 (20to30-fold) and PGE_2 (1.5-fold) output by a hormone responsive, in vitro, renal papilla preparation. The relative amount of PGE_1 increased from $< 5\%$ of PGE_2 in controls to 25-35% of PGE_2 in the papillae of 20:3ω6-supplemented animals. During the study renomedullary triglycerides in the 20:3ω6-supplemented animals increased 2.8-fold compared to animals fed an equal amount of a control fatty acid mixture, and in addition to a marked enrichment in 20:3ω6, also contained increased proportions of 20:4ω6 and longer chain polyenes. The increase in triglyceride content found in the renal medulla was not seen in the renal cortex or liver. There was no increase in renomedullary phospholipid content during the study, and phopholipids of treated animals contained increased proportions of 20:3ω6 and 20:4ω6, but not longer chain polyenes. The results indicate that enriching the prostaglandin precursor pool by feeding 20:3ω6 can alter the type and amount of prostaglandin released by the renal papilla, at least in vitro. Also, the selective changes in amount and long chain polyene content of renomedullary triglycerides during the study suggest some special functions for this lipid class in prostaglandin precursor metabolism.

INTRODUCTION

Renal prostaglandin synthesis may contribute to the regulation of renal blood flow, salt and water excretion, and blood pressure control (1,2). The renal medulla is the major site of renal prostaglandin biosynthesis (3,4), and renomedullary triglycerides are rich in prostaglandin precursor fatty acids compared to triglycerides of other tissues (5). While the role of prostaglandins in renal function is usually studied either by inhibiting their biosynthesis or by infusing exogenous prostaglandins and their precursor fatty acids, another perhaps more physiological approach would be to alter endogenous prostaglandin release by changing the renal prostaglandin precursor pool. It has been found, for instance, that renal papillae from essential fatty acid deficient rabbits contain lower amounts of PGE_2 than those from control rabbits (6). Feeding the ethyl ester of prostaglandin precursor fatty acids can also accomplish this and apparently can alter prostaglandin dependent parameters in the kidney, since feeding ethyl arachidonate (ethyl 20:4ω6) lowers the blood pressure of renal artery-clipped, hypertensive rats (7).

The diet can be supplemented with either arachidonic (20:4ω6) or dihomo-γ-linolenic (20:3ω6) acids, so one could theoretically manipulate the proportions of monoenoic and dienoic prostaglandins released by altering the composition of the precursor pool. Both arachidonic and dihomo-γ-linolenic acids are incorporated into the lipids of many tissues when their respective ethyl esters are fed to rats (8). Feeding ethyl dihomo-γ-linolenate to rabbits results in increased urinary excretion of the major urinary metabolite of the E type prostaglandins (9), which reflects both metabolized PGE_1 and PGE_2, and indicates increased whole-body prostaglandin synthesis.

The present study was carried out to see what effect feeding ethyl dihomo-γ-linolenate has on the proportion of monoenoic and dienoic prostaglandins formed by the renal medulla in vitro. The renal inner medulla does not normally produce thromboxanes (10) or prostacyclin (11) to an appreciable extent, or metabolize PGE_1 or PGE_2 (12). Measuring the PGE_1 and PGE_2 released, therefore, should give a good indication as to the utilization of their endogenous precursors by the prostaglandin synthetase complex. Since renomedullary lipids contain an unusual amount of long chain polyenes, including immediate prostaglandin precursors, the effect of feeding ethyl dihomo-γ-linolenate on the proportions as well as the fatty acid composition of several renomedullary lipid classes was also studied.

MATERIALS AND METHODS

Five white male New Zealand rabbits (2.7-2.9 kg) were fed diets supplemented with 1g/kg/day ethyl dihomo-γ-linolenic acid (gift of

Dr. John E. Pike, Upjohn Co., Kalamazoo, MI) absorbed into their chow for 25 days. Five controls received the same diet but with 1 g/kg/day of a mixture of fatty acid ethyl esters mimicking the composition of normal chow. Further details of the feeding are published elsewhere (9).

Immediately after exsanguination for platelet studies, the kidneys were removed, placed on ice and dissected. One papilla (~400-600 mg) from each animal was used for lipid analyses, and one was incubated as described by Danon et al. (13). After each 30 min of incubation, the buffer was decanted, frozen in a dry ice-acetone bath and stored at -70 C until extraction.

For bioassay, the buffer was first extracted twice with three volumes of petroleum ether (b.p. 30-60 C), acidified with 10% formic acid to pH 3.2-3.4, and extracted twice with three volumes of chloroform. The chloroform was dried under N_2 at room temperature and redissolved in 25 μl of 100% ethanol to which 250 μl of saline was added. An overall recovery averaged 80% calculated by recovery of tracer amounts of added 1-[^{14}C]-PGE_1. Total prostaglandin E was measured as PGE_2 equivalents on the rat fundus strip (13).

PGE_2 and PGE_1 also were isolated and analyzed by a stable isotope dilution method. The deuterated standards [$^2H_4$]-PGE_2 and [$^2H_4$]-PGE_1 were added to samples of incubation medium which were then acidified and the prostaglandins extracted into chloroform. After methylation of the extract with diazomethane, the methyl esters of PGE_1 and PGE_2 were separated by high performance liquid chromatography (HPLC) using a fatty acid μBondapak column (Waters Assoc., Milford, MA). PGE_2 methyl ester was further purified by subsequent HPLC on a μPorasil column (Waters Assoc.), and was finally analyzed by combined gas chromatography-mass spectrometry (GC–MS), under conditions reported previously as the PGE_2 methyl ester-methoxime-bisacetate (8). PGE_1 methyl ester was converted to PGB_1 methyl ester (to take advantage of the better GC properties of the latter), again purified by HPLC, derivatized to the PGB_1 methyl ester-trimethyl silyl ether (rather than the acetate, due to better MS sensitivity), and analyzed by combined GC-MS.

Fatty acid analysis of the lipid classes was accomplished by thin layer and gas liquid chromatography under the conditions described by Danon et al. (8), and fatty acid quantitation was accomplished by the addition of known amounts of standard compounds containing reference fatty acids to the samples prior to lipid extraction. Phospholipid fractionation by thin layer chromatography was done as in Oelz et al. (9).

All solvents used were reagent grade and angiotensin II (5-Ile) was purchased from Calbiochem, La Jolla, CA.

TABLE I

Prostaglandin Output by Renal Papillae of 20:3ω6-fed and Control Rabbits[a]

Minutes	20:3ω6-fed	Control
0 - 30	27.0 ± 6.6	25.2 ± 1.6
30 - 60	15.4 ± 2.1	12.3 ± 1.5
60 - 90	9.8 ± 3.1	6.5 ± 0.6
90 - 120	3.3 ± 0.3	2.6 ± 0.2
120 - 150	4.2 ± 0.6	3.0 ± 0.4
150 - 180	3.0 ± 0.2	2.5 ± 0.2

[a]Data expressed as PGE_2 equivalents, bioassayed on the rat fundus strip, in μg/g tissue/30 min. Values are mean ± SEM, n=5. The output by 20:3ω6-fed rabbits is significantly greater than control ($p<.025$) by analysis of variance for a split plot design, each animal representing the whole plot. Angiotensin II-Ile (100 ng/ml) was added at 120 min, and the resulting small increase in PGE_2 output is significant ($p<.05$) by student's paired t-test.

TABLE II

PGE_1 and PGE_2 Output During Incubation of Renal Papillae from Control and 20:3ω6 Fed Rabbits as Determined by Combined Gas Chromatography-Mass Spectrometry (See Text for Details)

	PGE_1		PGE_2	
Time (min)	Control	20:3	Control	20:3
0-30	0.14	4.79	14.96	18.62
30-60	0.10	2.45	6.70	9.40
60-90	0.08	1.79	3.55	5.28

[a]Values are means of 2 samples in each group, in μg per 30 min/papilla.

RESULTS

Table I shows the time course of prostaglandin E output by rabbit renal papillae as measured with the rat fundus strip. Throughout the incubation, papillae from the ethyl 20:3ω6-fed animals produced more prostaglandin than those from control animals. The viability of the preparation is indicated by the small but significant increase in prostaglandin output in response to angiotensin II. Angiotensin II has previously been shown to stimulate prostaglandin output by the perfused rabbit kidney (14) and the rat renal papilla in vitro (13).

Samples from the first three periods of two

TABLE III

Fatty Acid Composition of Some Renomedullary Neutral Lipids (As % Composition) of Rabbits Receiving a Diet Supplemented with Ethyl 20:3ω6 (20:3) and Control Rabbits

Fatty acids	Triacylglycerols		Mono- and diacylglycerols		Free fatty acids	
	Control	20:3	Control	20:3	Control	20:3
16:0	22.8 ± 1.6	15.7 ± 0.9[a]	17.4 ± 1.5	14.4 ± 1.1	23.5 ± 1.2	24.5 ± 1.3
16:1	5.4 ± 0.8	2.7 ± 0.4[b]	1.4 ± 0.2	1.6 ± 0.3	3.5 ± 0.7	4.4 ± 0.7
18:0	8.7 ± 0.8	6.5 ± 0.3[b]	23.8 ± 0.5	21.1 ± 1.2	23.1 ± 2.9	20.2 ± 2.4
18:1	26.8 ± 1.7	12.8 ± 1.1[c]	15.7 ± 0.9	12.7 ± 0.6[b]	19.1 ± 2.5	18.2 ± 0.9
18:2	21.6 ± 1.2	15.8 ± 0.7[a]	11.7 ± 0.9	9.4 ± 1.2	7.4 ± 1.9	4.6 ± 1.4
20:3	0.6 ± 0.2	19.8 ± 1.6[c]	2.1 ± 0.4	9.5 ± 2.4[d]	0.6 ± 0.2	2.5 ± 0.6[a]
20:4	3.5 ± 0.5	9.0 ± 0.7[c]	20.9 ± 1.9	24.0 ± 0.7	5.9 ± 1.2	7.3 ± 2.1
>20:4[e]	7.0 ± 2.0	13.5 ± 1.8[b]	8.7 ± 1.3	7.7 ± 2.1	<.5	<.5

[a]p < .01
[b]p < .05
[c]p < .001
[d]p < .02
[e]mostly 22:4.

TABLE IV

Fatty Acid Composition of Renomedullary Phospholipids (as % Composition) of Rabbits Receiving a Diet Supplemented with Ethyl 20:3ω6 (20:3) and Control Rabbits

Fatty acids	Phosphatidylcholines		Phosphatidylethanolamines		Phosphatidyl Serines & inositols	
	Control	20:3	Control	20:3	Control	20:3
<16:0	0.5 ± 0.1	0.6 ± 0.2	5.8 ± 2.0	7.2 ± 0.7	0.9 ± 0.1	1.0 ± 0.2
16:0	30.0 ± 1.2	32.8 ± 1.1	10.6 ± 1.3	8.8 ± 0.8	4.6 ± 0.2	5.3 ± 0.6
16:1	2.1 ± 0.1	2.4 ± 0.1[a]	2.8 ± 0.6	2.6 ± 0.1	0.5 ± 0.1	1.0 ± 0.1[d]
18:0	9.5 ± 0.4	9.0 ± 0.5	13.4 ± 0.9	14.5 ± 0.9	36.4 ± 0.6	37.3 ± 1.0
18:1	24.5 ± 0.8	19.6 ± 1.0[b]	16.4 ± 0.2	12.8 ± 0.7[d]	20.1 ± 0.8	15.8 ± 0.7[b]
18:2	21.0 ± 0.8	11.6 ± 0.8[d]	8.3 ± 0.3	4.5 ± 0.3[e]	8.9 ± 0.6	4.2 ± 0.2[d]
20:3	0.7 ± 0.1	8.5 ± 0.4[e]	1.1 ± 0.1	4.6 ± 0.3[e]	0.9 ± 0.1	8.3 ± 0.6[e]
20:4	9.9 ± 0.7	13.3 ± 0.9[c]	35.5 ± 0.5	41.6 ± 1.0[d]	24.9 ± 0.7	27.2 ± 1.2
>20:4[f]	1.1 ± 0.3	0.9 ± 0.1	5.2 ± 0.9	5.3 ± 0.8	1.8 ± 0.2	1.8 ± 0.4

[a]p < .05
[b]p < .01
[c]p < .025
[d]p < .005
[e]p < .001
[f]mostly 22:4.

renal papilla incubations in both the control and the ethyl 20:3ω6-fed groups were analyzed for PGE_1 and PGE_2 by combined GC-MS. These data are presented in Table II and show that the papillae from the ethyl 20:3ω6-fed group produced 20-30 times more PGE_1 and 25-50% more PGE_2 than those of control rabbits, increasing PGE_1 output from <5% of that of PGE_2 to 25-35% of PGE_2 production.

After 25 days of dietary enrichment with the ethyl ester of 20:3ω6, there was extensive incorporation of 20:3ω6 into all renomedullary lipids. This data is presented in Tables III and IV. It can be seen that every renomedullary lipid class of the 20:3ω6-fed animals showed increases in both the 20:3ω6 and 20:4ω6 suggesting some conversion of the former to the latter. In the phospholipid fractions, 20:3ω6 replaced mostly linoleic and oleic acids, indicating that it was being esterified largely to the β-position. The phospholipid arachidonate content was increased by about 20%. In the triglycerides, not only did the treatment with 20:3ω6 triple the proportion of arachidonic acid (20:4ω6), but it led also to sizable increases in longer chain polyunsaturates (mostly 22:4ω6). This increase in the longer chain polyunsaturates was not seen in the phospholipid

fractions. There was also much less selective displacement of fatty acids by the 20:3ω6 in the triglycerides. Although oleic acid was replaced the most, there were sizable and proportional decreases in all the other fatty acids shorter than 20 carbon atoms.

In addition to a marked increase in the proportion of prostaglandin precursors in the lipids of the treated animals, there was also a 2.8-fold increase in the amount of triglyceride in the renal papillae from these animals. Kidney papillae from control and 20:3ω6 fed animals contained 2.46 ± 0.29 and 6.90 ± 1.68 mg of triglyceride fatty acid per gram wet weight of tissue, respectively ($p < .05$ student's t test; mean ± SEM). The values for mg phospholipid fatty acid per gram wet weight of tissue were 7.64 ± 0.55 for controls and 7.60 ± 0.84 for the 20:3ω6-fed animals. Our control data are similar to those reported by Morgan et al. (5), with our samples containing somewhat more triglyceride and less phospholipid, probably because ours were samples of inner rather than whole medulla. The increase in amount of triglyceride with feeding 20:3ω6 compared to feeding a fatty acid mixture was only seen in the renal medulla and not in the renal cortex or liver.

DISCUSSION

It has been shown previously that feeding ethyl dihomo-γ-linoleic acid to rabbits increased whole body production of the E type prostaglandins as indicated by a rise in excretion of their major urinary metabolite (9). Since this treatment also increased the arachidonic acid content of plasma and all tissues examined except the platelet (9), the rise in urinary metabolite could be due to increased production of either PGE_1 or PGE_2. The present study shows that enriching renomedullary lipids with the precursor of the monoenoic prostaglandins results in greatly enhanced production of PGE_1 as well as some increase in PGE_2 output by the renal medulla, although the papillae from our 20:3ω6-supplemented animals still formed PGE_2 in considerably larger amounts than PGE_1.

In some biological actions, the corresponding members of the monoenoic and dienoic prostaglandin series are qualitatively similar, but in others, such as platelet aggregation (15), they can differ markedly. Also, the distribution of oxygenation products of dihomo-γ-linoleic acid will be quite different from that of arachidonic acid, since the former is reported to give a much higher proportion of lipoxygenase rather than cyclooxygenase products (16) and cannot be converted to prostacyclin. These differences raise the possibility that pharmacologic conversion to an increased oxygenation of dihomo-γ-linolenic acid could alter the results of physiologic activation of the "arachidonate cascade" in vivo.

The occurrence of relatively large amounts of long chain polyunsaturated fatty acids in renomedullary triglycerides compared to those of other tissues suggests that this lipid class may be serving a storage function for these fatty acids in the renal medulla that is unique among the tissues investigated. The relationship between such selective accumulation of particular fatty acids by renomedullary triglycerides and the high rate of prostaglandin output by this tissue is not clear. It has been shown that in rabbits the number of triglyceride-rich lipid droplets in renomedullary interstitial cells decreases in renal artery clipped hypertensive animals (17) and increases with indomethacin treatment (18), correlating with presumed increases and decreases in tissue prostaglandin output, respectively.

We have found changes in the types and amounts of prostaglandins produced by renal papillae of 20:3ω6-fed rabbits as well as marked differences between the effects of 20:3ω6 feeding on the amount and composition of renomedullary triglycerides and phospholipids. Eventually, such studies may allow an understanding of the control of prostaglandin precursor mobilization by specific acyl hydrolases, as well as the mechanisms whereby the biosynthesis of prostaglandins and the storage of their precursors are coordinated.

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Uptake and Utilization of 1-^{14}C Palmitic Acid by Heart Cells Treated with Fresh or Thermally Oxidized Fats[1]

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ABSTRACT

The effects of fractions isolated from thermally oxidized corn oil or olive oil on the metabolic acitivty of heart endothelial and muscle cells were studied. Rat heart cells in culture, exposed to thermally oxidized fat components, took up more exogenous 1-^{14}C-palmitic acid and incorporated more of it into the cell triacylglycerol fraction than when the cells were treated with fresh fats. Particularly with the heated corn oil compared to fresh corn oil, much less of the radioactivity from the labeled palmitic acid was deposited in the phospholipid fraction. Also, with heated corn oil when the incubation period was extended beyond 12 hr, there was a decline in the radioactivity retained in the triacylglycerol fraction of the heart muscle cells. When the fresh fats were compared for ^{14}C-radioactivity incorporation into the heart cells, the olive oil gave much higher values, indicating a distinct difference in response to the proportion of fatty acids supplied.

INTRODUCTION

Several workers have reported that the inclusion of oxidized fats in experimental diets resulted in pathological changes in certain organs of the test animals. The changes cited include growth depression, fatty infiltration in the myocardium, enlarged liver with increased lipid accumulation, and pyknotic hepatocellular nuclei (1-4). These effects are indicative of the toxic reactions of heated fat components in tissues active in the transport, synthesis and utilization of lipids.

Shue and co-workers (5) observed that the components of heated oil which did not adduct with urea reduced the rate of formation of $^{14}CO_2$ from palmitic acid by 30% in the liver. These data suggested that the non-urea-adducting fatty acids from heated fat impair the rate of oxidation of naturally occurring fatty acids. Dietary fats can have a great influence on the synthesis and metabolism of other fatty acids (6,7). Rao and co-workers (8) showed that oxidized fat components influenced the rate of incorporation of 1-^{14}C-acetate into different fatty acids in the triacylglycerol fraction of the livers of rats fed heated fat.

The purpose of this experiment was to investigate the effect of fresh or thermally oxidized fat components from two different types of edible fats, (corn oil and olive oil) on the uptake and utilization of 1-^{14}C-palmitic acid by heart muscle and endothelial cells.

MATERIALS AND METHODS

Preparation of Lipid Fractions

Commercially available corn oil (Mazola Corn Oil, Best Food Division, Canada Starch Company Ltd., Montreal) and olive oil (Pompeian Brand, Pompeian Olive Oil Corp., Baltimore MD) were heated in stainless steel beakers at 180 C for 72 hr with 8 hr of aeration every day. The distillable non-urea adductable fractions (DNUA) from each thermally oxidized fat was prepared by the method of Crampton et al. (9) with modifications (10).

Primary Heart Cell Culture

Primary cultures of heart muscle (M) and endothelial (E) cells were prepared from 2-5 day old rat hearts by the method of Rogers (11) as monolayered coverslip cultures in leighton tubes. Each culture tube was inoculated with 2 ml of a cell suspension (ca. 2x10^5 cells/ml of the medium). The cells were incubated for 3 hr in a culture medium (HB 597, Connaught Laboratories, Ltd., Willowdale, Ontario) with 5% fetal calf serum (GIBCO, Grand Island, NY) prior to separation of the muscle cells from the endothelial cells. Several slides were observed under a microscope to confirm a good separation. Four-day old cultures of heart endothelial and muscle cells were exposed to a medium containing fresh or heated fat fractions.

Addition of Lipid Fractions to the Culture Medium

Free fatty acids from the fresh fat controls (FF) or from the DNUA of the heated fats (HF) were prepared. One ml of the sample was combined with 4 ml of 2 N NaOH in a 250 ml

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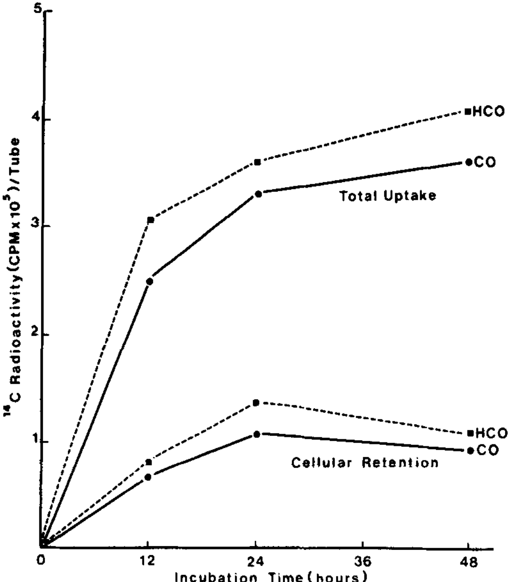

FIG. 1. Uptake and retention of [1-^{14}C] palmitic acid by heart endothelial cells treated with fatty acids of corn oil (CO) or heated corn oil (HCO).

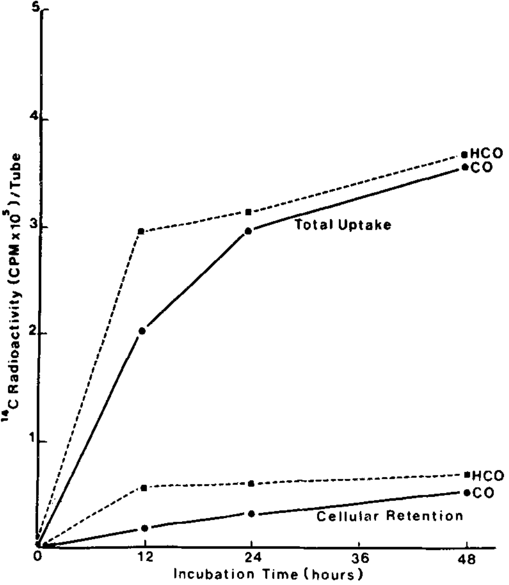

FIG. 2. Uptake and retention of [1-^{14}C] palmitic acid by heart muscle cells treated with fatty acids of corn oil (CO) or heated corn oil (HCO).

round bottom flask. The flask was flushed with nitrogen, stoppered, left at room temperature for 2 hr and shaken periodically. Concentrated HCl was added to the saponified mixutre while it was shaken, and checked for pH until it was ca. 1. The free fatty acids were extracted twice from the mixture with diethyl ether and water washed to pH 5. The solvent was evaporated in a rotary evaporator under nitrogen. The saponification procedure was repeated once. The sample was added to the culture medium in the form of an emulsion with bovine serum albumin (Fraction V, poor in unesterified fatty acids, from ICN Pharmaceuticals, Inc., Cleveland, OH) dissolved in phosphate buffered saline (PBS) from GIBCO (Grand Island, NY). The required concentration of each free fatty acid fraction, dissolved in hexane, was transferred to a 100 ml sterilized bottle, and the solvent was evaporated completely. The solution of bovine serum albumin (40 mg/ml of PBS) was added to the lipid fractions. A ratio of free fatty acid fraction to bovine serum albumin of 1:60 (w/w) was maintained. The bottle was screw capped and incubated at 40 C with occasional shaking for 2 hr. This incubation period was sufficient to obtain an emulsion of free fatty acids ready for administration into the culture medium. Fractions of FF and HF were added to the medium to give a concentration of 100 μg/ml.

Heart cells were incubated with FF or HF for 24 hr, and at the end of this period, the cultures were rinsed well with PBS and then exposed to a medium containing radioactive palmitic acid. The [1-^{14}C]palmitic acid (benzene solution $>$ 50 mCi/mMol, Amersham/searle Corp., Oakville, Ontario) was dissolved in hexane and combined with nonradioactive palmitic acid in a sterilized screw capped bottle. The solvent was evaporated under nitrogen. A sterilized solution of $KHCO_3$ (0.035 mg/ml) was added to the fatty acid mixture for neutralization and incubated at 40 C for 5 hr. This mixture was put in the culture medium in the form of the potassium salt bound to delipidized serum proteins (12). The preparation of delipidized protein, except filtration, was carried out at approximately -40 C, with all solvents and flasks chilled before use. Ethanol-ether (3:1, v/v) was chilled with dry ice for 2 hr. Fifty ml of fetal calf serum was added slowly to 1200 ml of this solvent with shaking. After 18 hr the contents of the flask were filtered through paper. The filter paper was washed thoroughly with diethyl ether to remove the remaining lipid, and air dried. The solvent extracted protein was dissolved in 50 ml of PBS and stored at -20 C. The concentration of serum protein solution used was 10% of the total medium. Each culture tube received 0.25 μCi of [1-^{14}C] palmitic acid and 2.5 μg of cold palmitic acid.

The total uptake of [1-^{14}C] palmitic acid,

its retention by the cells and incorporation into different lipid classes were determined.

Measurement of Cellular Uptake and Retention of 1-^{14}C-Palmitic Acid

The radioacitivity of the medium was determined at 0 hr (initial counts) and then at incubation periods of 12, 24 and 48 hr. The total uptake of [1-^{14}C] palmitic acid by the cells was determined by subtracting the counts left in the medium at 12, 24, and 38 hr from the counts present at 0 time. The total cellular retention of ^{14}C was determined by counting the radioactivity in the cellular fraction of each tube for each incubation period. The system was vented periodically to avoid accumulation of CO_2. A phenol red pH indicator was present in the medium. The difference between total uptake and retention at each of the time periods indicated the amount of loss of ^{14}C as a result of cellular oxidation of palmitic acid and its derivatives. Five replications were made for each treatment.

Extraction and Separation of Cellular Lipid

Cellular lipid was extracted by means of the Folch procedure (13) and fractionated by thin layer chromatography (TLC). Glass plates coated with Silica Gel G of 0.5 mm thickness and a solvent system consisting of heptane, isopropyl ether and acetic acid at a ratio of 60:40:3 by volume was used (14).

RESULTS

The uptake and retention of the radioactive palmitic acid by heart E and M cells in culture, treated with corn oil free fatty acids (CO) or with the heated corn oil fraction free fatty acids (HCO), are shown in Figures 1 and 2. The cells treated with HCO were able to take up more labeled fatty acid from the medium at each incubation period. Also, the retention of ^{14}C-radioactivity was significantly higher in the HCO treated cells compared to those treated with CO.

Similar observations were made (Figures 3 and 4) for heart E and M cells treated with free fatty acids from olive oil (OO) or heated olive oil (HOO), in that in every case there was a much higher uptake of exogenous [1-^{14}C]palmitic acid and greater retention of radioactivity with the HOO treatment.

The incorporation of ^{14}C fatty acid into the different lipid classes of the cells treated with HF was found to be different from that for the FF treated cells (Tables I and II). The heart E and M cells exposed to HCO (Table I) exhibited significantly higher incorporation of ^{14}C into the triacylglycerol (TG) fractions and lower levels in the phospholipid (PL) fractions than for the control (CO). However, when the incubation period was extended beyond 12 hr,

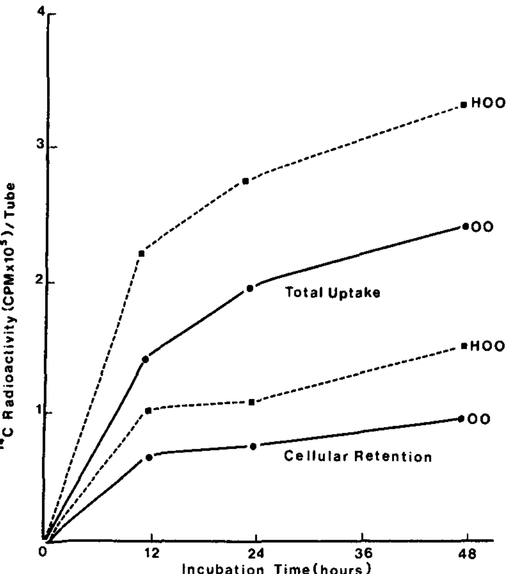

FIG. 3. Uptake and retention of [1-^{14}C] palmitic acid by heart endothelial cells treated with fatty acids of olive oil (OO) or heated olive oil (HOO).

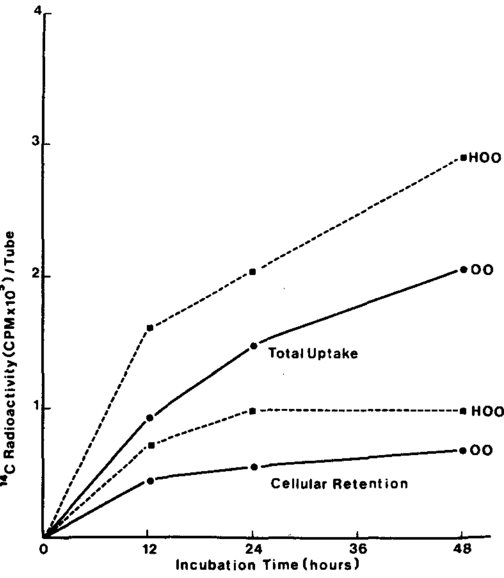

FIG. 4. Uptake and retention of [1-^{14}C] palmitic acid by heart muscle cells treated with fatty acids of olive oil (OO) or heated olive oil (HOO).

TABLE I

Percent Incorporation of ^{14}C-Radioactivity into Phospholipid and Triacylglycerol Fractions of Cells Treated with Free Fatty Acids from Fresh or Thermally Oxidized Corn Oil[a]

Lipid classes	Incubation time (hr)	Endothelial cells		Muscle cells	
		CO	HCO	CO	HCO
Phospholipid	12	64.1[a]	48.2[b]	80.4[a]	38.6[f]
	24	66.7[a]	39.7[c]	73.6[b]	43.8[e]
	48	70.1[a]	47.2[b]	65.1[c]	48.4[d]
Triacylglycerol	12	5.0[d]	33.9[a]	4.2[e]	40.2[a]
	24	7.7[c]	33.4[a]	6.7[de]	28.8[b]
	48	9.8[b]	35.0[a]	8.9[d]	23.4[c]

[a]Expressed as percentage of total radioactive counts present in cellular total lipid. Remainder of the counts not shown were in other lipid classes. Each value represents the mean of 5 determinations. Values with the same superscripts are not significantly different from each other ($P<0.05$) by Duncan's multiple range test.

TABLE II

Percent Incorporation of ^{14}C-Radioactivity into Phospholipid and Triacylglycerol Fractions of Cells Treated with Free Fatty Acids from Fresh or Thermally Oxidized Olive Oil[a]

Lipid classes	Incubation time (hr)	Endothelial cells		Muscle cells	
		OO	HOO	OO	HOO
Phospholipid	12	49.3[ab]	48.4[b]	46.2[a]	38.6[b]
	24	52.4[a]	39.1[d]	46.0[a]	38.4[b]
	48	42.0[cd]	44.2[c]	48.8[a]	38.0[b]
Triaclyglycerol	12	34.9[c]	37.4[bc]	31.7[b]	42.7[a]
	24	27.5[d]	41.6[a]	31.4[bc]	42.8[a]
	48	38.3[abc]	38.9[ab]	27.3[c]	44.3[a]

[a]Expressed as percentage of total radioactive counts present in cellular total lipid. Remainder of the counts not shown were in other lipid classes. Each value represents the mean of 5 determinations. Values with the same superscripts are not significantly different from each other ($P<0.05$) by Duncan's multiple range test.

there was a decline in the amount of radioactivity retained in the TG fraction of the M cells treated with HCO.

The M cells exposed to HOO (Table II) did not show any fall in radioactivity in the TG fraction with a prolonged incubation period. However, these cells did show a higher level of ^{14}C in the TG fraction and a lower level in the PL fraction compared with the controls. In contrast, the E cells treated with HOO did not show any consistent difference in the rate of incorporation of ^{14}C palmitic acid into the TG or PL fractions.

When the two fresh fat treatments were compared for ^{14}C incorporation into different lipid classes, the OO showed about 3 times as much activity in the TG fractions for both the E and M cells (Tables I and II).

DISCUSSION

A physiologically harmful substance exerts its toxic effect first at the molecular level where it disturbs metabolic reactions and may result in cell death. This research was undertaken to study the uptake and metabolism of a naturally occurring fatty acid (palmitic acid) by heart cells in culture treated with thermally oxidized fat components.

Palmitic acid is preferentially taken up by the myocardium and used for synthetic reactions or as an energy source (15). It also has been shown that palmitic acid is important in the beating activity of heart cells in culture (16). In our experiment, cells treated with fresh corn oil fatty acids utilized the majority of the radioacitive palmitic acid for PL synthesis, although incorporation of this fatty acid into other lipid classes also was observed.

The HF treated cells appeared to be different metabolically from the cells treated with FF. Changes observed in the cells due to the HF treatments were as follows: (a) a significantly higher uptake of exogenous 1-^{14}C-palmitic acid by the cells, and (b) although the radioacitivity was incorporated into all lipid classes, there

was an increased incorporation into the TG fraction and a reduced incorporation into the PL fraction compared with control cells treated with FF.

The mechanism by which fatty acids move from the surface binding sites to the interior of the cells has not been well established. Some of the available data are compatible with an energy independent diffusion phenomenon of the fatty acid through the cell membrane (17-21). In our study the higher uptake of 1-^{14}C-palmitic acid by the heart cells treated with HF could have resulted from an increased permeability of the cell membrane, allowing a rapid influx of the fatty acids into the cells. One might speculate that the higher rate of uptake of exogenous fatty acids by the HF treated cells is related to a stress reaction by the heart cells to meet their energy requirements, resulting in more exogenous fatty acids being oxidized by the cells. However, it was found that although the cells were able to oxidize the fatty acids, a significant amount of radioactivity was retained, especially by the cells treated with the HOO. The amount of radioacitivity retained relative to the total uptake decreased in the cells treated with HCO when the incubation was continued beyond 24 hr. This indicated that the fatty acid was being used by the cells more efficiently with time.

Indications based on the radioactive counts recovered in the different lipid fractions were that the HF treated cells had a greater tendency to synthesize TG than the control cells. Also, lower counts obtained in the phospholipid fractions of HF treated cells suggest that the phospholipid synthesis was inhibited. A tendency to synthesize more TG would result in a reduced availability of fatty acids for phospholipid synthesis. Impaired mobilization of the TG fatty acids might also be a factor. The heart M cells treated with HCO were high in TG at 12 hr, but showed a gradual reduction in the radioactive counts and a concurrent increase in the counts for the PL fraction with time. This indicates that the M cells were able to hydrolyze deposited TG which could have contributed to the relative increase in PL.

Oleic acid is known to cause fat accumulation in cultured cells (22), and, in the current study, this was revealed by increased TG synthesis (Table II) when OO was the treatment, compared with the TG synthesis in Table I when CO was used. The HOO tended to increase the TG values further, particularly for the M cells.

These results raise several questions regarding the possible mechanisms involved in the toxic effects of oxidized fats. The heart cells in this study not only represented a model for intact myocardium, but possibly for tissues involved in the absorption and metabolism of dietary fats. It would be of interest to pursue studies regarding the interaction of heated fat components with cell membranes. Any alteration in these membrane structures might affect enzyme activities.

ACKNOWLEDGMENTS

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Effect of Feeding Protected Cholesterol on Ruminant Milk Fat Secretion

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ABSTRACT

Feeding 1-2 g/day of cholesterol protected against ruminal hydrogenation caused a 20-30% drop in the secretion of milk fat by goats and cows. The effect was observed with goats fed conventional rations or with goats and cows fed rations supplemented with protected lipids, but was not observed with cows fed conventional rations, or when unprotected cholesterol and protected β-sitosterol was fed to these animals. The results suggest that this depression in milk fat is due to a decreased uptake of plasma triacylglycerol fatty acids by the mammary gland, induced by dietary cholesterol.

INTRODUCTION

The serum cholesterol of ruminant animals rises when supplemental fat is fed in a form that ensures the absorption of long chain fatty acids (1,2). The effect of these fat supplements on cholesterol metabolism has been studied in sheep and goats resulting in significantly enhanced sterolgenesis in the small intestine both in vivo and in vitro, while in vitro sterolgenesis appeared to be suppressed in the liver (3). These findings may reflect the increased requirement for cholesterol due to an induced response to aid in transport of the large amount of triglyceride in chylomicrons.

Cholesterol and other unsaturated sterols are hydrogenated by rumen fluid in vitro (4,5), and this hydrogenation resembles the intestinal hydrogenation in other species (6). Ruminal hydrogenation has also been demonstrated to occur in vivo in sheep fed cholesterol supplements (4,5). Consequently, the intestine of ruminants are not normally challenged with large amounts of dietary cholesterol.

The present series of studies were undertaken to evaluate the nature and the physiological significance of ruminal sterol hydrogenation by utilizing procedures previously developed to protect sterols against ruminal metabolism (4,5). This paper describes how the feeding of protected cholesterol supplements depressed the secretion of milk fat in lactating ruminants, and some initial studies designed to explain this cholesterol-induced suppression in milk fat secretion are presented.

MATERIALS AND METHODS

Protected Cholesterol (PC) Supplements

These were prepared by (a) dissolving cholesterol in chloroform (2:1, w/w); (b) mixing the cholesterol solution with warm (45 C) sunflower oil (containing butylated hydroxyl anisole (0.01-0.02%); (c) emulsifying this mixture with an aqueous 16% casein solution (ratio casein/oil/cholesterol, 4:3:1, w/w/w); (d) adding NaOH to give a pH of 8.5; (e) treating the alkaline emulsion with formaldehyde to induce gelation, and (f) drying the comminuted gel in a fluid bed drier (air temperature < 50 C) until all traces of chloroform were removed.

The effectiveness of this procedure for preparing PC supplements was verified by incorporating [4-^{14}C]cholesterol(sp. act. 24.6 mCi/mM) into the supplements and incubating the radiolabeled supplements with rumen fluid in vitro as previously described (4,5). The sterols extracted from the incubated rumen fluid reaction mixtures were separated by argentation thin layer chromatography (Ag^+TLC) (5% $AgNO_3$, Silica Gel G – solvent system; chloroform/acetone, 98:2, v/v), and the distribution of radioactivity was measured by radioscanning (using a Varian Berthold scanner, Varian Instruments Co., Palo Alto, CA).

Unprotected Cholesterol (UPC) Supplements

These were prepared by dissolving the sterol in sunflower oil (1:3, w/w) at 45 C and mixing the sterol/sunflower oil mixture with dry sodium caseinate (1:1, w/w). The ratio of casein/oil/cholesterol was the same as in the protected supplements (i.e., 4:3:1, w/w/w).

Protected Oil (PO) Supplements

These were prepared by using sunflower oil and casein or mixtures of sunflower seed kernel and casein using procedures previously described (7,8). For all of these supplements, the formaldehyde was added to the oil/protein/water emulsions prior to drying, and the formaldehyde treated gel was dried using a flash drier.

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Feeding and Management of Animals

Goats: Lactating Saanen goats were milked and individually fed once daily in the morning. The control diets were chpped alfalfa hay and crushed oats (1:1, w/w, 1500 g/day). The cholesterol supplements were mixed with ca. 50 g of crushed oats and given prior to the main bulk of feed to ensure complete consumption.

Cows: Lactating Holstein and Friesian cows were fed and milked twice daily in the morning and afternoon. The control diet consisted of chopped alfalfa hay and crushed oats (2:1, w/w, 12 kg/day). Cholesterol supplements were mixed with ca. 50 g of crushed oats and given twice daily prior to the main bulk of feed to ensure complete consumption.

Milk Sampling

The weight of milk produced was recorded at each milking, and an aliquot was taken for subsequent chemical analyses. The aliquot from the morning and evening milking of cows was a fixed proportion of the total yield, and the two aliquots were then pooled to give a representative sample of the daily milk production. All milk samples were stored at 1-5 C for up to 2 days pending analysis.

Milk Protein and Nonfat Solids

Milk protein and nonfat solids were determined by the standard methods (9).

Milk Fat and Fatty Acids

The fat content of milk was measured by the Babcock method (10). For analysis of the milk fatty acids, another milk sample was extracted by the Roese-Gottlieb procedure (10), and the extracted fat was interesterified with borontrifluoride in butanol (11).

The butyl esters of milk fatty acids were analyzed by temperature-programmed gas liquid chromatography (GLC) (Varian instrument Model 1200). The column temperature was programmed to rise from 110 C to 195 C at a rate of 10 C per min. The column was of stainless steel, 2 m long, 2 mm O.D., containing 20% DEGS absorbed onto silanized acid washed chromosorb W (60-80 mesh; Applied Science Laboratories, State College, PA). The output from the GLC was integrated electronically, and the results were converted to weight percentage of fatty acids using the appropriate fatty acid correction factors to eliminate the contribution of the butyl group.

Knowing the weight of milk produced, the fat content and the fatty acid composition, it was possible to calculate the daily secretion of individual fatty acids.

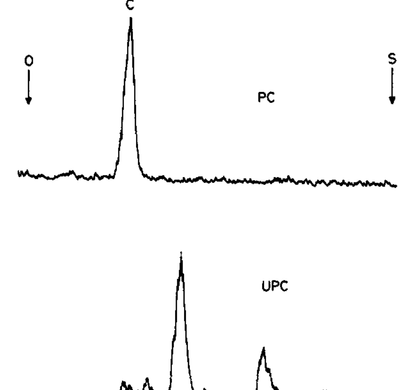

FIG. 1. In vitro ruminal metabolism of [4-^{14}C] cholesterol. Radioscan of [^{14}C] cholesterol and its metabolites extracted from in vitro rumen incubation mixtures. Ten ml of rumen fluid was incubated anaerobically for 20 hr with 40 mg of Protected Cholesterol (PC) or Unprotected Cholesterol (UPC) preparations containing 1 μCi of [4-^{14}C] cholesterol. 0 – origin; S – solvent front; C = cholesterol.

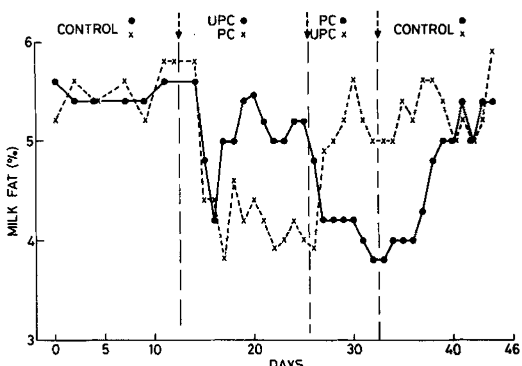

FIG. 2. Effect of Feeding Protected Cholesterol (PC) or Unprotected Choelsterol (UPC) Supplements on the Fat Content of Milk from Two Goats. Sixteen g (equivalent to 2 g cholesterol) of each supplement was fed daily. The average daily milk production in kg for each dietary period was: for goat A (●——● 0.73, 0.89, 1.01 and 1.14 during the first control, UPC, PC and last control period, respectively; and for goat B (X——X) 1.04, 1.03, 1.02 and 1.11 during the first control, PC, UPC and last control period, respectively.

The transfer of protected dietary 18:2 into milk was also calculated by expressing the net increase in daily milk secretion of 18:2 as a *percent* of the daily intake of 18:2 in the PO supplement.

Cholesterol Content of Plasma and Milk

Blood samples (20 ml) were taken from each goat and cow at regular intervals throughout the experiment and placed into tubes contain-

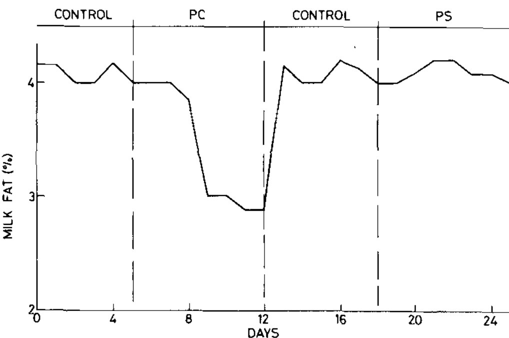

FIG. 3. Effect of Feeding Protected Choelsterol (PC) and Protected β-Sitosterol (PS) Supplements on the Fat Content of Goat's Milk. Protected β-sitosterol and protected cholesterol were fed at the rate of 16 g/day (equivalent to 2 g of sterol). The average daily milk production in kg for each dietary period was 1.50, 1.58, 1.46 and 1.49 during the first control, PC, second control and PS periods, respectively.

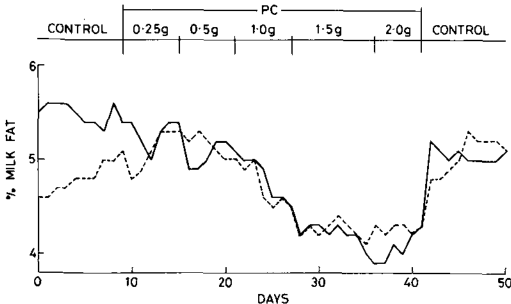

FIG. 4. Effect of Graded Levels of PC Supplements on the Fat Content of Goat's Milk. PC supplementation was such as to provide 0.25-2.0 g cholesterol per goat per day. The average daily milk production in kg for each dietary period was: for goat A (—) 0.79, 0.81, 0.78, 0.89, 0.91, 0.88 and 0.75; and for goat B (---) 1.12, 1.10, 1.14, 1.18, 1.34, 1.26 and 1.14; during the first control, 0.25 g PC, 0.5 g PC, 1.0 g PC, 1.5 g PC, 2.0 g PC and last control period, respectively.

ing a 10% EDTA solution (0.5 ml). Plasma was obtained after centrifugation. The plasma and milk cholesterol concentrations were measured using the Technicon Autoanalyzer (12).

Faecal Fat Extraction

Goats were confined to individual metabolism cages. The total daily faecal and urine output was measured and subsampled. (The fat content of goat faeces was estimated by chloroform extraction of the dried faeces). Each sample of dried faeces (5 g) was put into a Soxhlet thimble, 0.5 ml of 2N HCl was added and refluxed with chloroform for 8 hr. The chloroform extract was washed once with water, the solvent removed by rotary evaporation and the weight of fat measured after further drying in the oven at 80 C.

RESULTS

Ruminal Metabolism of Sterols

In Figure 1 the Ag^+TLC radioscan of nonsaponifiable lipids extracted from 20 hr incubated rumen fluid reaction mixtures containing [^{14}C]labeled PC and UPC preparations is shown. The UPC was extensively metabolized to less polar components, while the PC remained unchanged, indicating its protection against ruminal metabolism.

Milk Protein and Nonfat Solids

There was no effect of protected or unprotected cholesterol supplementation on the protein or nonfat solids content of milk at the 5% level.

Milk Fat

The results of a crossover feeding trial using two goats fed 2 g/day of PC or UPC is shown in Figure 2. The feeding of UPC had no effect on the milk fat content (except for a transient depressing effect in goatA [●—●]), but the PC supplement caused a marked decrease in the milk fat content of both goats. The milk fat content rapidly returned to control levels when the PC was replaced with UPC or when both the goats were returned to control rations.

In another study a goat was fed 2 g/day of PC followed by 2 g/day of protected sitosterol (PS). In contrast to a decreased milk fat following the feeding of PC, there was no change in milk fat content after feeding PS (Figure 3).

The effect of feeding increasing quantities of PC on the fat content of goat's milk is shown in Figure 4. Feeding PC supplements at the rate of 0.25 or 0.5 g cholesterol/day had little effect on the fat content of milk, but when fed in amounts equal to, or greater than 1.0 g cholesterol/day, the PC caused a reduction in milk fat content. This effect was maximal at 1.5 g cholesterol/day, since at doses of 2 g cholesterol/day there was no further reduction in milk fat content.

Figure 5 shows the effect of feeding PC supplements (2 g cholesterol/day) to a goat which was also receiving a supplement of PO. Feeding the PO and PC supplements together caused no alteration in the milk fat content, but, as in previous studies (13) when PO supplement was fed, there was an immediate rise in the fat content. A similar effect was observed when PC supplementation also

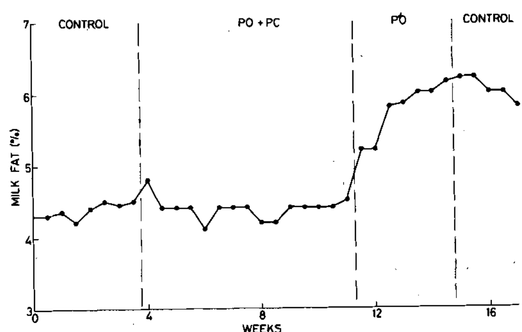

FIG. 5. Effect of Feeding PC and PO Supplements on the Fat Content of Goat's Milk. The PC supplement was fed at the rate of 16 g/day (equivalent to 2 g of cholesterol), and the PO supplement was fed at the rate of 200 g/day. The average daily milk production in kg for each dietary period was: 1.30, 1.41, 1.26 and 1.08 during the first control, PO + PC, PO and last control period, respectively.

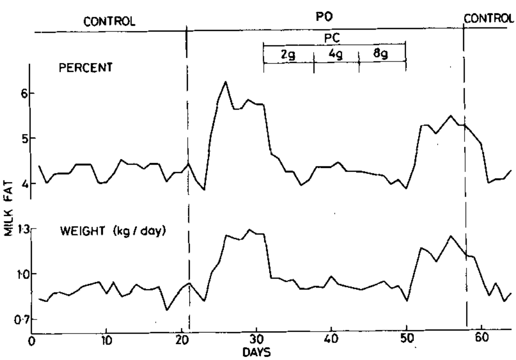

FIG. 6. Effect of Feeding PC Supplements on the Milk Fat Response to the Feeding of PO Supplements to a Cow. The control diet contained chopped alfalfa (8 kg) and crushed oats (4 kg). The PO supplemented diet contained PO (1.5 kg equivalent to ca. 0.6 kg oil). PC supplements were fed to a Holstein cow at the rates of 16, 32 and 64 g/day (equivalent to 2 g, 4 g and 8 g of cholesterol, respectively).

eliminated the PO-induced elevation in the fat content and yield of cow's milk (Figure 6). Although not shown in Figure 6, changing the nature of the oil in the PO supplement from sunflower to tallow did not alter the effect.

Milk Fatty Acids

The effects of PC and PO supplementation on the daily secretion of individual fatty acids in goats was similar to cow's milk. The response in cow's milk is shown in Figure 7. PO supplementation caused a marked elevation in the secretion of 18:2, lesser increases in the secretion of 18:1 and 18:0 and slight decreases in the secretion of 16:0 and 14:0. On the other hand, PO supplementation had no consistent effect on the secretion of the medium and short chain fatty acids (< 14:0). PC supplementation tended to reverse the PO supplementation effect, as it caused a marked drop in the secretion of 18:2, 18:1 and 18:0; there also appeared to be a small drop in the secretion of 16:0 and only a marginal drop in 14:0. Figure 7 also shows that there were no effects of PC supplementation on the secretion of short and medium chain length fatty acids (< 14:0).

During PO supplementation the increased 18:2 in the milk fat comes entirely from the PO (sunflower oil) supplement, and, knowing the amounts, consequently it was possible to calculate the efficiency of transfer of 18:2 from the diet into milk. The results in Figure 8 show that PC supplementation caused a 33% decrease in the efficiency of 18:2 transfer from the diet into milk.

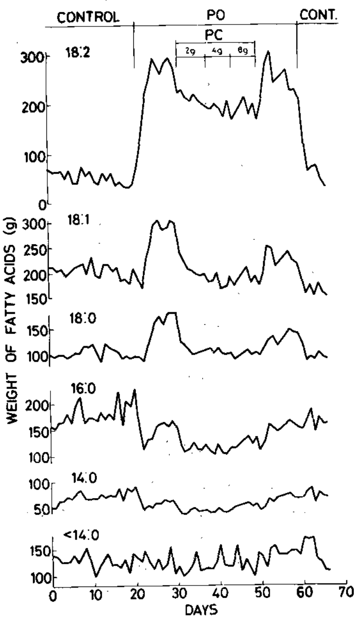

FIG. 7. Effect of Feeding PC Supplements on the Daily Secretion of Cow's Milk Fatty Acids. See Figure 6 for details of diets.

Cholesterol Content of Plasma and Milk

The plasma cholesterol of goats on a control diet supplemented with PC for three weeks was elevated by 17.8% above the mean control level (107 mg/100 ml). This response was similar to that obtained with sheep (5). Supplementing the basal diet with UPC did not effect the plasma cholesterol levels. In animals fed PO supplements for seven weeks, there was a 74% increase above the mean control level (86

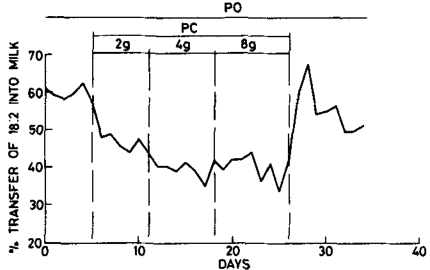

FIG. 8. Effect of Feeding PC Supplements on the Transfer of 18:2 from the Diet into Milk. See Figure 6 for details of diets. The percent transfer of 18:2 into milk fat was calculated as described in the Methods.

TABLE I

Effect of Feeding PC Supplements on the Faecal Excretion of Fat from Goats

Treatment	Faecal fat	
	%	(g/day)
Control	6.5 ± 0.5	65.1 ± 5.7
PO + PC	9.2 ± 0.5	77.3 ± 4.5
PO	8.9 ± 0.4	70.8 ± 3.7
Control	7.2 ± 0.5	63.5 ± 4.5

mg/100 ml) in goats and 22.3% in cows (mean control level 412 mg/100 ml). This was similar to that found by other workers (2). Supplementation of PO with PC for four weeks further enhanced the plasma cholesterol to 28% above PO level (150 mg/100 ml) in goats and 8.5% in cows (PO level, 504 mg/100 ml).

Milk cholesterol levels were not significantly effected with PC supplementation.

DISCUSSION

Ruminants normally consume about 0.2% of their ration as unsaturated phytosterols, and in some cases may also receive appreciable amounts of cholesterol in meat or blood meal supplements. Dietary unprotected cholesterol had no effect on ruminant milk fat secretion (Figure 2), and this may be partially due to anaerobic fermentation of cholesterol in the foregut (4,5). In contrast, feeding small amounts of cholesterol protected against ruminal hydrogenation by encapsulation in a matrix of formaldehyde-treated casein caused a decrease in the fat content of milk from goats fed control rations or from goats and cows fed rations supplemented with protected sunflower oil (Figures 2-6). In a goat study, there was also no effect of feeding equivalent amounts of protected β-sitosterol (Figure 3).

The maximum cholesterol-induced depression in the milk fat content was 20-30%, and this was observed with as little as 1.5 g/day of cholesterol (goats – Figure 4) or 2.0 g/day (cows – Figure 6). There was no effect of cholesterol supplementation on the yield of milk, the nonfat solids, the protein or the cholesterol content of milk (at the 5% level). Hence, the effect is specifically related to the secretion of milk fat and the elevation of plasma cholesterol. The latter appeared to be related to length and level of cholesterol supplementation in these feeding trials.

Long chain fatty acids of milk TG are derived primarily from circulating plasma triglycerides (14,15). These studies showed that it was this particular group of fatty acids that were affected when PC supplements were fed to a cow (Figure 7). The secretion of short and medium chain length fatty acids mainly synthesized in the mammary gland was unaffected by PC supplementation. Similar effects were observed by analyzing the fatty acid composition of milk from goats fed the PC supplements (16). The PC-induced suppression of milk fat was also observed with cows fed protected tallow supplements, but interestingly it was not possible to demonstrate a PC-induced depression in milk fat of cows fed control diets; this was so even when PC was fed at levels up to 16 g/day of cholesterol to both Jersey and Holstein cows (unpublished results).

The cause of the cholesterol-induced milk fat depression is not clear, and there appear to be no previous reports of any such analogous effect in monogastric species. The evidence does, however, suggest that the milk fat depression is due to a reduced secretion of the longer chain length fatty acids derived from the plasma. Since most of these fatty acids are in turn derived from dietary fats, it is possible that PC induced a suppression of fat absorption. This is not supported by the analysis of faecal fats from goats fed PO and PC. A slight decrease (6.5 g/day) in absorption of fat (Table I) was observed. However, this difference was not significant and does not account for the decrease in secretion of milk fat (17.5 g/day6), especially when the transfer rates of dietary lipid to milk are taken into account (Ca. 60%). Consequently, it is possible that the effect is at some stage of mammary uptake of plasma lipids.

Studies by Brumby (17) have shown that cholesterol addition to lipoproteins inhibits the activity of mammary gland lipoprotein lipase in vitro, and it is possible that a similar effect is produced in vivo when additional cholesterol is fed in a form which escapes hydrogenation in the rumen. While this would help explain the

role of cholesterol in milk fat suppression, it does not explain the apparent difference between goats and cows on control diets. This would indicate a need for further studies to examine the lipoprotein fractions of blood serum in order to follow the transport mechanism of dietary cholesterol and its relationship with lipid metabolism.

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COMMUNICATIONS

Behavior of Gangliosides on Dialysis

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ABSTRACT

Mixed brain gangliosides or individual ganglioside GM_1 were dissolved in one of the following solvents: (a) water, (b) 0.1 M aqueous KCl, or (c) methanol-aqueous 0.1 M KCl at concentrations ranging from 10^{-8} to 10^{-3} M, and were submitted to dialysis against distilled water for up to 4 days. No significant loss of gangliosides on dialysis was observed when gangliosides were dissolved in water or 0.1 M aqueous KCl, but a loss occurred when the ganglioside solution contained methanol; the loss was greater at the lowest ganglioside concentration. However, losses ceased to occur after 1 day when methanol was removed from the dialysis sac.

INTRODUCTION

Gangliosides are a family of complex lipids with a lipophilic and a large hydrophilic region. Owing to these chemical properties, dialysis against water has been an important step in the purification of gangliosides (1-3). After Kanfer and Spielvogel reported (4) that dialysis led to a loss of gangliosides, several alternative procedures such as precipitation with trichloroacetic acid-phosphotungstic acid (5) or gel filtration on Sephadex column (6) have been explored. However, none of these methods has given a quantitative recovery, and one (5) produced chemical artifacts.

The observation by Kanfer and Spielvogel (4) that losses occur when solutions of gangliosides in methanol-0.1 M aqueous KCl are dialyzed against distilled water led us to reexamine the problem. Our aim was (a) to provide definite evidence for or against use of dialysis in ganglioside purification, and (b) to define, if feasible, precise conditions for using dialysis for this purpose.

In the present study, we were able to confirm the results of Kanfer and Spielvogel (4), but we could also show that significant losses of ganglioside do not occur during dialysis, regardless of ganglioside concentration, provided the ganglioside is initially dissolved in aqueous solution.

MATERIALS AND METHODS

Solvents were redistilled before use. Water was twice distilled on a glass apparatus. Silica gel for column chromatography (0.05-0.2 mm, 70-230 mesh ASTM) and silica gel precoated thin layer plates were from Merck GmbH. Dialysis tubing (0.22 in. and 0.25 in. width) was purchased from Visking and A. Thomas. The same results were obtained with both types of tubing, regardless if the tubings were simply washed or boiled for 15 min.

Gangliosides were extracted and purified from calf brain according to Tettamanti et al. (3). The purified ganglioside mixture contained less than 0.5% phosphorus contamination and showed the following molar compositions of individual gangliosides: GM_1, 19%, GD_{1a}, 35%; GD_{1b}, 12%; GT_{1b}, 22%; G_{Q1}, 8%; minor gangliosides, 4%. The ganglioside nomenclature is that of Svennerholm (7), and according to the recommendations of IUPAC-IUB: GM_1 = II^3NeuAc-$GgOse_4$Cer; GD_{1a} = II^3NeuAc,IV^3NeuAc-$GgOse_4$Cer; GD_{1b} = II^3 $(NeuAc)_2$-$GgOse_4$Cer; GT_{1b} = II^3 $(NeuAc)_2$,IV^3NeuAc-$GgOse_4$Cer; G_{Q1} = II^3 $(NeuAc)_3$, IV^3NeuAc-$GgOse_4$Cer. The average molecular weight was 1820 daltons. Ganglioside GM_1 was purified from the same ganglioside mixture by column chromatography on silica gel. Several runs were required to obtain a final product with > 98% purity. [^{3}H]labeled ganglioside GM_1 was prepared by the galactose oxidase-sodium [^{3}H]-borohydride method of Suzuki and Suzuki (8), as modified by Ghidoni et al. (9) in order to obtain the higher specific activity of 0.15 mCi/nmole.

Gangliosides were determined, as bound N-acetylneuraminic acids (NeuAc), according to Svennerholm (10). Thin layer chromatography (TLC) of gangliosides was done on precoated thin layer plates at 18-20 C; the solvent system was chloroform-methanol-0.3% aqueous KCl, 60:35:8, v/v (2 hr run). Ganglioside fractions were made visible by exposure to iodine vapors and were scraped off the plate and analyzed.

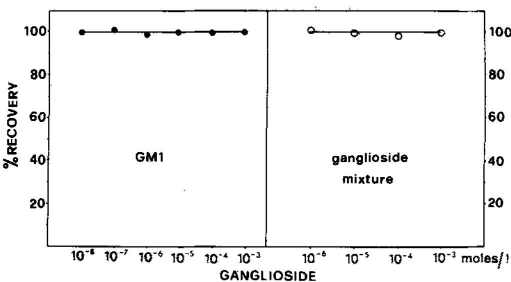

FIG. 1. Recovery of ganglioside GM_1 and brain ganglioside mixture, dissolved in distilled water (or 0.1 M aqueous KCl), after dialysis against redistilled water. Dialysis time: 4 days.

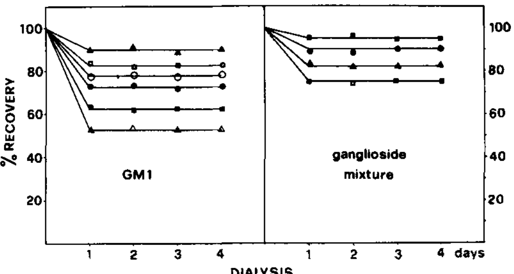

FIG. 2. Loss of ganglioside GM_1 and brain ganglioside mixture, dissolved in methanol-0.1 M aqueous KCl (1:1, v/v) on dialysis against redistilled water. GM_1 concentration: 10^{-8}M, 10^{-7}M, 10^{-6}M, 10^{-5}-M, 10^{-4}M, 10^{-3}M. Ganglioside mixture concentration: 10^{-6}M, 10^{-5}M, 10^{-4}M, 10^{-3}M.

The following was considered when gangliosides of a particular and constant physical form were to be dialyzed: (a) gangliosides occur in water solution as monomers and micelles (11); (b) the process of association-dissociation of micelles upon dilution is likely to be slow (12); and (c) the dimension and shape of the micelles may be different according to the conditions used for solubilizing gangliosides. Therefore, the standard conditions used for preparing ganglioside samples were as follows: to a known amount of ganglioside GM_1 or of ganglioside mixture, dissolved in water, $[^3H]GM_1$ ganglioside was added (the ratio of labeled to cold GM_1 did not exceed 1:20), and the sample was lyophilized. The residue was completely dissolved with chloroform-methanol, 2:1, v/v (from 2 to 10 ml) in order to dissolve gangliosides as free monomers. The mixture was then evaporated to dryness and the residue dissolved with 5 ml of one of the following solvent systems: (a) distilled water; (b) 0.1 M aqueous KCl; or (c) methanol-0.1 M aqueous KCl, 1/1, v/v. Each mixture was dialyzed for different periods of time at 4 C against 100 vol of distilled water, changed every 8 hr, under constant stirring. At the end of dialysis, the content of each dialysis sac was measured and the ganglioside content determined as radioactivity bound to GM_1. When the ganglioside concentration was above 50 μg/ml, direct colorimetric assay (10) was also performed. Each experiment was carried out in triplicate. The radioactivity in the dialyzate was measured by a Packard TriCarb 2425 liquid scintillation spectrometer in a system containing 0.1-0.2 ml of sample and 10 ml of emulsifier (Instagel, Packard) with an efficiency of ca. 40%.

The composition of the ganglioside mixture before and after dialysis was assayed by TLC under the experimental conditions described above. When the starting ganglioside concentration was 10^{-6} M, the whole content of the sac was spotted on the plate. The content of methanol in the mixture, or in the dialyzate, was determined by gas chromatography using a 20 m capillary column packed with SE52 at 100 C (carrier gas, N_2, 1 cm/min; electron capture detector, DANI 3900 gas chromatography).

RESULTS AND DISCUSSION

The behavior of gangliosides on dialysis was studied as a function of ganglioside concentration and dialysis/time.

Figure 1 shows that no loss of ganglioside occurred upon prolonged dialysis (up to 4 days) regardless of ganglioside concentration, when the starting solution of ganglioside was pure water or 0.1 M aqueous KCl. The range of ganglioside concentration was from 10^{-8} to 10^{-3} for pure GM_1, and from 10^{-6} to 10^{-3} M for the ganglioside mixture. Both ranges include ganglioside concentrations at which gangliosides are present as free monomers (MW 1500-1800 daltons), namely an assumed critical micellar concentration of 10^{-4} M for the ganglioside mixture (13,14), and not higher than 10^{-5} M for ganglioside GM_1 (unpublished results). The presence of salts (KCl) did not significantly effect the behavior of gangliosides during dialysis. Experiments in which solutions of gangliosides in 0.1 M aqueous KCl were dialyzed against the same 0.1 M KCl did not show passage of gangliosides across the dialysis sac either.

Conversely, when gangliosides, both pure GM_1 and the mixture, were dissolved in methanol-0.1 M aqueous KCl, 1:1, and submitted to dialysis, an appreciable loss of ganglioside occurred (Fig. 2). The loss was higher

(ca. 50%) at the lowest ganglioside concentrations. Maximum loss was reached after dialysis for 1 day, and no additional losses occurred thereafter. Under our experimental conditions, methanol contained in the starting solution disappeared from the sac after overnight dialysis and was recovered in the dialyzate. We assume that the loss of gangliosides from the sac ceased after methanol had been removed. In water solution, the ganglioside molecules in the monomeric state apparently interact with the dialysis membrane, which does not take place in the presence of polar organic solvents.

The distribution of individual ganglioside components in the ganglioside mixture did not change during dialysis. Even after dialysis for 4 days, no degradation of the ganglioside was observed.

In conclusion, gangliosides dissolved in water or in low ionic strength aqueous solutions do not dialyze through common cellulose dialysis tubings, even as free monomeric molecules. This is also true for long periods of dialysis (3-4 days) as they are commonly used in ganglioside purification. Because the presence of polar organic solvents such as methanol causes ganglioside loss on dialysis, it is advisable to remove any traces of organic solvents from samples prior to submitting them to dialysis.

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Fatty Acid Composition of Unfertilized and Fertilized Eggs of the Sea Urchin, *Arbacia punctulata*

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ABSTRACT

Fatty acid compositions of polar lipids and triacylglycerols isolated from eggs of the sea urchin, *Arbacia punctulata,* were examined before and 15 min after insemination. The main fatty acids present in polar lipids and triacylglycerols were palmitate, palmitoleate, eicosaenoate, arachidonate and eicosapentaenoate, while palmitate and palmitoleate predominated in triacylglycerols. There were no significant changes in weight percentrage of individual fatty acids of either polar lipids or triglycerides of eggs after insemination.

INTRODUCTION

Minutes after fertilization, sea urchin eggs undergo striking structural and metabolic changes. Some of these sequelae may be due to alterations in egg lipid metabolism, including the change in plasma membrane fluidity (1), and the postfertilization burst of oxygen consumption (2). Although the total lipid content of sea urchin eggs does not change to any appreciable extent until the mesenchyme blastula stage (3,4), turnover of certain lipid species may be stimulated (5,6). There is also some suggestion that the fatty acid composition of egg lipids changes during early stages of development (7). To determine whether egg lipids are altered soon after fertilization, we examined fatty acid patterns of polar lipids and triacylglycerols from eggs of *Arbacia punctulata* before and 15 min following insemination.

MATERIALS AND METHODS

Sea urchins of the species *A. punctulata* were studied at the Marine Biological Laboratories, Woods Hole, MA during the summer of 1977. Eggs shed following injection of 0.5 M KCl into the body cavity were washed with filtered sea

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TABLE I

Fatty Acid Composition of Unfertilized and Fertilized *A. Punctulata* Eggs % by wt ± S.D.

	Phospholipids (N=5)			Triacylglycerols (N=6)	
Fatty acid	Unfertilized	Fertilized	Fatty acid	Unfertilized	Fertilized
16:0[a]	10.63 ± 4.26	12.64 ± 2.79	14:0	4.37 ± 4.57	3.22 ± 3.23
16:1	1.41 ± 1.26	1.43 ± 1.19	14:1	0.55 ± 0.94	0.90 ± 0.73
16:2	1.78 ± 0.89	1.37 ± 0.41	15:0	1.65 ± 1.58	1.97 ± 1.61
16:3, 17:0?	2.29 ± 1.85	1.56 ± 1.09	15:1	0.61 ± 0.69	1.54 ± 1.75
16:4?	2.95 ± 2.24	4.38 ± 1.39	16:0	21.98 ± 6.43	18.22 ± 3.81
18:0	5.41 ± 1.56	5.64 ± 1.57	16:1	12.48 ± 1.75	11.44 ± 3.68
18:1	3.52 ± 0.78	4.16 ± 1.35	16:2	4.54 ± 1.87	2.95 ± 1.97
18:2	3.55 ± 1.27	2.77 ± 1.85	18:0	3.90 ± 0.21	4.56 ± 0.86
18:3	0.34 ± 0.54	0.36 ± 0.87	18:1	8.00 ± 4.04	8.21 ± 2.04
18:4	7.50 ± 3.31	6.85 ± 1.53	18:2	0.70 ± 0.41	3.16 ± 4.98
20:1	10.33 ± 2.38	9.09 ± 3.27	18:3	1.93 ± 0.56	1.56 ± 0.90
20:2	2.70 ± 3.12	2.55 ± 1.86	18:4	2.73 ± 0.96	2.46 ± 0.60
20:4	19.94 ± 2.26	21.37 ± 1.07	20:0	2.03 ± 0.95	2.19 ± 1.02
20:5	15.10 ± 2.84	17.37 ± 1.96	20:1	7.96 ± 1.35	9.30 ± 1.87
22:1	1.88 ± 3.65	0.39 ± 0.96	20:2	2.10 ± 0.95	2.58 ± 0.93
22:5	1.53 ± 2.67	Trace	20:4	5.85 ± 1.46	6.53 ± 1.24
22:6	5.76 ± 2.50	4.39 ± 2.35	20:5	8.69 ± 1.87	9.47 ± 1.26
24:1	2.46 ± 3.53	0.89 ± 1.21	22:4	0.56 ± 0.76	0.68 ± 0.59
			22:5	0.60 ± 0.69	0.10 ± 0.25
			22:6	6.45 ± 1.26	7.30 ± 0.92
			24:1	0.74 ± 0.82	1.19 ± 0.63

[a]no. carbon atoms: no. of double bonds.

water and passed through a 125 mesh nylon screen to remove the jelly coat. Two percent suspensions (v/v) of eggs in filtered sea water were fertilized with fresh sperm or incubated without sperm at 18 C. After 15 min, eggs were pelleted by centrifugation at 500 x g for 1 min, washed three times with filtered sea water, then extracted with chloroform-methanol (2:1, v/v) according to the method of Folch et al (8). More than 95% of eggs in the inseminated vessels had been fertilized as judged by elevation of fertilization envelopes.

Lipid extracts were washed with 0.2 vol of 0.05% $CaCl_2$, and the resulting lower phases were washed two additional times with Folch "upper phase." Polar lipids, triacylglycerols and sterol esters were isolated by thin layer chromatography (9), and fatty acid methyl esters were prepared by transesterification of lipid fractions in methanolic-HCl (9). A Perkin-Elmer 940 gas liquid chromatograph equipped with flame ionization detector and 1.8 m stainless steel column (3.2 mm OD) packed with 10% EGSS-X on Gas Chrom P (100/120 mesh) was used routinely to analyze fatty acid compositons. Column temperature was programmed between 170-195 C with temperature increasing 1 C/min starting 2 min after sample injection. A Packard chromatograph equipped with flame ionization detector and 1.8 m glass column (2 mm ID) packed with 10% SP-2330 on Chromosorb WAW (100/120 mesh) (Supelco, Bellefonte, PA), run isothermally at 185 C, was used to confirm identity of fatty acid methyl esters. Fatty acids were identified by comparing relative retention times with those of authentic standards and by plots of log retention times vs. carbon number. Peak areas were determined by triangulation. Statistical analysis was carried out using the *t*-test for paired samples.

RESULTS AND DISCUSSION

The predominant fatty acids in polar lipids and triacylglycerols of *A. punctulata* eggs were palmitate, palmitoleate, eicosaenote, arachidonate and eicosapentaenoate (Table I). These are the main fatty acids present in eggs and gonads of other species (4,7,10,11). Polar lipids were enriched with arachidonate and eicosapentaenoate, while the triacylglycerols were enriched with palmitate and palmitoleate. The sterol esters, which are only a minor component of sea urchin egg lipids (3,4), contained palmitate (16.6% by wt), oleate (16.3% by wt), eicosapentaenoate (12.7% by wt), docosahexaenoate (10.9% by wt), eicosaenoate (8.9% by wt) and stearate (8.3% by wt) as the main fatty acids. The quantities of free fatty acid and partial glycerides present in egg extracts were insufficient for fatty acid analyses.

No significant differences were found in weight percentages of individual fatty acids in either the polar lipids or triaclyglycerols when unfertilized and fertilized eggs were compared. ($p>0.05$). Kozhina et al. (4) and Chelomin et al. (10) found what they considered only minor changes in fatty acid compositions of *Strongylocentrotus intermedius* eggs following fertilization. In contrast, Barber and Mead (7) reported that there was a decline in the weight percentage of saturated species in triglycerides and an increase in the percentage of saturated fatty acids in polar lipids of membrane ghosts from *Strongylocentrotus purpuratus* eggs after fertilization. However, no statistical analysis of these changes was provided. Our study demonstrates that the fatty acid composition of sea urchin eggs is not acutely changed following fertilization, despite the dramatic metabolic alterations which occur during this time (12).

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15-Methyl-1,2-hexadecanediol, a Major Constituent of Hamster Surface Wax

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ABSTRACT

Long chain 1,2-alkanediol diesters comprise about 15-20% of the acetone soluble skin surface wax of golden Syrian hamsters. The constituent 1,2-alkanediols, obtained through acidic methanolysis, were fractionated by preparative gas liquid chromatography of their isopropylidene derivatives. The major component (57%) was identified by nuclear magnetic resonance and mass spectrometry as 15-methyl-1,2-hexadecanediol.

INTRODUCTION

The lipid biochemistry of mammalian sebaceous glands is of interest because these glands produce diester waxes and other lipids not commonly found in mammalian cells (1). It has long been known that the constituent 1,2-alkanediols of wool wax (2,3) and other mammalian surface lipids (4-6) contain branched chain compounds. Although early investigations of wool wax have shown the presence of both *iso-* (2) and *anteiso-* (3) compounds, the structures of most branched chain alkanediols of mammalian diol diesters remained unidentified (1,4-6). We now report the positive identification of 15-methyl-1,2-hexadecanediol and a series of other *iso*-branched diols as constituents of hamster diol diester wax.

EXPERIMENTAL PROCEDURES

Surface wax (20-25 mg per hamster) was obtained by dipping golden Syrian hamsters into acetone. After removal of the solvent under reduced pressure, the diol diesters were isolated by preparative thin layer chromatography (TLC) on 0.5 mm layers of Silica Gel H (Merck) using hexane-benzene (1:1, v/v); the fraction was eluted from the adsorbent with diethyl ether and repurified by TLC with hexane-benzene (3:2, v/v). The diol diesters were hydrolyzed in HCl/methanol (5% w/v) at 80 C for 3 hr, and the diols were isolated by preparative TLC using hexane-diethyl ether (1:1, v/v). They were converted to their isopropylidene derivatives by reaction with anhydrous acetone at room temperature for 3 hr in the presence of catalytic amounts of perchloric acid. The acetone solution was applied to layers of Silica Gel H and the reaction products isolated by chromatography in hexane-diethyl ether (9:1, v/v).

Analytical gas liquid chromatography (GLC) was done with a Varian Aerograph 1200 equipped with flame ionization detector. A 6' x 1/8" aluminum column packed with 10% EGSS-X on Gas Chrom P, 100-120 mesh and an 8' x 1/8" column of 3% OV-101 on 100-120 mesh Gas Chrom Q (Applied Science Laboratories, Inc, State College, PA) were operated isothermally at 180-210 C. For preparative GLC a Victoreen 4000 instrument equipped with thermoconductivity detector and heated outlet was used with a 4' x 1/4" column packed with 10% OV-101 on 80-100 mesh Supelcoport (Supelco, Inc, Bellefonte, PA), at 235 C.

^{1}H-NMR spectra were recorded with a Varian CFT-20 instrument operating at 79.54 MHz. $CDCl_3$ was used as solvent and for field-frequency locking. Chemical shifts are expressed in ppm downfield from internal TMS (δ

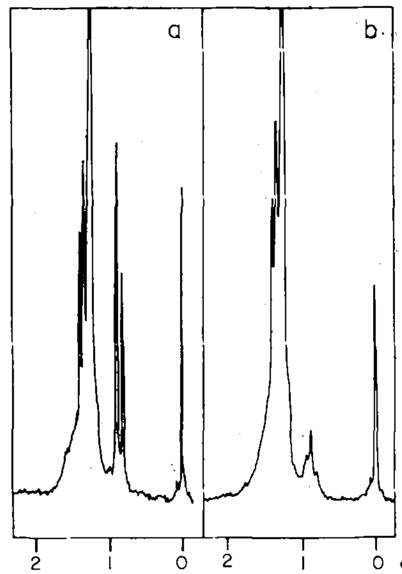

FIG. 1. Diagnostic regions in the ^{1}H-NMR spectra of isopropylidene derivatives of (a) 15-methyl-1,2-hexadecanediol (hamster) and (b) *n*-heptadecanediol (synthetic).

TABLE I

Relative Retention Times of Methyl Esters of Fatty Acids (Me) and Isopropylidene Derivatives of 1,2-Alkanediols (IPD)

No. of C-atoms[a]	EGSS-X (180 C)		OV-101 (200 C)	
	Me	IPD	Me	IPD
n 16	1.00	1.00	1.00	1.00
i 17	---	1.15	---	1.25
a 17	1.23	---	1.28	---
n 17	1.32	1.33	1.40	1.40
i 18	1.52	1.54	1.74	1.74
n 18	1.75	1.76	1.97	1.96
i 19	---	2.05	---	2.44
a 19	2.13	---	2.52	---
n 19	2.32	2.34	2.74	2.74
i 20	2.64	2.69	3.43	3.42
n 20	3.05	3.06	3.86	3.84
i 21	---	3.51	---	4.74
a 21	3.71	---	4.92	---
n 21	4.02	4.05	5.32	5.33

[a]n = normal, i = iso-branched, a = anteiso-branched.

0.0). Mass spectra were taken with a Hitachi Perkin-Elmer RMU-6D instrument equipped with the direct insertion system M6-150; ionization potential was 70 eV.

RESULTS AND DISCUSSION

During structural analyses of mammalian diester waxes (7), we observed two series of homologous 1,2-alkanediols as constitutents of hamster surface wax. Analysis by GLC of their isopropylidene derivatives showed one major straight chain constituent, n-hexadecanediol (16.2% of the total diols), and a branched chain constituent tentatively identified as 15-methyl-1,2-hexadecanediol (57.4%); other minor components amounted to less than 5% each of the diols (7). The branched chain component was isolated by preparative GLC and identified by its NMR spectrum (Fig. 1).

In the nuclear magnetic resonance (NMR) spectrum, the methyls of the terminal isopropyl group appeared as a distinct doublet centered at 0.86 δ, J = 6.2 Hz. In contrast, the terminal methyl group of the isopropylidene derivative of a synthetic (8) 1,2-*n*-heptadecanediol appeared as an apparent triplet at 0.88 δ. In each case the isopropylidene methyl groups characteristically appeared as two singlets at 1.35 and 1.39 δ due to their different environments. The mass spectrum of the isopropylidene derivative of the branched chain diol was of little value in establishing its structure but confirmed its molecular weight. As expected (9), the only significant peak (56%) in the high mass unit region was at m/e 297 (m-15) due to loss of a methyl group from the dioxolane ring. Essentially the same spectrum was obtained from the straight chain isomer.

In early work, Horn and Hougen (2) isolated straight chain and *iso*-branched 1,2-alkanediols from unsaponifiable matter of wool wax by distillation and crystallization and identified them by comparison with authentic standards and by degradation to fatty acids of known structure. Later, Downing et al. (3) prepared a fraction of 1,2-alkanediols from the same material by chromatography and converted the diols to hydrocarbons. GLC analysis revealed mostly *iso*- and *anteiso*-branched structures (3).

In our work on diol diesters of hamsters and mice (7), we did not detect *anteiso*-branched 1,2-alkanediols. Their isopropylidene derivatives should be separable by GLC from those of straight chain and *iso*-branched isomers as are those of fatty acid methyl esters (10). We observed that relative retention times in GLC within series of straight chain and branched chain methyl esters were very similar to those within the corresponding series of isopropylidene derivatives. Such data are compared in Table I. Thus, GLC retention times of methyl esters of straight chain, *iso*- and *anteiso*-branched fatty acids can aid in the identification of diols.

The predominance of 15-methyl-1,2-hexadecanediol as a constituent of hamster surface diol diesters is of biochemical interest. It is generally believed that mammalian diester waxes are produced by the sebaceous glands rather than by the epidermis or by microorganisms (11). The 1,2-alkanediols are probably

derived from the corresponding 2-hydroxy fatty acids (12) by enzymic reduction. However, neither 15-methylhexadecanoic acid nor its 2-hydroxy analog are major components of hamster surface wax (7).

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Alpha Tocopherol Levels in Various Regions of the Central Nervous Systems of the Rat and Guinea Pig

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ABSTRACT

The alpha tocopherol contents of various discrete anatomical regions in the central nervous system of adult rats and guinea pigs were assayed using a liquid chromatographic method. All parts of the guinea pig nervous system had lower alpha tocopherol contents per gram wet, dry or lipid weights than the corresponding areas in the rat. In both animals the distribution of alpha tocopherol did not correspond to the distribution pattern of total lipid. There was also a rostral to caudal concentration gradient with respect to alpha tocopherol content; gray matter from cerebral hemisphere has the highest concentration and cervical spinal cord the least. In both animals alpha tocopherol contents per gram dry weight or lipid weight were higher in gray matter areas when compared with white matter areas. The low concentration of alpha tocopherol in spinal cord could make this region more susceptible to damage from deficiency than the rest of the central nervous system.

INTRODUCTION

A deficiency of vitamin E in animals is known to result in several pathological changes in the central nervous system. A detailed morphological study of the effect of vitamin E deficiency on the nervous systems of various laboratory animals was done by Einarson and Telford (1). They found from their study of suckling rats, adult rats, mice, guinea pigs, rabbits and monkeys that the nerve cells undergo pathological changes as a result of vitamin E deficiency.

Several investigators have observed the occurrence of neuroaxonal dystrophy in the brains of rats deficient in vitamin E (1-4). In the adult rat, both demyelination and axonal lesions were observed in the posterior fasciculi. The accumulation of lipofuscin pigment in the neurons of vitamin E deficient rats is also a well known phenomenon (1). The mechanism of production of such pathological changes and a potential role for vitamin E in nervous system function is still unknown. One of the initial steps in understanding the neurochemistry of vitamin E is to determine the distribution of the substance in various areas of the nervous systems of different species of experimental animals. This paper reports results of studies on the steady state levels of alpha tocopherol, the most active of all the naturally occurring tocopherols, in different areas of the central nervous systems of adult rat and guinea pig.

MATERIALS AND METHODS

Male Sprague-Dawley rats were raised on normal control diets. Four rats weighing 350-400 g were sacrificed by decapitation after overnight fasting, and the brains were carefully removed. The following regions were dissected out as described by Glowinski and Iversen (5): cerebellum, medulla oblangota including the pons, thalamus and subthalamus plus caudate (head). After these regions were removed under a dissecting microscope, the corona radiata was followed and the white matter removed from the cerebral hemispheres. A sample of the remainder of the cortex was taken as gray matter. A sample of cervical spinal cord also was obtained. Brain and cervical spinal cord samples from four adult guinea pigs raised on normal guinea pig diet were similarly dissected out.

The dissected nervous tissue samples were immediately frozen in liquid nitrogen and kept frozen at -70 C. All analyses were conducted within 3 weeks. Each sample was divided into two roughly equal parts. One portion was used for determination of the alpha tocopherol content, and the other was used for the determination of water content by dessication of the samples to constant weight under vacuum (6). Alpha tocopherol levels were determined by a liquid chromatographic procedure developed in this laboratory (7). Using corresponding samples from similar adult rats and guinea pigs, total lipid contents were determined gravimetrically after chloroform-methanol extractions of the tissue samples (8).

RESULTS AND DISCUSSION

The alpha tocopherol content of the various

TABLE I

Alpha Tocopherol Levels in Various Areas of the Central Nervous Systems of the Rat[a] and Guinea Pig[a] Expressed as Micrograms Per Gram Wet, Dry and Lipid Weights

Areas analyzed	Micrograms alpha tocopherol/gram					
	Wet weight		Dry weight		Lipid weight	
	Rat	Guinea pig	Rat	Guinea pig	Rat	Guinea pig
	mean ± S.E.	mean ± S.E.	mean ± S.E.	mean ± S.E.	mean ± S.E.	mean ± S.E.
Gray matter from cerebral hemispheres	13.2 ± 0.2 (13.0 – 13.6)[b]	9.4 ± 0.3 (8.8 – 10)[b]	64 ± 0.4 (63 – 65)[b]	48 ± 1.3 (45 – 51)[b]	169 ± 1.7 (165 – 174)[b]	141 ± 3.9 (131 – 150)[b]
Thalamus plus head of caudate nucleus	12.1 ± 0.3 (11.5 – 12.6)	9.4 ± 0.2 (8.9 – 9.8)	53 ± 0.9 (51 – 55)	42 ± 1.0 (40 – 45)	116 ± 2.3 (111 – 121)	119 ± 2.5 (113 – 124)
Cerebellum	9.2 ± 0.1 (9.0 – 9.5)	7.0 ± 0.1 (6.8 – 7.3)	42 ± 0.7 (41 – 44)	31 ± 0.6 (30 – 33)	97 ± 1.2 (95 – 100)	86 ± 1.3 (84 – 90)
White matter from cerebral hemispheres	13.4 ± 0.2 (13.1 – 13.9)	9.5 ± 0.5 (8.3 – 10.6)	50 ± 0.8 (48 – 52)	32 ± 1.1 (29 – 35)	104 ± 1.5 (102 – 108)	76 ± 3.8 (66 – 85)
Medulla plus pons	12.4 ± 0.2 (11.8 – 12.8)	8.2 ± 0.3 (7.9 – 8.9)	44 ± 0.7 (42 – 45)	30 ± 1.2 (29 – 34)	79 ± 1.5 (76 – 82)	69 ± 2.1 (66 – 75)
Cervical spinal cord	11.1 ± 0.2 (10.8 – 11.4)	5.4 ± 0.2 (5.0 – 5.7)	32 ± 0.9 (30 – 34)	17 ± 0.6 (16 – 18)	53 ± 0.7 (52 – 55)	34 ± 1.1 (31 – 36)

[a]Tissues from four animals were used.

[b]The values within brackets give the range of results obtained.

TABLE II

Statistical Analysis of the Difference in Mean Alpha Tocopherol Concentrations in Micrograms per Gram Dry Weight Among Some of the Areas in the Central Nervous Systems of the Rat and Guinea Pig

Neuroanatomical areas compared	P value for rat	P value for guinea pig
Gray vs White	<0.005	<0.005
Gray vs thalamus and caudate	<0.005	Not significant
Gray vs cerebellum	<0.005	<0.005
Gray vs medulla and pons	<0.005	<0.005
Gray vs spinal cord	<0.005	<0.005
White vs thalamus and caudate	Not significant	<0.01
White vs cerebellum	<0.01	Not significant
White vs medulla and pons	<0.02	Not significant
White vs spinal cord	<0.001	<0.005
Thalamus and caudate vs medulla and pons	<0.01	<0.005
Thalamus and caudate vs cerebellum	<0.005	<0.005
Thalamus and caudate vs spinal cord	<0.001	<0.005
Medulla and pons vs spinal cord	< 0.001	<0.005
Cerebellum vs medulla and pons	<0.05	Not significant
Cerebellum vs spinal cord	<0.001	<0.001

areas of the central nervous systems of rats and guinea pigs expressed as micrograms per gram wet, dry and lipid weights are given in Table I. In both animal species, there is a roastral to caudal gradient in alpha tocopherol levels. Results of statistical comparisons of mean alpha tocopherol levels per gram dry weight of different areas of the central nervous systems of rat and guinea pig are given in Table II. Similar comparisons of alpha tocopherol levels per gram lipid showed significant differences in all cases except white matter vs. medulla pons in the guinea pig.

An examination of Table I reveals that there is no neuroanatomical region in either the rat or guinea pig which has a particularly high concentration of alpha tocopherol with respect to the rest of the brain. This contrasts with the levels of a transmitter substance like dopamine, which is highly concentrated in the striatum (9). The wide spread distribution pattern of alpha tocopherol in the central nervous systems of these animals suggests that the compound is functionally involved with the general metabolic integrity of brain.

Vitamin E deficiency results in a wide spectrum of pathological changes which are species specific (10). Myopathy, which is one of the most widely occurring changes due to vitamin E deficiency, responds to treatment with vitamin E to varying degrees depending upon species (11). Telford (11) points out that muscles of most animals regenerate quite readily following tocopherol therapy, whereas in man or adult rat, the myopathy is irreversible. The role of the nervous system in the pathogenesis of such nutritional myopathy has not been elucidated. Hence, a comparison of the regional distribution of alpha tocopherol in the central nervous systems of adult rats and guinea pigs was performed. In general, the patterns of distribution of alpha tocopherol in both species are very similar. In both species gray matter areas have higher levels of alpha tocopherol per gram dry weight than white matter areas. This observation is especially interesting since Einarson and Telford (1) found that nerve cells are primarily the site of pathology in the central nervous systems of vitamin E deficient animals, indicating that alpha tocopherol plays a functional role in maintaining the structural and/or metabolic integrity of the nerve cell body. In both rats and guinea pigs, gray matter from the cerebral hemispheres has the highest level of alpha tocopherol per gram dry weight or lipid weight. Levels of tocopherol in guinea pig brain are generally lower than those of rat brain, and the alpha tocopherol level in the guinea pig spinal cord is the lowest compared with all other central nervous system areas. Interestingly, Einarson and Telford (1) found that, in guinea pigs with pronounced vitamin E deficiency, the neuropathological changes which could be characterized as liquifaction or disso-

lution of the nerve cells were most marked in the cervical and lumbo-sacral enlargements of the spinal cord. Recent studies in this laboratory using human autopsy brain have shown that the spinal cord alpha tocopherol levels are the lowest among all the central nervous system regions examined (12).

ACKNOWLEDGMENTS

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The Optical Rotation of a Major Component of Plant Cutin[1]

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ABSTRACT

The major component of cutin from the fruit of both tomato and papaya, dihydroxypalmitate, is shown to have plain positive rotation and is, therefore, assigned L configuration in analogy to other known hydroxy fatty acids.

Cutin, a biopolyester, which covers the aerial surfaces of all plants, is composed of long chain hydroxy and epoxy fatty acids (1). The most common major monomer of cutin is a mixture of dihydroxypalmitic acids which have one hydroxyl moiety on C-16 and a second hydroxyl group on either carbon 7,8,9, or 10 (2,3). Dihydroxypalmitate from cutin is usually a mixture of positional isomers present in a ratio characteristic of its source (4). This note is the first report that these dihydroxypalmitates are optically active.

Cutin was isolated from the fruits of tomato and papaya as previously reported (3,5). Following depolymerization of the cutin with 14% boron trifluoride in methanol (6), the methyl dihydroxypalmitate fraction was isolated by silica gel column chromatography (3.5 x 68 cm, ethyl ether-hexane-methanol, 10:5:1), and UV absorbing material was removed with the aid of decolorizing carbon to yield a pure fraction as shown by gas liquid chromatography (GLC) on 5% OV-1 (yield 20% and 40% by weight from tomato cutin and papaya cutin, respectively). The purified mixture of methyl esters was derivatized with N,O-*bis*-(trimethylsilyl)-acetamide, and the composition of isomers was estimated from the relative intensities of the α-cleavage fragments generated in the gas chromatography/mass spectrometry (GC/MS) (6). The relevant portions of these mass spectra and the major fragments expected from the predominant isomers are shown in Figure 1. The dihydroxypalmitate fraction isolated from tomato cutin contained 79% 10,16-, 12% 9,16-, and 9% 8,16-positional isomers. On the other hand, the dihydroxypalmitate from papaya

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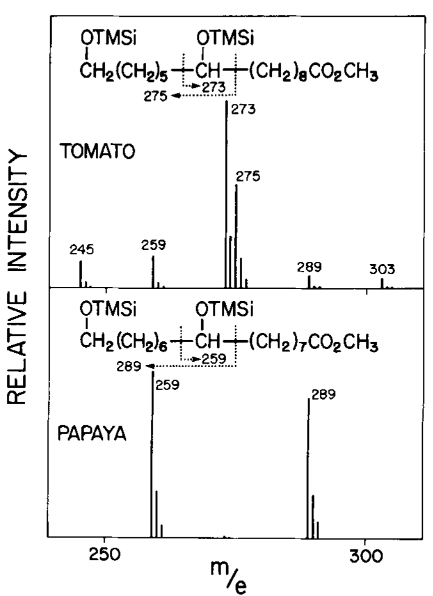

FIG. 1. Partial mass spectra of trimethylsilyl derivatives of methyl dihydroxypalmitates from cutin of tomato and papaya fruit.

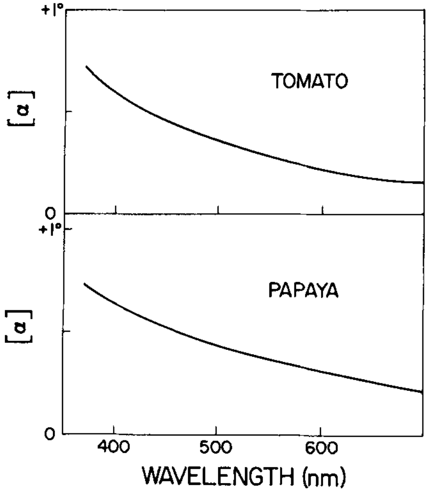

FIG. 2. ORD spectra of methyl dihydroxypalmitates from cutin of tomato (C = 13.0, $CHCl_3$) and papaya (C = 12.7, $CHCl_3$) fruit.

cutin contained 98% 9,16- and 2% 10,16-dihydroxy acids. This high isomeric purity is unique among cutins which have been examined and is the reason that papaya was chosen for this study (3). Optical rotary dispersion (ORD) spectra of the two methyl dihydroxypalmitate fractions were recorded on a Jasco Model ORD/UV-5, and the results are given in Figure 2. The tomato and papaya fractions gave an $[\alpha]_D^{20}$ of +0.26 C and +0.30 C, respectively. Although these methyl dihydroxypalmitates clearly gave a plain positive ORD spectra, their absolute configuration cannot be firmly assigned. However, the rotation of a series of analogous compounds from seed oils (7,8) would indicate that the conformation of dextrarotatory mid chain hydroxylated fatty acids is *L*, and therefore it appears that the dihydroxypalmitate of cutin from papaya and tomato fruits is of *L* configuration. It seems clear that one of the critical enzymes involved in cutin biosynthesis, ω-hydroxypalmitate hydroxylase (9,10), acts in a stereospecific manner.

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Rapid Transmethylation of Microgram Amounts of Phosphatidylcholine on Potassium Methoxide/Celite Columns[1,2,3]

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ABSTRACT

A rapid and convenient method for the determination of acyl groups in phosphatidylcholine (PC) has been developed. Transmethylation reactions were carried out on potassium methoxide-impregnated Celite microcolumns that were readily prepared from Pasteur pipettes. The methods were tested on microgram amounts of synthetic L-α-phosphatidylcholines (di-14:0; di-18:2; α-16:0-β-14:0) and on egg yolk lecithin, in methylene chloride or hexane solution. Transmethylation of these lipids occurred rapidly at room temperature. Gas liquid chromatographic (GLC) analysis of the product methyl esters demonstrated that the reaction was neither selective for one acyl position of PC over the other, nor sensitive to the amount of unsaturation within the acyl group. The results of the acyl group analysis of natural egg yolk lecithin compared favorably with the results from an established procedure.

INTRODUCTION

This paper addresses the analysis of the acyl groups of phospholipids and, in particular, the largest subclass, phosphatidylcholine (PC). In standard procedures (1-3), methyl esters for gas liquid chromatographic analysis (GLC) are generated from milligram amounts of PC in methanol in the presence of basic or acidic catalysts. Especially rapid transesterification occurs in a solution of sodium or potassium methoxide in methanol (4-6). The ester products then are extracted from the reaction medium, concentrated by solvent evaporation, and finally injected onto a GLC column.

In earlier research with neutral lipids, Schwartz demonstrated the advantages of derivatization via microcolumn procedures (e.g., 7-10), including the feasibility of transmethylation of microgram amounts of triglycerides on a potassium methoxide/Celite microcolumn (11). The present research was undertaken to (a) expand upon the microcolumn transmethylation technique, (b) refine the microcolumn preparation procedure in order to facilitate column packing and impart long term stability and storability, and (c) demonstrate the effectiveness of the technique with regard to polar lipids and, particularly, PC. The following results with PC demonstrate the convenience and simplicity of this transmethylation method. A newly developed, prepacked and disposable microcolumn need only be snapped open and treated with a few micrograms of lipid, such as isolated from a thin layer chromatographic (TLC) spot, and then eluted of product esters for GLC analysis.

EXPERIMENTAL PROCEDURES

Reagent on Celite

Celite diatomaceous earth (6.0 g analytical grade, Fisher Scientific Co., Pittsburgh, PA) was ground thoroughly and rapidly with powdered potassium methoxide (1.0 g, Ventron Corp., Danvers, MA). The resulting mixture was transferred into a screw cap vial.

Column Preparation

The Schwartz procedure (11) was modified to simplify column preparation and to maintain reagent activity over prolonged storage conditions. The new procedure is outlined in Figure 1. A glass capillary (i.d. 11.5 mm) with an attached funnel was obtained by shortening a 9 in. Pasteur pipette. The residual wide end was sealed at one end and used later as a disposable collection vessel for column eluate. A small plug of glass wool was inserted into the capillary via the funnel end by means of forceps and a wire tamper. Discarded plungers from microliter syringes were convenient tampers. Anything introduced into the capillary must not be contaminated with fingerprints, which are a rich source of lipids in microgram quantities. The plugged capillaries were dried in an oven at 120 C and then transferred into a dry

[1]Presented at AOCS 68th Annual Meeting, New York, May 1977.

[2]Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

[3]Chloroform and carbon disulfide have been classified by OSHA as carcinogens and/or toxic materials; they should be handled with care under proper ventilation.

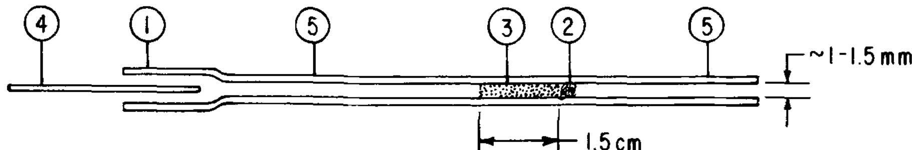

FIG. 1. Microcolumn preparation.

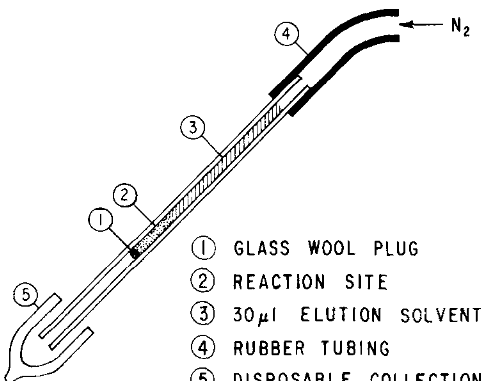

FIG. 2. Elution of reaction products.

box for subsequent packing. Sufficient amounts of the reagent/Celite mixtures were added via the funnel ends so that a 1.5 cm column resulted after subsequent tamping with the wire plunger. Finally the dry columns were removed from the dry box and immediately sealed at both ends by means of a small flame. The resulting sealed columns (9 cm long) were stored in vials at room temperature for future use.

Lipid Solutions

The following synthetic L-α-phosphatidyl cholines were used as model compounds: α-palmitoyl-β-myristoyl- (Supelco Inc., Bellefonte, PA), dimyristoyl- (Sigma Chemical Co., St. Louis, MO), and dilinoleoyl- (PL Biochemicals, Inc., Milwaukee, WI). Purity was ascertained by silica gel TLC using each of two solvent systems (chloroform[3]-methanol-acetic acid-water, 25:15:4:2 and chloroform-methanol-ammonium hydroxide 10:5:2, by volume). Egg yolk lecithin (Sigma Chemical Co., St. Louis, MO) served as a natural PC. Solutions of these lipids were prepared in methylene chloride or hexane (Nanograde, Mallinckrodt, Inc., St. Louis, MO) to contain 2.5 μg/μl. Individual solutions of $(14{:}0)_2$ PC and $(18{:}2)_2$ PC were analyzed for their lipid content by quantitative determination of their phosphorus content (12). These solutions then were combined to give a new solution with equal weights of each lipid.

Potassium Methoxide Reactions

A prepacked potassium methoxide/Celite microcolumn was scored and snapped open at both ends. The PC solution (1 to 10 μl) was introduced with a 10 μl syringe. The open column ends then were sealed temporarily with Critoseal vinyl plastic putty (made for sealing microhematocrit tubes, A.H. Thomas Co., Philadelphia, PA). Two to 6 min later the putty-sealed ends were scored and snapped off, and the methyl esters were eluted with methylene chloride or carbon disulfide (30 μl), as depicted in Figure 2. Elution was expedited by application of nitrogen under pressure. Prolonged contact with the potassium methoxide led to reduced yields, probably due to slow concomitant saponification (13). The eluate was collected in a small disposable vessel made from the cut-off wide end of a Pasteur pipette or in some cases simply removed from the lower end of the column with a 10 μl syringe. The eluate was then introduced into the GLC inlet. Prior concentration by evaporation was avoided (*cf.* below). In the case of one 2 min reaction, the column was acidified by drawing hydrogen chloride vapor over it, then eluted

TABLE I

Transmethylation of Synthetic PC by Microcolumn[a]

Reaction	Normalized values			
	14:0	16:0	18:2	Standard deviation
α-16:0-β-14:0 in CH_2Cl_2, 6 min, 25 C	47.0	53.0	---	±1.5
Calculated FID response[b]	46.7	53.3	---	---
Equal weights of $(14{:}0)_2$ PC and $(18{:}2)_2$ PC in hexane, 2 min, 25 C	48.6	---	51.4	±1.3
Calculated FID response[b]	48.8	---	51.2	---

[a]25 μg in 10 μl solvent.
[b]Flame ionization detector, cf text (14,15).

TABLE II

Transmethylation of Egg Yolk Lecithin by Microcolumn

Ester[a]	Normalized values by KOMe/Celite method[b]	Method of Luddy et al.[c]
16:0	33.16 ± 0.61	33.29 ± 0.53
16:1	1.18 ± 0.10	1.12 ± 0.06
18:0	14.59 ± 1.41	13.72 ± 0.73
18:1	28.69 ± 1.27	30.54 ± 0.24
18:2	15.58 ± 0.73	15.94 ± 0.18
20:4	4.58 ± 0.82	3.75 ± 0.29
Unidentified	1.18 ± 0.18	0.75 ± 0.13
22:6	1.12 ± 0.09	0.90 ± 0.19

[a]In order of appearance.
[b]Mean ± standard deviation for 4 runs @ 30 μg lipid, 6 min, 25 C.
[c]1 run @ 2 mg lipid, 4 aliquots for GLC (5).

with methylene chloride and then methanol. TLC analysis of the eluate showed the absence of PC and its monoacylated hydrolysis product, lysophosphatidylcholine.

Gas Liquid Chromatography

GLC separations of methyl myristate from either methyl palmitate or methyl linoleate were achieved on a Hewlett-Packard 5750 instrument equipped with a flame ionization detector (FID). An EGA/phosphoric acid column was used (7.5% stabilized ethylene glycol adipate and 2% phosphoric acid on 90-100 mesh Anakrom ABS; 8 ft by 1/8 in. silanized stainless steel). The column was maintained at 130 C for 4 min and then programmed up to 200 C at 6°/min. Separation of the complex mixtures of methyl esters derived from transmethylation of egg yolk lecithin was done on a Perkin-Elmer Sigma 3 gas chromatograph in the FID mode. In that case, separations were achieved on a wall-coated, open tubular, 50 meter glass capillary column with a stationary phase of diethylene glycol succinate (Perkin-Elmer No.009-7765). The column was maintained at 140 C for 4 min and then programmed up to 190 C at 4°/min. Peak areas were determined with a Supergrator 2 programmable computing integrator (Columbia Scientific Industries, Austin, TX). Integral values of FID-generated signals were considered to be proportional to the number of noncarbonyl carbon atoms; for long chain fatty esters, these values are approximately proportional to the mass (14,15). Retention times were compared to those of standard esters (Nu-Chek Prep., Inc., Elysian, MN).

Evaporation Experiment

The α-16:0-β-14:0 PC (25 μg) in methylene chloride (10 μl) was allowed to react on a potassium methoxide/Celite microcolumn in the usual way. The eluate was collected, and an aliquot was immediately withdrawn and analyzed by GLC. The remainder was exposed to a constant stream of nitrogen; at intervals of 2, 4, and 6 min, the solution was reconstituted by addition of methylene chloride, and aliquots were injected onto the GLC column.

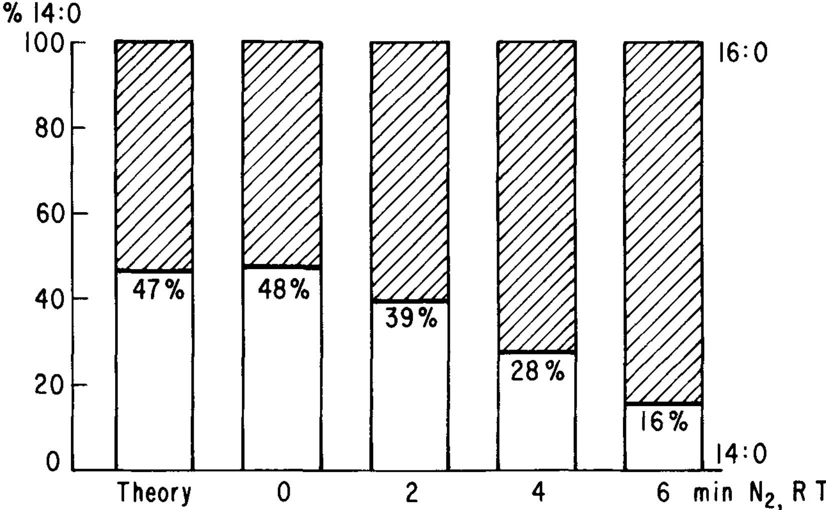

FIG. 3. Effect of N_2 evaporation on GLC results. (Reaction of 25 mg model PC on KOMe/Celite. Bars illustrate normalized ratios of 14:0/16:0 detected by GLC. Percentage of 14:0 within bars.)

RESULTS AND DISCUSSION

Seven samples of the α-16:0-β-14:0 PC (6 x 25 μg, 1 x 2.5 μg) and five samples of the $(14{:}0)_2$ PC/$(18{:}2)_2$ PC equal weight mixture (5 x 25 μg) were analyzed for their total acyl group content by reaction on potassium methoxide/Celite microcolumns. Results are given in Table I. The data are in good agreement with the expected values, and demonstrate a lack of selectivity for saturated vs. unsaturated acyl groups and for α-acyl vs β-acyl groups.

Five samples of egg yolk lecithin (30 μg in 10 μl CH_2Cl_2) were analyzed for acyl content after transmethylation on potassium methoxide/Celite microcolumns. The results are listed in Table II and correlate well with data obtained by use of the procedure of Luddy et al. (5) on 2 mg of the same lipid.

All results were substantially altered whenever the eluate of esters was subjected to evaporation under vacuum or nitrogen stream. To illustrate the effect of the nitrogen stream, the eluate from a single reaction of 25 μg of the α-16:0-β-14:0 PC on a potassium methoxide/Celite microcolumn was sampled prior to evaporation and then after 2, 4, and 6 min under a constant nitrogen stream at room temperature. Results are illustrated in Figure 3. The severity of product loss through selective evaporation of these methyl esters was unexpected, though Ackman and Burgher (16) warned of moderate losses of 14:0 over 16:0 during evaporation of milligram amounts of mixtures. Indeed, a call for avoidance of such evaporation would be contrary to several published methods (1-3). The selective evaporation of 14:0 over 16:0 was, to be sure, exaggerated at the microgram level and was even noticed in samples that were exposed only to room air currents. The problem was obviated by injection of aliquots of the unevaporated eluate directly into the GLC inlet at a relatively low column temperature. After dissipation of the large solvent peak, the esters were allowed to elute by a subsequent programmed increase in column temperature.

Another problem involved the use of chloroform as the solvent during reaction. The 0.75% ethanol that is typically added to chloroform by the manufacturer as a stabilizer was sufficient to give rise to considerable amounts of ethyl ester byproduct. This may be understood better by comparing the molar amount of methoxide in a typical microcolumn (13 μmol) with the molar amount of ethanol in 10 μl of lipid solution (2.4 μmol). For each μmol of ethoxide, there are only 5.5 μmol of methoxide. A related problem recently was reported in the literature (17).

The data in Tables I and II demonstrate that by heeding the warnings concerning fingerprints, evaporation, and chloroform, one may use the potassium methoxide microcolumn procedure to establish the acyl compositon of phosphatidylcholine. Standard deviation calculations on repetitive runs demonstrate, furthermore, that the results are reproducible. The advantages gained by use of this new procedure are, of course, amplified when multiple analyses are required.

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Rapid Enzyme-Induced Hydrolysis of Microgram Amounts of Phosphatidylcholine on Phospholipase A_2/Celite Columns[1,2]

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ABSTRACT

A method has been developed to hydrolyze microgram amounts of phosphatidylcholine (PC) regiospecifically. Hydrolysis of the *sn*-glycerol 2-acyl group occurs rapidly on microcolumns of immobilized phospholipase A_2 on Celite 545 diatomaceous earth. Close to 90% reaction occurs within the first 5 min. Acyl group analysis then may be accomplished by gas liquid chromatography (GLC) of the resulting fatty acids. Hydrolysis of α-16:0-β-14:0 PC demonstrated consistent selectivity for 14:0 liberation, with small amounts of 16:0 probably indicative of acyl scrambling during the synthesis of the PC. Hydrolysis of an equal weight mixture of $(14{:}0)_2$PC and $(18{:}2)_2$PC demonstrated nonpartiality of the immobilized enzyme for either a saturated or unsaturated substrate. The new methodology offers a convenient and sensitive alternative to the presently used procedures.

INTRODUCTION

Phospholipase A_2 (phosphatide 2-acyl hydrolase, E.C. No. 3.1.1.4) specifically catalyzes the hydrolysis of the *sn*-glycerol 2-acyl group of glycerophosphatides such as phosphatidylcholine (PC) to yield a free fatty acid and a lysoglycerophosphatide (1,2).

Standard methodology for such hydrolyses requires multiphase contact between an ether phase containing milligram amounts of lipid and a buffered aqueous phase containing the enzyme and the necessary calcium ion cofactor. Generally the heterogeneous mixture is shaken at least 1 hr, followed by extraction of the lipid residue, separation of the liberated free fatty acid, LPC, and unreacted PC by thin layer chromatography (TLC), esterification of the fatty acid or transesterification of the LPC, and finally analysis of the resulting methyl esters by gas liquid chromatography (GLC) (3-6).

The present research was influenced by recent reports on the immobilization of lipase enzymes (7-10) and the conviction that analysis of *microgram* amounts of lipids is a feasible, rapid, and convenient alternative to conventional methodology. Prior work demonstrated that microgram amounts of phosphatidylcholine could be analyzed for total acyl group content by transmethylation on microcolumns of potassium methoxide/Celite diatomaceous earth (11). It was envisioned, therefore, that phosphatidylcholine could be analyzed for its 2-acyl group by hydrolysis on microcolumns containing immobilized phospholipase A_2. Immobilization by adsorption of an aqueous solution of the enzyme onto Celite would allow a large surface area of the aqueous enzyme to contact the lipid in organic phase.

EXPERIMENTAL

Preparation of Enzyme Solution

Phospholipase A_2 (toxic; handle with care) from *Crotalus terrificus terrificus* venom was obtained in 50% aqueous glycerol, 1 mg/ml, activity 200 μ/mg (Boehringer Mannheim GmbH). This was diluted 1:1 with a Tris buffer solution that was adjusted to pH 7.5 by addition of hydrochloric acid and made 4 mM in calcium ion. The buffer solution was prepared by dissolving Tris (J.T. Baker Chemical Co., Phillipsburg, NJ, $(HOCH_2)_3CNH_2$, 0.605 g) in water (25.0 ml), then adding 0.1 N hydrochloric acid (40.6 ml) and $CaCl_2 \cdot 2H_2O$(39 mg). The resulting "working solution" of the enzyme was similar in constitution to that in the modified Brockerhoff procedure reported by Christie (6).

Celite Microcolumn

Large Pasteur pipettes were shortened to give glass capillaries with built-in funnels, as reported previously (11). A small plug of glass wool was inserted two-thirds the way down the capillary, and a 1.5 cm microcolumn of carefully washed (4 x by conc. HCl, 4 x by distilled water) Celite 545 diatomaceous earth (Fisher Scientific Co., Pittsburgh, PA) was tamped in place above the plug. Measured aliquots of the phospholipase A_2 working solution (generally 2 μl) were carefully added to the top of the Celite within the column by microliter syringe. If de-

[1]Presented at the AOCS 68th Annual Meeting, New York, May 1977.

[2]Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

TABLE I

Regioselectivity of Enzymic Hydrolysis of α-16:0 -β-14:0 PC

Enzyme working solution, μl	Reaction time, min	Number of experiments	% 14:0[a]	mole % 14:0[b]
2	5	8	88.8 ± 1.6	90.1
2	30	5	88.2 ± 0.7	89.6
4	5	4	88.4 ± 1.0	89.8
4	30	4	86.8 ± 1.2	88.4
6	60	3	89.6	90.6

[a]Flame ionization detector response as mean ± standard deviation; [14:0/(14:0 + 16:0)] x 100.

[b]FID response is proportional to n-1 carbon atoms in a C_n fatty acid (15).

sired, these columns then could be sealed at both ends with a small flame and stored in a freezer for later use. They retained their activity for at least several months when prepared and stored this way.

Preparation of Lipid Solution

Synthetic L-α-phosphatidylcholines served as model compounds: α-palmitoyl-β-myristoyl- (Supelco, Inc., Bellefonte, PA); dimyristoyl– (Sigma Chemical Co., St. Louis, MO); dilinoleoyl– (PL Biochemicals, Inc., Milwaukee, WI). Solutions were prepared in Nanograde methylene chloride (2.5 μg/μl), purities were confirmed by TLC (11), and concentrations were determined by phosphorus analysis (12). Individual solutions of $(14{:}0)_2$PC and $(18{:}2)_2$PC of known concentrations were combined to give a new solution that contained equal weights of each lipid.

Reaction Conditions

A prepacked enzyme microcolumn was snapped open at both ends. The lipid solution (1.0 to 10 μl) was deposited onto the column with a 10 μl syringe. The reaction was allowed to proceed at room temperature (generally 5 min for GLC analysis of released free fatty acid, but up to 1 hr for maximum hydrolysis). Then the liberated free fatty acids were eluted from the column with a portion of ether (30-50 μl). This eluate was free of contaminants (by TLC) and ready for GLC analysis. In order to establish the extent of reaction, the residual material was eluted with methanol (50 μl) and analyzed by TLC.

Gas Liquid Chromatography

Product mixtures of myristic and palmitic acids were analyzed directly on an EGA/phosphoric acid column using conditions identical to those reported earlier for methyl esters (11). Mixtures of myristic and linoleic acids were converted to their methyl esters by treatment with an ether solution of diazomethane prior to GLC analysis (13,14).

Thin Layer Chromatography

Commercial plates of Silica Gel H and 5% ammonium sulfate were used (Redi-Coat L/S, Supelco, Inc., Bellefonte, PA). Plates were developed in one direction in order to separate the reactant PC from the product LPC. Optimum development required a predevelopment with acetone and then the use of chloroform-methanol-acetic acid-water, 25:15:4:2 by volume. Plates were visualized by charring in an oven at 180 C, at which temperature the incorporated charring agent, ammonium sulfate, pyrolyzed to sulfuric acid. Phosphorus-containing spots could also be visualized by spraying the plate with a diluted molybdate spray (12) or with Phospray (Supelco). Nondestructive visualization was accomplished by contact with iodine vapor.

Quantitative Analysis of PC and LPC

The Vaskovsky-Kostetsky-Vasendin phosphorus analysis procedure was used (12). The heteropolyphosphomolybdate blue was measured in a Bausch and Lomb Spectronic 88 spectrophotometer at 830 nm; values were compared to KH_2PO_4 standards. In typical runs, PC and LPC TLC spots were scraped and digested in 0.2 ml 70% $HClO_4$ in a block heater at 180 C for about 20 min. These data were compared with values obtained for blank scrapings of equivalent R_f and for identical aliquots of the reagent PC solution.

RESULTS AND DISCUSSION

Separation of PC and LPC by TLC

The assessment of the extent of reaction demanded a clean isolation of the reactant from the reaction mixture. This was achievable by (a) elution of the mixture from the microcolumn with methanol, (b) application of the eluate to

TABLE II

Extent of Reaction

Reaction time, min	A_2 per column, µl[a]	Number of experiments	Residual PC, %[b]
0[c]	---	6	92.8 ± 6.8
1	2	1	20.2
1	4	1	14.9
5	2	5	11.0 ± 1.8
5	4	5	12.6 ± 1.7
30	2	5	8.9 ± 1.1
30	4	4	7.9 ± 0.7
60	2	5	4.5 ± 1.3
60	4	5	3.3 ± 1.4
60	6	5	4.4 ± 1.0

[a]Volume of working solution of enzyme.
[b]See text for explanation. Multiple runs are reported as the mean ± standard deviation.
[c]Unreacted PC was applied to the plate and then analyzed in the presence of the adsorbent.

a TLC plate, (c) predevelopment of the plate with acetone to separate glycerol from the phospholipids, and (d) development in the acidic solvent system to separate LPC from PC.

Acyl Group Analysis

The selectivity of the new procedure was tested in two ways: (a) the solution that contained equal weights of $(14{:}0)_2$PC and $(18{:}2)_2$ PC was subjected to the reaction conditions in order to test the selectivity for unsaturated vs. saturated acyl groups; (b) the solution of α-16:0-β-14:0 PC was used to test the regioselectivity of the process for the β-acyl group.

The mixture of $(14{:}0)_2$PC and $(18{:}2)_2$PC was subjected to seven replicate 30 min reactions. GLC analysis of the methyl esters of the resulting fatty acids showed the 14:0 content to be 48.7 ± 1.6% by peak area, compared with the expected value of 48.8%, using the correction factors of Ackman (15). This indicated that the procedure was not selective for unsaturated vs. saturated acyl groups.

In order to test regioselectivity, several series of reactions were performed on aliquots of the α-16:0-β-14:0 PC in methylene chloride (25 µg of PC in 10 µl was applied to each column). GLC should have detected no palmitic acid if the model PC had palmitoyl groups only in the α-position and if no acyl group migration had occurred. Actual data are given in Table I. Regardless of the amount of enzyme present or the time of reaction, ca. 90 mole % myristic acid (14:0) was detected (molar mean ± standard deviation for the five sets in Table I = 89.7 ± 0.9) vs. 10% of the unexpected palmitic acid (16:0). Since specificity for the 2-acyl group did not diminish with longer reaction times and since it is shown in the accompanying publication (11) that this PC contained a 50:50 molar ratio of 14:0 to 16:0, the most likely cause for the appearance of 16:0 by enzymic hydrolysis was acyl group scrambling. Since the extent of scrambling did not increase with increasing reaction times, such scrambling probably occurred during the synthesis of the PC from α-16:0 LPC. In fact, some scrambling has been reported to have occurred in every mixed acid PC synthesis for which such analysis has been attempted (16,17). Furthermore, in an ongoing project in this laboratory, α-16:0-β-14:0 PC that was synthesized by a novel acylation of pure α-16:0 LPC showed 7% scrambling by application of the same microcolumn procedure that consistently showed 10% scrambling in the present report (18).

Extent of Reaction

Data on the rate of consumption of PC are given in Table II and were obtained as follows: for each experiment, three reactions of α-16:0-β-14:0 PC (25 µg in 10 µl CH_2Cl_2) were allowed to proceed concurrently and in an identical manner for a specific duration. The combined eluates were applied to a TLC plate and the residual PC was isolated by development of the plate. The combination of three reaction mixtures was necessary for accurate analysis of the traces of PC that remained after the first few minutes of reaction. The PC spot was analyzed for phosphorus content and compared with the phosphorus content of the initial and unreacted PC solution (i.e., of 3 x 10 µl) In order to assess the precision or repeatability of the procedure, each experiment generally was repeated several times, and the results were reported as mean values ± standard deviation.

Varying the amount of enzyme produced virtually no change in reaction rate. The reac-

tion approximated first order kinetics, independent of enzyme concentration, with a rate constant about 1.4 sec^{-1} (derived from $\ln[PC]-\ln[PC_o] = -kt$). The first order behavior is typical of many enzyme reactions involving a single substrate at low concentration (19). The reaction was rapid. Whereas over 95% of the PC was consumed within 1 hr, almost 90% reacted within the first 5 min.

For routine enzymic analyses, then, these reactions on microcolumns are a convenient and sensitive alternative to the methodology in use today. When the new procedure is invoked along with the total acyl group analysis by transmethylation on microcolumns (11), detailed structural assignment of the total acyl content of the lipid may be accomplished on microgram amounts of starting material.

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Transport of Diacylalkylglycerols in Chylomicrons and Very Low Density Lipoproteins of Rat Intestinal Lymph Following Intragastric Administration of 1,3-Dioctadecenoyl-2-Hexadecylglycerol[1]

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ABSTRACT

The triacylglycerol (TG) analog 1,3-dioctadecenoyl-2-hexadecyl glycerol was used in the study of the transport of dietary lipids by lipoprotein fractions of rat intestinal lymph. 1,3-Diacyl-2-alkyl glycerols (DAG) are hydrolyzed by pancreatic lipase to form 2-alkyl glycerols and free fatty acids. These hydrolysis products are then absorbed, and DAG are resynthesized within the intestinal mucosa. Intestinal lymph of rats was collected following intragastric administration of 1,3-dioctadecenoyl-2-hexadecyl glycerol. The DAG to TG ratios in very low density lipoprotein (VLDL) and chylomicron fractions were determined as a measure of the incorporation of lipid of dietary origin. The ratio of DAG to TG in the VLDL-2 (S_f 12-100) fraction ranged from 0.06 to 0.56 indicating a significant amount of DAG transported relative to TG. The glyceryl ether to TG ratio increased with mean lipoprotein volume from the VLDL-2 fraction to the chylomicron (S_f>400) fraction. The correlation between glyceryl ether to TG ratio and average volume and between the amount of DAG per ml of original lymph and average volume within the chylomicron fraction was 0.99. Thus, the amount of dietary fat transported was correlated with the size of the chylomicrons produced. The glyceryl ether to TG ratio was positively correlated with the average volume of the lipoprotein fractions isolated (chylomicrons, chylomicron rich (S_f>100), VLDL-1 (S_f 100-400) and VLDL-2) (r=0.87). These results suggest that the size of the lipoproteins produced by the intestine is determined by the amount of fat available for transport and that particles of larger diameter are formed by the addition of lipid of dietary origin to existing VLDL.

INTRODUCTION

Triacylglycerols (TG) produced in the intestinal mucosa of fasting animals are secreted into the lymph in very low density lipoproteins (VLDL) (1) while the mucosa of fed animals secretes both VLDL and chylomicrons. The relationship between VLDL and chylomicrons and their role in the transport of dietary fat is, however, not clearly understood. Lipid for the production of the endogenous TG incorporated into VLDL of fasting animals is derived from the intestinal lumen (2,3) and is synthesized primarily via the *sn*-3-glycerolphosphate (*sn*-3-GP) pathway (4-6) since 2-acylglycerol (2-MG) is not present in substantial amounts. In fed animals, 2-MG is produced by the hydrolysis of dietary TG in the intestinal lumen and substantial amounts of TG are reformed in the intestinal mucosa by the 2-MG pathway of TG biosynthesis (7). After fat feeding, the proportion of VLDL and chylomicrons produced appears to be related to the size of the fat load (8,9). The current study was undertaken to investigate the role of chylomicrons and VLDL of intestinal origin in the transport of TG synthesized from 2-MG, the product of the lipolysis of dietary fat in the intestinal lumen.

Interpretation of results obtained using TG in the study of intestinal metabolism is complicated by the instability of the 2-MG produced by the lipolysis of TG in the intestinal lumen. 2-MG can isomerize to 1- or 3-MG before or after absorption, or be completely deacylated with resultant difficulty in data interpretation. Several investigators have demonstrated that 1,3-diacyl-2-alklglycerols (DAG) are metabolized similarly to their TG analogs. The acyl groups of DAG are cleaved by pancreatic lipase (10). The resulting 2-alkylglycerols (2-AG) are absorbed by the intestinal cells and partially oxidized to free fatty acids and glycerol (11). The remainder of the 2-AG is reesterified to form DAG.

DAG were administered in this study since their hydrolysis products 2-AG appear to be useful analogs for the naturally occurring 2-MG without the complication of acyl migration (for review of the use of alkylglycerides in metabolic studies, see reference 12). The transport of glyceryl ethers by lipoprotein fractions in lymph has not, to our knowledge, been studied.

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MATERIALS AND METHODS

Benzylidene glycerol and hexadecyl p-toluenesulfonate (M.P. 48.0-48.5 C), were synthesized (13,14) and reacted as described by Gupta and Kummerow (15). The product was purified by crystallization from hexane following cleavage of the benzylidene group (16). 2-AG, pure by thin layer chromatography (TLC), was dissolved in chloroform and reacted with a 0.5 molar excess of oleoyl chloride in the presence of pyridine. Purification of the DAG was accomplished in a manner analogous to that used for TG, by crystallization 3 x at -25 C from 95% ethanol-acetone (90:10, v/v) (13). The Buchner funnel, filter flask, filter paper and solvents used to wash the crystals were kept at -45 C. Polar contaminants were removed on a column of neutral alumina (17). Gas liquid chromatography (GLC) analysis of methyl esters derived from the DAG showed the product to contain 99% oleate and 1% stearate.

Male Sprague Dawley rats, 320 to 340 g, obtained from Holtzmann Co., Madison, WI, were given free access to rat cow until two weeks prior to cannulation. Rat chow was then given ad libitum at 9:00 p.m. and removed at 9:00 a.m. The evening prior to cannulation, 200 ml of 5% sucrose was given ad libitum, in place of feed. The rats used in the following experiments weighted between 400 and 500 g.

Nembutol, 35 mg/kg, was administered intraperitoneally and the intestinal lymph duct cannulated (18). The antrum of the stomach was punctured with a needle, and the tapered end of a 25 cm section of polyethylene tubing (2.08 mm OD), inserted 1 cm, was glued in place with Eastman 910 adhesive (Eastman Organics, Kingsport, TN). The stomach was repositioned and the incision closed. The animals were maintained in restraining cages similar to those described by Bollman (19).

Approximately 8 hr after cannulation, 1,3-dioctadecenoyl-2-hexadecyl glycerol (ca. 300 mg/kg body weight) was administered through the stomach cannula. The rats were infused intragastrically, with isotonic saline at 2.0 ml/hr for the remainder of the collection period. Lymph collection was continued into tubes maintained in wet ice, until free of DAG. This was ascertained by periodically applying lymph from the tip of the cannula to a Silica Gel G TLC plate and developing it with petroleum ether-diethyl ether (75:10 v/v). Visualization was with iodine vapors. Thimerosol, adjusted to pH 7.0 (20), was added to yield a final concentration of 0.0025%. The lymph was then centrifuged for 15 min at 2000 g and

TABLE I

Mean Diameter and Volume of Lipoprotein Fractions Isolated from Rat Intestinal Lymph Following Intragastric Administration of 1,3-Dioctadecenoyl-2-Hexadecyl Glycerol

Fraction	Chylomicron		Chylomicron rich		VLDL-1		VLDL-2	
Sample[a]	Diameter[b]	Volume[c]	Diameter	Volume	Diameter	Volume	Diameter	Volume
A-1	1198 ± 39	128	867 ± 26	47	647 ± 13	17	479 ± 12 (498)[d]	6.4 (8.0)[d]
A-2	1435 ± 26	170	1099 ± 34	100	773 ± 10	26	439 ± 10 (477)[d]	5.2 (6.6)[d]
B-1			1036 ± 37	68			443 ± 13	6.0
B-2	1648 ± 45	293	1090 ± 39	106	773 ± 11	26	467 ± 11 (443)[d]	6.3 (6.6)[d]
C-1			973 ± 31	69			554 ± 14	11.2

[a]Chylomicron, (S_f>400); Chylomicron rich, (S_f>100); VLDL-1, very low density lipoproteins (S_f 100-400); VLDL-2, (S_f 12-100). A-1, B-1 and C-1 are initial collections; A-2 and B-2 are second collections from the same animals following clearance of the initial sample from the lymph.

[b]Mean diameter, Å ± standard error of the mean.

[c]Mean volume ($Å^3$ 10^{-7}).

[d]Size of VLDL-2 determined in lymph separated into chylomicron, VLDL-1 and VLDL-2 fractions.

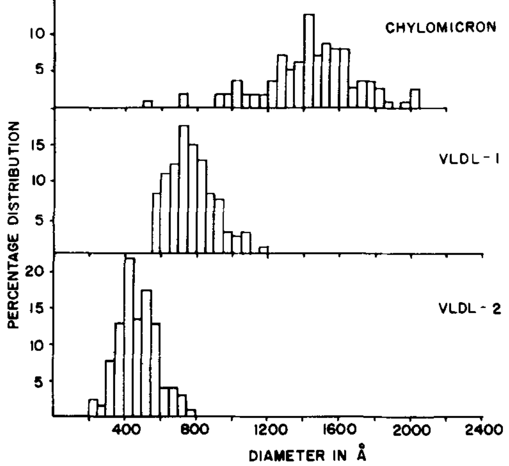

FIG. 1. Size distribution of chylomicrons, VLDL-1 and VLDL-2 isolated from rat intestinal lymph following intragastric administration of 1,3-dioctadecenoyl-2-hexadecylglycerol. (Sample A-2).

the precipitated coagulated material discarded. Following clearance of the initial sample of DAG, certain rats were used for a second or third experiment. These animals are identified, in the tables, with the suffix 2 or 3.

Chylomicron rich ($S_f > 100$) and VLDL-2 (S_f 12-100) particles were isolated by flotation through 5 ml of a NaCl solution (d-1.006 g/ml) by centrifugation for 35 min at 15,000 RPM in a Beckman 40.3 rotor. The top 1 ml, washed through 5 ml of the NaCl solution, contained chylomicron rich lipoproteins. VLDL-2 was isolated (following removal of the chylomicron rich fraction) by centrifucation for 15 hr at 40,000 RPM in a Beckman SW 50.1 rotor.

Separation into chylomicrons (S_f>400), VLDL-1 (S_f 100-400) and VLDL-2 was as follows: five ml of lymph was overlaid with a solution of NaCl (d-1.006 g/ml) and centrifuged for 30 min at 17,500 RPM in a Beckman 50 Ti rotor. The top 0.5 ml washed 2 x by flotation (d-1.006 g/ml) contained chylomicrons. VLDL-1 was isolated following removal of chylomicrons by flotation (d-1.006 g/ml) for 1 hr at 35,000 RPM in a Beckman 50 Ti rotor. The top 0.5 ml was recentrifuged twice as above yielding a fraction designated VLDL-1. VLDL-2 was concentrated after removal of VLDL-1 by centrifugation for 15 hr at 40,000 RPM in an SW 50.1 rotor. The top 1 ml contained VLDL-2. Samples were prepared for electron microscopy as described by Fraser (9).

Lipids were extracted (21) and TG and DAG separated on 20 x 20 cm, 0.5 mm TLC plates of Silica Gel G developed in petroleum ether-diethyl ether (75:10, v/v). Visualization was with iodine vapors. The TG and DAG bands were scraped and eluted through sintered glass funnels with chloroform-methanol (90:10, v/v). Methyl esters were prepared at room temperature with 2.5 N methanolic $NaOCH_3$ (22) following addition of methyl heptadecanoate as an internal standard. All GLC analyses were conducted with an F&M Model 810 GLC equipped with dual hydrogen flame detectors. Separations were made by temperature programming a 6 ft x 1/8 in. stainless steel column packed with 10% SP-2330 on 100/200 mesh Chromosorb W-AW (Supelco, Inc., Bellefonte, PA). The initial temperature of 175 C was maintained for 5 min followed by a heating rate of 6 C/min until the final temperature, 240 C, was reached. Peak areas were determined with a Disc Integrator and normalized using the internal standard. Moles of fatty acids were calculated by dividing the peak areas by the molecular weight of the component methyl esters. Moles of TG and DAG were derived by dividing the total moles of methyl esters in the samples by 3 and 2, respectively.

RESULTS

Lymph was free of glyceryl ethers 14.2 hr (SD±2.3 hr) following intragastric administration of 1,3-dioctadecenoyl-2-hexadecyl glycerol. Lipoproteins from all lymph samples collected were separated into chylomicron rich and VLDL-2 fractions, whereas, due to limitations on the amount of lymph, only three samples were fractionated into chylomicrons, VLDL-1, and VLDL-2 (Table I). The average diameter of the chylomicron rich, VLDL-1 and VLDL-2 fractions were 1427 Å, 1013 Å, 731 Å and 474 Å, respectively, with corresponding volumes ($Å^3 10^{-7}$) of 197, 78, 23, and 7.0 (Table I). Individual lipoprotein preparations were relatively heterogeneous (Fig. 1), with the chylomicron diameters ranging from 950 to 2100 Å, VLDL-1 from 575 to 1200 Å and VLDL-2 from 200 to 800 Å.

TG transported in lymph ranged from 3.2 to 9.3 mg/ml and the DAG from 0.2 to 7.4 mg/ml (Table II). The TG concentration was relatively constant for four samples ranging from 7.6 to 9.3 mg/ml and for the total collected from 107 to 156 mg. Two samples contained an average of only 3.5 mg of TG/ml, with a total collection of ca. 7 to 8 mg each. Total DAG transported ranged from 28 to 130 mg for four samples, while two samples contained 0.4 and 4.7 mg, respectively.

The molar ratio of DAG to TG in the unfractionated lymph ranged from 0.06 to 0.82

TABLE II

Amount of Triacylglycerols and Diacylalkyl Glycerols Collected in Rat Intestinal Lymph Following Intragastric Administration of 1,3-Dioctadecenoyl-2-Hexadecyl Glycerol

	Triacylglycerols			Diacylalkylglycerols		
Sample[a]	mg/ml	mg/hr	Total	mg/ml	mg/hr	Total
A-1	7.6	5.9	106.8	2.0	1.60	28.2
A-2	9.3	9.9	129.3	2.7	2.90	38.1
A-3	7.6	8.2	123.2	3.2	3.48	52.2
B-1	3.2	0.6	6.7	0.2	0.04	0.4
B-2	9.0	11.1	155.9	7.4	9.2	129.6
C-1	3.7	0.6	8.2	2.1	0.3	4.7

[a]A-1, B-1 and C-1 are initial collections, A-2 and B-2 are second collections and A-3 is a third collection from the same animal. Lymph was free of glyceryl ethers prior to another administration of 1,3-dioctadecenoyl-2-hexadecyl glycerol.

TABLE III

Molar Ratio of Diacylalkyl Glycerol to Triacylglycerol in Intestinal Lymph and Lipoprotein Subfractions of Lymph from Rats Following Intragastric Administration of 1,3-Dioctadecenoyl-2-Hexadecyl Glycerol

Fraction	Original lymph	Chylomicron rich	VLDL-2[a]	Chylomicron	VLDL-1[a]	VLDL-2[a]
Sample[b]						
A-1	0.26	0.64	-[c]	0.60	0.67	0.29
A-2	0.29	0.52	0.44	1.79	0.89	0.53
A-3	0.42	0.62	0.23	0.52	0.28	0.06
B-1	0.06	0.16	0.47	-[d]	-[d]	-[d]
B-2	0.82	1.42	0.44	3.84	0.61	0.56
C-1	0.57	0.70	0.41	-[d]	-[d]	-[d]

[a]VLDL, very low density lipoproteins.
[b]A-1, B-1 and C-1 are initial collections, A-2 and B-2 are second collections and A-3 is a third collection from the same animal. Lymph was free of glyceryl ethers prior to another administration of 1,3-dioctadecenoyl-2-hexadecyl glycerol.
[c]Sample lost in preparation.
[d]Not determined.

(Table III). In the chylomicron rich and chylomicron fractions, the ratios were always higher than in the original lymph. The VLDL-2 fraction, isolated by either procedure, had a lower ratio than the corresponding chylomicron or chylomicron rich fractions in all cases except B-1, where the ratio in the chylomicron rich fraction was 0.16 and in the VLDL-2, 0.47. The ratio in the VLDL-1 fell between that of the VLDL-2 and chylomicrons, except in sample A-1, where the ratio in VLDL-1 was higher than that of the chylomicrons. In three samples, none of the ratios in the isolated lipoproteins were lower than those of the original lymph, while in three samples, they were.

It is apparent that the VLDL-2, VLDL-1 and chylomicron fractions differ markedly in both mean volume and ether to TG ratio (Fig. 2), with the VLDL-1 fraction falling intermediate between the chylomicron and VLDL-2 samples in both respects. Within the VLDL-2 and VLDL-1 fractions, there is no correlation of DAG to TG ratio with mean lipoprotein volume, while in the chylomicron fraction, the correlation between the ratio and mean volume is very high (r-0.99).

The VLDL-1 lipoproteins have higher DAG to TG ratios and larger volumes than the VLDL-2 particles, although there is considerable variation in the ratio at constant volume within each fraction. This variation is not unexpected since different amounts of DAG were transported in relation to TG in the different animals. Sample A-1 had a lower ratio in the origianl lymph (0.26) than B-2 (0.82) (Table III). This trend was followed in the lipoprotein fractions in that the former (A-1) had a lower ratio in VLDL-2 (0.29) and chylomicrons (0.60) and the latter a higher ratio, 0.56 and 3.84, respectively. There is a definite correlation between the DAG to TG ratio and lipoprotein volume from the VLDL-2 through the VLDL-1 to the largest chylomicrons. A correlation (r=0.72) exists between the size and ratio

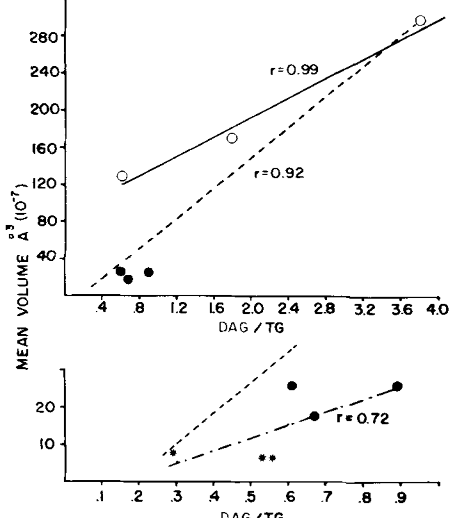

FIG. 2. Relationship of mean volume (Å)^{3}10^{-7} to molar diacylalkylglycerol to triacylglycerol ratios (DAG/TG) for chylomicrons (○), VLDL-1 (•) and VLDL-2 (*) isolated from rat intestinal lymph following intragastric administration of 1,3-dioctadecenoyl-2-hexadecyl glycerol.

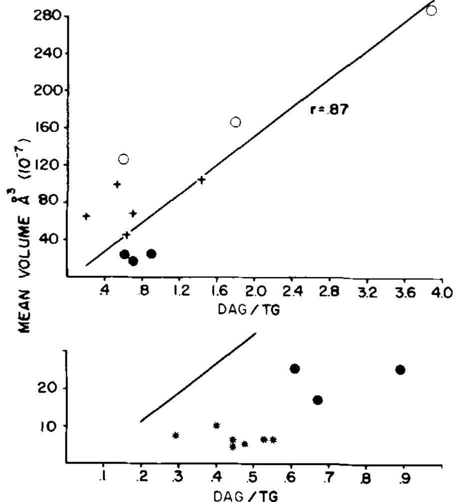

FIG. 3. Relationship of mean volume (Å)^{3}10^{-7} to molar diacylalkylglycerol to triacylglycerol ratios (DAG/TG) for chylomicrons (○), chylomicron rich (+), VLDL-1 (•), and VLDL-2 (*) isolated from rat intestinal lymph following intragastric administration of 1,3-dioctadecenoyl-2-hexadecyl glycerol.

in the VLDL-2 and VLDL-1; however, a stronger correlation (r=0.92) exists for the entire population of lipoproteins (Figure 2).

In Figure 3, the mean volume is plotted vs. the DAG to TG ratio for all the lipoprotein fractions collected. The correlation of the ratio with average fraction volume for the entire population is 0.87.

DISCUSSION

The size of the chylomicrons (1200-1650 Å) and chylomicron rich (879-1100 Å) lipoprotein fractions isolated (Table I) agree favorably with published values. Windmueller and Spaeth (23) collected lymph from isolated perfused rat intestine following intraluminal administration of soybean oil and determined the mean chylomicron diameters to be between 1000 and 2000 Å. Chylomicrons isolated from thoracic duct lymph of rats fed corn oil or cream had mean diameters between 1500 and 2000 Å (24).

Ockner et al. (25) collected 2.2 ml of intestinal lymph per hr from rats that were infused with isotonic saline at a rate of 5 ml/hr. In the current study, intestinal lymph flow rates of 0.8 to 1.2 ml/hr were obtained in four of the runs reported in Table II. These lower flow rates of lymph obtained were expected due to the lower rate of infusion of isotonic saline (2 ml/hr) used in this study.

Total neutral glycerides (TG and DAG) transported in the lymph ranged from 7.5 to 20.3 mg/hr for four samples (Table II). This is similar to results reported by Ockner et al. (25), where 5.9 mg of TG was transported per hr in intestinal lymph of rats infused with saline and up to 19.45 mg/hr when infused with oleic acid and monoolein. Lymph from two of the animals (Table II) contained considerably lower amounts of neutral glyceride per ml, 3.4 and 5.8 mg respectively, as well as lower total amounts of neutral glyceride obtained over the 11 to 14 hr collection period. This probably resulted from collection of only a portion of the lymph produced. The flow rate did, however, remain constant throughout the entire collection period. The lipoproteins isolated from the lymph of these two animals have a similar size (Table I) and DAG/TG ratio in the VLDL-2 fractions (Table III) as that collected from the animals with higher flow rates. It is, therefore, likely to be representative of the total lymph produced.

The molar ratio of DAG to TG (Table III) in fractions of intestinal lymph isolated by ultracentrifugation were higher than in the original lymph for samples A-1, A-2, and B-1. This could have resulted from failure to isolate a class of lipoproteins which had a low DAG to TG ratio. In the isolation procedure, used lipoproteins of d>1.006 were not retained. Fraser and Courtice (26) reported a lipoprotein fraction of d>1.019 in rabbit's intestinal lymph

which carried ca. 15% as much TG as VLDL. This percentage remained relatively constant with differing fat loads, which primarily increased the TG content of chylomicrons. Windmueller and Spaeth (23) reported a fraction of d>1.006 in lymph produced by isolated rat intestine.

The data obtained did not indicate that VLDL-TG of fed animals was synthesized exclusively by the *sn*-3-GP pathway of TG biosynthesis, since DAG were present in the VLDL fractions. The amount of DAG relative to TG increased with the size of the lipoprotein fractions reflecting the addition of a high percentage of lipid of dietary origin. This is reflected in the correlation between the DAG to TG ratio and the average volume of the various fractions. This suggests that the larger lipoprotein particles were formed by the addition of a high percentage of fat of dietary origin (Figs. 2 and 3) to VLDL particles which contained largely, but not exclusively, endogenous lipid.

Since there was a large difference in mean volume of the chylomicron and VLDL-1 fractions, extrapolation from VLDL to chylomicrons is hazardous; however, it is interesting to note that the correlation between the DAG to TG ratio and volume for the entire population of particles in Figure 2 was 0.92. This suggests, but does not prove, that chylomicrons were formed from VLDL by the addition of DAG.

Data for all the fractions isolated (Fig. 3) lends additional support to this possibility. The data presented do not rule out the possiblility that the *sn*-3-GP pathway supplies all of the TG for the smallest VLDL. The presence of DAG in the VLDL-2 could be due to the heterogeneity of the VLDL-2 fraction (Fig. 1).

Within the chylomicron fraction (Fig. 2), the correlation between the DAG to TG ratio and lipoprotein volume was 0.99. The volume of the chylomicrons produced was also highly correlated (r=0.99) with the mg of DAG transported per ml of lymph and the DAG to TG ratio in the original lymph (r=0.98), but not with the amount of TG transported (r=0.57). Thus, the size of the chylomicrons produced was directly related to the amount of exogenous lipid available for transport.

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The Effect of Elaidic Acid Incorporation Upon the Lipid Composition of Ehrlich Ascites Tumor Cells and of the Host's Liver

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ABSTRACT

The incorporation of elaidic acid into Ehrlich ascites tumor cells (EATC) upon feeding the host an elaidic acid-rich diet has been investigated in the present study. The EATC lipids contained only one-half the concentration of elaidic acid found in the lipids of either the host livers or of livers from normal mice. On the other hand, elaidic acid incorporation into tumor cells was close to that of ascites fluid. This incorporation was mainly into phospholipids; the highest into choline phospholipids and ethanolamine phospholipids. Some changes in the EATC fatty acid composition were noted due to this incorporation. EATC phospholipids had reduced polyunsaturated fatty acids as compared with oleic acid-grown cells. The same was true with respect to ascites fluid phospholipids, but neutral lipids were not altered. Tumor development was accompanied by an increase in elaidic acid of the host's liver. Elaidic acid incorporation into tumor cells resulted in a reduction in the amount of all major lipids in the tumor. In contrast, elaidic acid had no effect on lipid composition of livers from normal mice and-tumor bearing mice, and also had no effect upon the lipids of the ascites fluid that bathes the tumor cells. The incorporation of elaidic acid into the lipids of EATC, normal liver and host liver did not affect the relative composition of phospholipids in these tissues. The development of the tumor did result in decreases in triacylglycerols and esterified cholesterol, and increases in phospholipids and free cholesterol in the livers of host animals.

INTRODUCTION

Although most naturally occurring unsaturated fatty acids are in the *cis* form, food processing such as hydrogenation results in production of *trans* fatty acids (1,2). It has been estimated that between 18 and 55% of the fatty acids in margarines are in the *trans* form (2). These data indicate that most American diets contain unnatural fatty acids.

Trans fatty acids are known to be incorporated into animal tissues (3-6). The percentage of *trans* fatty acids incorporated depends on the amount in the diet (7). In human tissues, the highest percentages of these fatty acids were found in liver and adipose tissue (8). Kummerow (9) reviewed the subject of *trans* fatty acids regarding their absorption, metabolism and effect on biomembranes. This information was obtained from work that has been done using normal animals and nonmalignant cells.

Ehrlich ascites tumor cells (EATC) grow in the peritoneal cavity of CBA mice suspended in ascites fluid (10). The ascites fluid originates from the host (11) and provides the major lipid supply to the tumor cells. Ascites lipids are in the form of free fatty acids (12) and various lipoproteins (13).

Liepkalns and Spector (14) found that the fatty acid composition of the EATC can be altered by feeding tumor-bearing mice diets containing different kinds of fats. This dietary alteration induces changes in the fatty acid composition of plasma membrane phospholipids (15,16), nuclear membranes (17), and neutral lipids that are present in the cytoplasm (18).

Since information is very limited about *trans* fatty acid incorporation in tumor cells and how this incorporation might affect tumor lipids, the present study was designed to investigate changes in cellular fatty acids that might occur due to the in vivo incorporation of *trans* fatty acids into the tumor cells. Elaidic acid was used because it is commercially available and because

TABLE I

The Composition of the Basal Semisynthetic Diet

Ingredient	% by weight
Corn starch	30.0
Sucrose	29.6
Casein	26.0
Celufil	6.0
Mineral mix[a]	4.0
Corn oil	2.0
Fatty acids[b]	2.0
Vitamin mix[c]	0.2
Choline chloride (70%)	0.1
DL-methionine	0.1

[a]Bernhart and Tomarelli (19).
[b]Elaidic acid or oleic acid.
[c]Provided per kilogram of diet: Thiamine HCl, 25.0 mg; riboflavin, 16.0 mg; Ca pantothenate, 20.0 mg; pyridoxine HCl, 6.0 mg; biotin, 0.6 mg; folic acid, 4.0 mg; menadione, 5.0 mg; vitamin B_{12}, 0.02 mg; ascorbic acid, 250.0 mg; niacin, 150 mg; vitamin A, 10,000 IU; vitamin D_3, 600 IU, α-tocopherylacetate, 50 IU.

TABLE II

Fatty Acid Composition of Ehrlich Ascites Tumor Cells
Grown in Animals Fed Either the Oleic of the Elaidic Diet

Fatty[a] acid	Composition (%)											
	CE[b]		TG		CP		EP		SP+IP		Sph.	
	Oleic	Elaidic	Oleic	Elaidic	Oleic	Elaidic	Oleic	Elaidic	Oleic	Elaidic	Oleic	Elaidic
14:0	3.6 ± 0.8[c]	4.2 ± 0.4	1.5 ± 0.4	1.9 ± 0.1	tr[d]	0.8 ± 0.1	0.6 ± 0.2	tr	2.3 ± 0.4	3.8 ± 1.1	0.9 ± 0.1	0.6 ± 0.1
14:1	1.4 ± 0.7	1.2 ± 0.3	tr ±	0.5 ± 0.1	tr	0.5 ± 0.1	tr	tr	1.9 ± 0.3	3.0 ± 1.1	tr	tr
16:0	23.8 ± 1.8	27.3 ± 1.3	24.7 ± 1.2	25.8 ± 1.4	4.5 ± 1.2	5.1 ± 0.6	11.6 ± 0.9	11.2 ± 0.7	27.1 ±5.2	32.5 ± 3.3	29.5 ± 2.9	28.4 ± 2.5
16:1	5.0 ± 0.3	5.7 ± 0.4	4.5 ± 0.5	4.1 ± 0.9	1.5 ± 0.4	2.3 ± 1.5	4.8 ± 1.1	5.7 ± 0.1	10.1 ± 2.2	9.3 ± 1.1	3.8 ± 0.3	4.4 ± 0.7
18:0	6.8 ± 1.1	8.7 ± 0.4	11.2 ± 0.7	8.8 ± 0.7	68.3 ± 0.8	63.6 ± 5.1	23.4 ± 3.4	23.4 ± 1.5	27.4 ± 7.4	15.3 ± 2.7	17.4 ± 1.8	15.4 ± 0.4
18:1(*trans*)	3.2 ± 0.9	5.5 ± 0.7	1.0 ± 0.1	3.3 ± 0.2	0	4.9 ± 0.2	0	6.2 ± 0.7	0	5.4 ± 0.6	0	3.7 ± 0.2
18:1(*cis*)	42.2 ± 3.9	36.6 ± 1.2	42.3 ± 1.9	39.0 ± 1.9	14.3 ± 0.3	15.4 ± 2.0	40.3 ± 0.5	38.2 ± 1.7	22.3 ± 0.9	23.8 ± 1.4	35.0 ± 1.6	34.0 ± 0.2
18:2 + 20:0	0.9 ± 0.1	0.9 ± 0.2	9.8 ± 1.4	8.6 ± 0.6	2.3 ± 0.5	1.2 ± 0.3	7.5 ± 0.7	8.1 ± 1.1	1.5 ± 0.1	1.2 ± 0.2	5.7 ± 0.3	7.1 ± 0.5
18:3 †[e]	3.9 ± 1.1	2.7 ± 0.3	2.7 ± 0.2	2.4 ± 0.3		0.8 ± 0.2					2.3 ± 0.5	2.1 ± 0.7
18:3(n-6)	4.3 ± 0.5	3.0 ± 0.2	1.4 ± 0.4				1.4 ± 0.2	1.1 ± 0.2	5.0 ± 1.3	2.6 ± 0.4	1.1 ± 0.1	0.9 ± 0.2
20:3	1.8 ± 0.4	3.0 ± 1.2	tr	1.6 ± 0.3	0.7 ± 0.2	tr	0.5 ± 0.1	tr	0.8 ± 0.1	tr	tr	
20:4	1.3 ± 0.2	1.6 ± 0.5	0.9 ± 0.1	1.1 ± 0.2	6.7 ± 1.4	3.7 ± 0.6	8.1 ± 2.0	5.0 ± 0.4	1.1 ± 0.6	2.8 ± 0.3		
20:5	1.3 ± 0.1	tr		0.9 ± 0.1								
22:5			2.0 ± 0.8									
24:0											3.3 ± 1.0	2.4 ± 0.8
24:1											0.6 ± 0.4	1.3 ± 0.4
Saturatedq	34.2	40.2	37.4	36.5	72.8	69.2	35.6	34.6	56.8	51.6	56.8	53.9
Monounsaturated	51.8	49.0	47.8	46.9	15.8	23.1	45.1	50.1	34.3	41.5	41.1	45.5
Polyunsaturated	14.0	10.8	14.8	16.6	11.4	7.7	19.3	15.1	8.9	6.9	3.1	0.6

[a]Chain length: number of double bonds.
[b]Abbreviations are: CE, cholesteryl esters; TG, triacylglycerols; CP, choline phospholipids; EP, ethanolami ne phospholipids; SP+IP, serine phospholipids + inositol phospholipids and Sph., sphinogomyelin.
[c]Values are means ± S.E. of 3 samples.
[d]<0.5% of fatty acids.
[e]† (n-3) + 20:1.

TABLE III

Fatty Acid Composition of Tumor Ascites Fluids Developed in Animals Fed Either the Oleic or Elaidic Diet

Fatty[a] acid	Composition (%)					
	CE[b]		TG		PL	
	Oleic	Elaidic	Oleic	Elaidic	Oleic	Elaidic
14:0	7.0 ± 1.4[c]	6.1 ± 0.4	1.5 ± 0.1	1.4 ± 0.1	2.4 ± 0.1	3.1 ± 0.1
14:1	3.0 ± 0.6	3.2 ± 0.4	0.5 ± 0.1	0.7 ± 0.1	1.1 ± 0.2	1.5 ± 0.5
16:0	34.3 ± 2.2	25.8 ± 1.9	24.1 ± 2.1	25.6 ± 1.1	46.0 ± 5.4	40.5 ± 1.9
16:1	8.7 ± 0.6	7.8 ± 0.2	6.2 ± 1.1	4.6 ± 0.7	4.2 ± 0.4	4.9 ± 0.2
18:0	10.8 ± 1.1	7.8 ± 0.9	2.2 ± 0.2	3.0 ± 0.2	16.7 ± 1.5	17.6 ± 0.6
18:1(*trans*)	tr[d]	6.0 ± 1.1	1.7 ± 0.4	2.4 ± 0.4	tr	4.2 ± 0.6
18:1(*cis*)	19.1 ± 0.5	18.2 ± 0.7	52.5 ± 2.6	45.3 ± 2.3	17.9 ± 2.1	16.4 ± 1.8
18:2+20:0	6.0 ± 1.7	5.5 ± 2.8	8.0 ± 0.6	9.1 ± 0.8	5.5 ± 1.0	5.8 ± 0.3
18:3(n-3)+20:1			0.5 ± 0.2	0.8 ± 0.3	1.1 ±0.2	0.8 ± 0.2
18:3(n-6)			0.5 ± 0.2	0.5 ± 0.1		
20:2			0.6 ± 0.2	0.5 ± 0.1	2.9 ± 0.4	3.1 ± 0.6
20:4	3.2 ± 0.6	7.3 ± 3.1	1.0 ± 0.4	0.8 ± 0.1	1.8 ± 0.8	2.1 ± 0.4
20:5	3.6 ± 1.3	5.9 ± 0.4	tr			
Saturated	52.1	39.7	27.8	30.0	65.1	61.2
Monounsaturated	30.8	35.2	60.9	53.0	21.1	27.0
Polyunsaturated	17.1	25.1	11.3	17.0	13.7	11.8

[a]Chain length: number of double bonds.
[b]Abbreviations: see footnote of Table II; PL, phospholipids.
[c]Values are means ± S.E. of 3 samples.
[d]< 0.5% of fatty acids.

the mammalian system lacks the ability to synthesize the essential fatty acid out of the 18:1 fatty acids. Consequentially, intrepretation of the results was much easier.

EXPERIMENTAL PROCEDURES

Animals and Diets

CBA weanling male mice were used. Animals were fed the basal semisynthetic diet (Table I) containing 2% of either elaidic acid (*trans* 18:1) or oleic acid (*cis* 18:1) (United States Biochemical Corp., Ohio). The elaidic acid used was of high purity (99% elaidic acid + 1% stearic acid), while the oleic acid was made of 81.8% oleic acid, 5.8% palmitoleic acid, 3% palmitic acid, 2.9% elaidic acid, 1.8% linoleic acid, 1.4% stearic acid and minor fractions of 14:0 and 14:1. The double bond position in oleic and elaidic acid is assumed to be in the $\Delta 9$ position; however, this has not been established.

Both diets contained 2% Mazola Corn Oil (Best Foods, N.J.) to supply the essential fatty acids to the animals. The resulting fatty acid composition for the oleic acid diet was as follows: 14:0, 3.7%; 14.1, 2%; 16:0, 12%; 16:1, 6.6%, 18:1, 4.2%; 18:1 (*trans*), 5.4%; 18:1 (*cis*), 48.6% and 18:2, 17.5%. Fatty acid composition of the elaidic diet was as follows: 16:0, 6.3%; 18:0, 2.5% 18:1 (*trans*), 62.1%, 18:1 (*cis*), 19% and 18:2, 9.5%.

Experimental Design

Two groups of animals, six animals in each, received the experimental diets for 4 weeks. This experiment was repeated twice. The Ehrlich ascites tumor cells were harvested 14 days after transplantation while the animals were receiving the experimental diets. The Ehrlich ascites tumor strain used was obtained from Dr. Arthur Spector, University of Iowa. The maximum chromosome number in these cells as determined by a Chromosome Microtest Kit (Difco Lab, Michigan) was found to be 45. The mean survival time for the CBA mice carrying the tumor is 14 days. The methods used for tumor transplantation and harvesting are those of Spector et al. (20). Cells were separated from the ascites fluid, washed (21) and then resuspended in saline. Cell free ascites fluid was centrifuged at 2,000 x g for 5 min at 4 C. Livers of tumor-bearing animals were washed, blotted and homogenized for 15 sec in saline on ice using a Tissumizer (Tekmar Co., Ohio). In a separate experiment, livers of normal animals (five in each group) that were fed the experimental diets for the same period (42 days) were treated as described above and served as controls for the host livers.

Chemical Analyses

Protein was assayed in aliquots of liver homogenate and cell suspensions (22). Lipids

were extracted from tissues by the method of Folch et al. (23). Free and esterified cholesterol (24), phospholipids (25), and triacylglycerol (26) were assayed in the lipid extracts. The chemical analyses of EATC and ascites fluid were done on all individual samples of the two experiments except in the case of triacylglycerols and phospholipid assays where 6-9 samples were used. In the last instance, half of the samples used were selected at random from each experiment. For the liver samples, all the chemical analyses were done on five samples selected at random from the tumor-bearing animals of the second experiment and on all individual samples of the nontumor-bearing animals in the third experiment.

Thin layer chromatography was used to separate the neutral lipids (27) and phospholipid fractions (28). The methods of Raheja et al. (25) were used to elute the lipids from the silica after separation. A factor of 25 was used to estimate the total phospholipid content from the inorganic phosphorus.

For fatty acid analysis, three lipid extract samples of EATC fluid and livers were selected at random from each experimental group. Fatty acid methylation of different lipid fractions was done according to Morrison and Smith (29) after saponification. Fatty acid methyl esters were separated using a 20 ft x 1/8" ID stainless steel column packed with 10% Apolar-10C on 100/200 Gas-chrom Q (Applied Sciences, PA). The GLC conditions were as follows: column temperature, 230 C and injector and detector temperature, 260 C. Nitrogen was used as carrier gas. The retention times for elaidic acid and oleic acid under these conditions were 32.4 and 35.1 min, respectively. Identification of the peaks was accomplished using authentic fatty acid methyl ester standards obtained from Applied Science (PA) and Supelco (PA). The areas under the peaks were measured by triagulation. Under the isothermal conditions used in fatty acid analysis it was difficult to quantitate fatty acids having retention times longer than 22:5 where the peaks become very broad. Thus, the fatty acid analysis obtained does not include values for 22:6 fatty acids which are assumed to be found in very small concentration in EATC, fluids and livers.

RESULTS

Incorporation of Elaidic Acid into EATC Lipids

The fatty acid composition of different lipid fractions of tumor cells grown in animals fed either the oleic or the elaidic diet is given in Table II. It is clear from these data that feeding the elaidic acid diet to the host has resulted in the incorporation of elaidic acid in all cellular lipid fractions, being the highest in the ethanolamine phospholipids and the lowest in the triacylglycerol fractions. If these values are to be expressed on the basis of the elaidic acid percentage of the total octadecenoate, the highest concentration of elaidic acid (24%) was found to be in choline phospholipids and the lowest of 8% of the total octadecenoate was found in the triacylglycerols. Only the neutral lipids of the EATC grown in animals fed the oleic acid diet reflected the small amount of elaidic acid present in the diet.

Ethanolamine phospholipids, sphingomyelins and neutral lipids of elaidic acid-grown cells contained lower concentration of oleic acid than those of oleic acid-grown cells. This was more noticeable in the neutral lipids than the phospholipid fractions mentioned. In most lipid fractions, elaidic acid-grown cells contained a lower concentration of polyunsaturated fatty acids as compared with the oleic acid feeding. However, the cause and effect cannot be ruled out from this experiment since the elaidic acid diet contained lower concentrations of polyunsaturated fatty acids as compared with the oleic acid diet.

Incorporation of Elaidic Acid into the Ascites Fluid Lipids

Examination of the fatty acid composition of ascites fluid lipids (Table III) obtained from animals fed either the elaidic acid or oleic acid diet revealed that elaidic acid was incorporated in all lipid fractions upon feeding the host a diet in elaidic acid. The ascites fluid fatty acids obtained from animals fed the oleic acid diet contained a small percentage of elaidic acid in their lipid fractions with the highest percentage in the triacylglycerols. The enrichment of elaidic acid in lipid fractions of ascites fluid obtained from animals fed elaidic acid was accompanied by a decrease in the concentration of saturated fatty acids in both the cholesteryl ester and phospholipid fractions and also by a decrease in concentration of monounsaturated in triacylglycerols. Similar to the tumor cells, elaidic acid incorporation into the ascites phopholipids resulted in a decrease in their polyunsaturated fatty acid concentration. Both cholesteryl esters and triacylglycerol fractions contained higher concentrations of polyunsaturated fatty acids in elaidic acid rich ascites fluid as compared with oleic acid ascites. Triacylglycerols contain, by far, the highest relative concentration of monoenoic acids when tumor-bearing mice were fed diets rich in either oleic or elaidic acid.

TABLE IV

Fatty Acid Composition of Normal Livers of Mice Fed Either the Oleic of Elaidic Acid Diet

Fatty[a] acid	Composition (%)											
	CE[b]		TG		CP		EP		SP+IP		Sph.	
	Oleic	Elaidic	Oleic	Elaidic	Oleic	Elaidic	Oleic	Elaidic	Oleic	Elaidic	Oleic	Elaidic
14:0	5.9	5.1	1.5 ± 0.6[c]	1.7 ± 0.2	tr[d]	tr	tr	tr	tr	tr	tr	tr
14:1	tr	3.2	0.6 ± 0.2	0.9 ± 0.2	tr	tr	tr	tr	tr	tr	tr	tr
16:0	36.8	31.8	23.3 ± 2.5	26.6 ± 0.6	32.0 ± 2.7	22.7 ± 0.5	25.2 ± 1.5	20.3 ± 1.0	29.3 ± 1.1	29.5 ± 2.1	27.5	27.0
16:1	17.6	11.5	7.7 ± 0.6	7.0 ± 0.5	5.8 ± 0.7	4.2 ± 1.0	4.9 ± 0.4	5.5 ± 1.2	6.0 ± 0.4	5.0 ± 0.3	4.9	7.4
18:0	17.6	9.6	1.8 ± 0.2	2.0 ± 0.2	13.5 ± 0.5	15.1 ± 2.0	22.8 ± 1.4	18.8 ± 3.3	31.1 ± 3.7	26.8 ± 1.5	17.3	12.5
18:1 (*trans*)	0	1.9	0	2.3 ± 0.4	0	11.8 ± 3.2	0	10.3 ± 0.9	0	12.0 ± 0.2	0	5.3
18:1 (*cis*)	17.6	29.9	51.1 ± 1.6	50.5 ± 1.2	33.5 ± 3.9	30.8 ± 2.2	35.9 ± 1.9	33.8 ± 4.5	24.7 ± 1.2	22.6 ± 1.3	35.3	30.9
18:2+20:0	4.4	7.0	3.7 ± 0.9	3.6 ± 0.3	9.1 ± 1.5	7.6 ± 0.9	2.7 ± 0.7	3.4 ± 0.4	2.9 ± 0.4	1.7 ± 0.3	2.7	7.4
18:3(n-3)+20:1			4.5 ± 0.3	3.1 ± 0.5	1.2 ± 0.4	1.6 ± 0.4	1.4 ± 0.2	1.5 ± 0.1	1.8 ± 0.4	0.7 ± 0.2	5.2	2.9
18:3(n-6)			0.7 ± 0.4	tr	0.7 ± 0.5	0.6 ± 0.1	0.7 ± 0.1	tr	1.3 ± 0.1			
20:3			0.8 ± 0.4	1.0 ± 0.1	0.5 ± 0.1	1.0 ± 0.4	tr ±	tr	1.4 ± 1.2		1.4	1.1
20:4	tr	tr	4.3 ± 1.5	1.1 ± 0.5	2.8 ± 0.4	4.4 ± 0.9	6.1 ± 1.0	4.9 ± 1.0	1.4 ± 0.1	tr		
24:0											1.8	1.6
24:1											2.7	2.0
Saturated	60.3	46.5	26.6	30.3	45.5	37.8	48.0	39.1	60.4	56.3	49.3	48.5
Monounsaturated	35.2	46.5	59.4	60.7	40.1	46.8	40.8	49.6	30.7	39.6	48.1	48.5
Polyunsaturated	4.5	7.0	14.0	9.0	14.4	15.4	11.2	10.3	8.9	4.1	2.6	3.0

[a]Chain length: number of double bonds.
[b]See footnote of Table II for abbreviations.
[c]Values are means ± S.E. of 3 samples. Those without S.E. are the averages of pooled samples from 3 animals.
[d]< 0.5% of fatty acids.

TABLE V

Fatty Acid Composition of Liver Lipids of Tumor-bearing Mice Fed Either the Oleic of Elaidic Acid Diet

Fatty[a] acid	Composition (%)											
	CE[b]		TG		CP		EP		SP+IP		Sph.	
	Oleic	Elaidic	Oleic	Elaidic	Oleic	Elaidic	Oleic	Elaidic	Oleic	Elaidic	Oleic	Elaidic
14:0	11.0	14.5	2.0 ± 0.4[c]	3.5 ± 0.6	tr[d]	tr	tr	tr	tr	tr	tr	tr
14:1	4.9	6.2	1.4 ± 0.1	2.8 ± 0.2	tr	tr	tr	tr	tr	tr	tr	tr
16:0	36.6	39.3	23.8 ± 1.3	27.2 ± 2.0	30.4 ± 1.2	28.1 ± 0.9	35.0 ± 1.8	28.6 ± 1.2	30.6 ± 3.2	27.5 ± 1.7	37.0	31.1
16:1	12.8	11.0	6.6 ± 0.2	6.6 ± 0.5	6.8 ± 0.8	4.3 ± 0.5	5.6 ± 0.2	3.4 ± 0.6	4.0 ± 0.3	4.4 ± 0.8	4.8	8.3
18:0	13.4	12.4	4.0 ± 1.8	4.3 ± 0.7	19.7 ± 2.1	17.6 ± 2.4	25.5 ± 2.8	23.7 ± 1.9	44.7 ± 3.0	35.1 ± 1.6	21.4	21.3
18:1 (*trans*)	0	1.4	0	7.9 ± 2.0	0	10.8 ± 5.8	0	15.9 ± 4.8	0	12.7 ± 0.5	0	9.2
18:1 (*cis*)	21.3	15.2	53.0 ±1.8	36.2 ± 2.8	29.0 ± 0.8	25.2 ± 2.3	26.8 ± 2.3	18.4 ± 3.2	18.1 ± 1.4	15.6 ± 1.0	15.3	12.8
18:2+20:0			4.5 ± 0.4	5.1 ± 0.5	8.5 ± 0.5	8.8 ± 2.5	2.4 ± 0.9	2.5 ± 0.2	0.9 ± 0.5	1.6 ± 0.3	1.9	1.6
18:2(n-3)+20:1			1.0 ± 0.1	1.3 ± 0.2	1.6 ± 0.2	0.8 ± 0.4	0.9 ± 0.4	0.5 ± 0.1	tr	tr	3.2	2.6
18:3(n-6)			tr	3.8 ± 2.7	1.6 ± 0.6	0.6 ± 0.3	0.5 ± 0.1	tr	6.0 ± 0.4	tr		
20:3			1.1 ± 0.5	0.9 ± 0.3	0.6 ± 0.1	tr	tr	tr	tr		1.7	0.8
20:4	tr	tr	1.7 ± 0.6	tr	1.8 ± 0.6	2.0 ± 0.7	3.0 ± 0.5	6.4 ± 1.2	0.3 ± 0.4	1.8 ± 0.4		
24:0											2.4	1.4
24:1											10.9	10.1
Saturated	61.0	66.2	29.8	35.0	50.1	45.7	60.5	52.3	75.3	62.6	62.7	55.4
Momousaturated	39.0	33.8	61.0	53.5	35.8	40.3	32.4	37.7	22.1	32.7	34.2	43.0
Polyunsaturated	tr	tr	9.2	11.5	14.1	14.0	7.1	10.0	2.6	4.7	2.1	1.6

[a]Chain length: number of double bonds.
[b]See footnote of Table II for abbreviations.
[c]Values are means ± S.E. of 3 samples. Those without S.E. are the averages of pooled samples from 3 animals.
[d]< 0.5% of fatty acids.

TABLE VI

Effect of Feeding Elaidic Acid-Rich Diet to the Tumor-bearing and Nontumor-bearing Mice on the Lipids of Ehrlich Ascites Cells, Fluid and Livers

Dietary fatty acid	Lipid content (μg/mg protein ± S.E.)			
	Free cholesterol	Cholesteryl ester	Triacylglycerols	Phospholipids
	Tumor Cells			
Oleic	10.0 ± 0.1(12)[a]	8.0 ± 0.6(12)	71.0 ± 2.7(8)	64.6 ± 3.9(12)
Elaidic	7.4 ± 0.1(12)[b]	6.2 ± 0.6(12)[c]	50.1 ± 5.9(9)[d]	48.0 ± 5.0(12)[e]
	Ascites Fluid			
Oleic	30.1 ± 3.8(11)	46.6 ± 5.9(11)	281.5 ± 44.4(6)	58.5 ± 9.0(6)
Elaidic	30.7 ± 2.9(12)	42.0 ± 3.9(12)	290.5 ± 62.2(7)	60.9 ± 9.4(6)
	Livers (tumor-bearing animals)[f]			
Oleic	10.0 ± 0.5(5)	3.5 ± 0.6(5)	27.0 ± 3.5(5)	67.5 ± 4.4(5)
Elaidic	9.5 ± 0.5(5)	3.9 ± 0.4(5)	19.8 ± 0.9(5)	78.1 ± 1.9(5)
	Livers (nontumor-bearing animals)			
Oleic	5.6 ± 0.2(5)	29.6 ± 0.6(5)	46.8 ± 3.4(5)	43.0 ± 3.3(5)
Elaidic	5.3 ± 0.2(5)	26.5 ± 1.2(5)	58.9 ± 5.4(5)	35.4 ± 3.8(5)

[a]Number of samples.
[b]Significantly different from oleic acid-grown cells ($P<0.001$).
[c]Significantly different from oleic acid-grown cells ($P<0.05$).
[d]Significantly different from oleic acid-grown cells ($P<0.01$).
[e]Significantly different from oleic acid-grown cells ($P<0.02$).
[f]Hepatic lipids of the tumor-bearing animals are significantly ($P<0.05$) different from those of nontumor bearing animals.

Incorporation of Elaidic Acid into Hepatic Lipids of Normal and Tumor-bearing Mice

The fatty acid composition of hepatic lipids of normal and tumor-bearing animals fed the experimental diets are given in Tables IV and V, respectively. It is clear from these data that livers of tumor-bearing animals fed elaidic acid contained a higher concentration of elaidic acid in most of their lipid fractions as compared with livers from normal animals raised on the same diet. Livers of oleic acid-fed animals, normal as well as tumor-bearing, possessed no detectable amounts of elaidic acid in their lipid fractions. As in the case with the EATC, the phospholipid fractions contained the highest concentration of elaidic acid as compared with the neutral lipids upon feeding elaidic acid. The incorporation of elaidic acid in liver tissues (normal and tumor-bearing) was a much higher percentage as compared with the corresponding lipid fractions of EATC. Regardless of the diet, livers from tumor-bearing mice had a higher percentage of saturated fatty acids than those of normal mice. Moreover, the oleic acid concentration of most hepatic lipids decreased as the tumor developed. No consistent pattern can be observed regarding monounsaturated and polyunsaturated fatty acid concentrations in different lipid fractions due to the elaidic acid feeding.

Effect of Elaidic Acid Incorporation into EATC on Tumor Lipid Content

The lipid composition of EATC grown in animals fed either the oleic or the elaidic acid diet are given in Table VI. Feeding the elaidic acid-rich diet to the host resulted in a significant decrease in the major tumor lipids as compared with those grown in animals fed the oleic acid diet. This reduction ranged from 22.5% in cholesteryl esters to 29.4% in triacylglycerols. In spite of these decreases in cellular lipids, the ratio of phospholipid/free cholesterol was unchanged.

On the other hand, this dietary manipulation of the host did not affect the relative composition of the tumor cell phospholipids. The phospholipid composition for elaidic acid grown cells was as follows: choline phospholipids, 46.2%; ethanolamine phospholipids, 23.5%; serine-plus inositol phospholipids, 15.2%; sphingomyelin, 13.4% and lysolecithins, 1.7%. Similar values were obtained in cells grown in mice fed the oleic acid diet.

Effect of Elaidic Acid Incorporation into Ascites Fluid Lipids on Its Lipid Content

The data obtained on the effect of elaidic acid feeding to the host on the ascites lipids are given in Table VI. There was no significant effect of elaidic acid feeding on the lipid content of ascites fluid.

Effect of Elaidic Acid Incorporation on the Hepatic Lipid Content of Normal and Tumor-bearing Animals

The effect of elaidic acid feeding on hepatic lipids in normal animals and tumor-bearing animals is given in Table VI. Elaidic acid incorporation had no significant effect on the hepatic lipid content of normal or tumor-bearing animals. Likewise, the hepatic phospholipid pattern was not affected by the dietary treatment or the development of the tumor. The phospholipid composition of livers from tumor-bearing animals fed the oleic diet was as follows: choline phospholipids, 48.3%; ethanolamine phospholipids, 28.1%; serine-plus inositiol phospholipids, 16.4%; sphingomyelin, 4.8% and lysolecithins, 2.6%. Similar data were obtained for livers of tumor-bearing animals fed the elaidic acid diet. These values were not significantly different from those of livers of normal animals fed the two different diets. Independent of the presence of elaidic acid in the diet, the presence of the tumor caused an increase in the host's hepatic phospholipids and free cholesterol, and a decrease in hepatic triacylglycerols and esterified cholesterol. The reduction in neutral lipids was very prominent in the esterified cholesterol fraction which comprises 80% of total liver cholesterol in the mouse (30). As the tumor develops, this lipid fraction is reduced to 12%-15% of its normal levels. On the other hand, hepatic free cholesterol has almost doubled with the growth of the tumor.

DISCUSSION

The present study indicates that EATC do incorporate elaidic acid upon feeding the host an elaidic acid-rich diet. The level of this incorporation was about one-half of that of the host liver, and very close to that of the ascites fluid. This difference might reflect a difference in the rate of elaidic acid oxidation by tumor cells as compared with the host liver. Alternatively, the incorporation of elaidic acid into different tissues might be under selective control mechanism as suggested by Decker and Mertz (31). Most of the elaidic acid accumulation was in the phospholipid fractions of EATC, normal liver and host liver. Using radioisotopes, Munsch and Pascaud (32) showed the preference of elaidic acid to accumulate in phospholipids, whereas oleic acid accumulates in rat liver triacylglycerols. The present study also showed that the choline phospholipid fraction possessed the highest concentration of *trans* 18:1 in their octadecenoic acids, as compared with other phospholipids. Spector and Steinberg (33) found that choline phospholipids of EATC represent the pool of fatty acids which turns over most rapidly.

Information on the incorporation of *trans* fatty acids in tumor cells is limited. While this manuscript was in preparation, Wood et al. (34) demonstrated the incorporation of *trans* isomers of octadecenoic acid into hepatoma lipids when the host received a partially hydrogenated safflower oil diet. The *trans* fatty acid incorporation into Ehrlich cell lipids in the present study was much higher than reported by Wood et al. (34); 24% of total octadecenoic acid content in some phospholipid fractions as compared with 17% in their hepatoma study. This difference might be due to several factors; i.e., the level of *trans* fatty acid in the diet, the duration of feeding and the type of tumor used. The effect of *trans* fatty acid incorporation in EATC phospholipids on the membrane functional properties warrants further study. Changes in physical properties of artificial membranes (35), alteration in swelling properties of mitochondria (31), membrane permeability (36), and membrane enzyme activities (37) were found to be associated with *trans* fatty acid incorporation into membranes.

The incorporation of elaidic acid in EATC was associated with a decrease in phospholipid polyunsaturated fatty acid concentration without affecting the relative concentration of phospholipid species. The decrease in phospholipid polyunsaturated fatty acids concentration of EATC might be caused by either of two possible mechanisms. The first one is the primary effect of elaidic acid incorporation, while the other mechanism may be the lower concentration of polyunsaturated fatty acids in the elaidic diet as compared with the oleic diet. The data on liver and ascites fluid, however, exclude the last possibility since their polyunsaturated fatty acid concentration was not affected by elaidic acid incorporation. Decker and Mertz (31) found that elaidic acid incorporation in nonmalignant tissues was associated with a decrease in their total polyunsaturated fatty acid concentration.

The resistance of EATC and liver phospholipid classes content ot the present dietary manipulation was in agreement with other reports. These reports showed that even with severe fatty acid manipulation. i.e., essential fatty acid deficiency, the relative concentrations of phospholipids are stable in tumor and nonmalignant tissues (38-40).

It was noticed from these studies that, with much lower levels of elaidic acid incorporation in EATC than those of liver, there was a significant decrease in tumor lipids. Such an effect, however, on hepatic lipids was absent regard-

less of the presence or absence of the tumor. The same was true with the ascites fluid since it originated from the host and not from the tumor (12).

Trans fatty acids are shown to inhibit lipid metabolism in an in vitro mammalian system (41). Elaidic acid, also, has been shown to have a lower rate of β-oxidation by rat heart mitochondria as compared with oleic acid (42,43). Ehrlich ascites cells are known to accumulate lipids as they grow in the peritoneal activities of mice (44). Removal of aged cells by aspiraion increases the rate of tumor growth which results in the disappearance of their cytoplasmic stored lipid droplets. Moreover, King et al. (44) reported that lipids of Ehrlich ascites cells as well as L-strain fibroblasts increase during retardation of cell division and protein synthesis. In the present study, tumors from animals fed a diet rich in *trans* 18:1 fatty acid exhibit decreased lipid content. Therefore, one might expect differences in cell division and protein synthesis between tumor cells that grow in animals fed either the *cis* or *trans* 18:1 resulting in altered tumor growth rates. In vitro studies will be more appropriate to test this hypothesis.

In present study, the hepatic esterified cholesterol and triacylglycerols of the host decrease in the presence of the tumor as compared with livers of normal animals. This is in agreement with the studies of Ruggieri et al. (38) and Carruthers and Kim (45), using hepatoma and transplantable mammary carcinoma, respectively. On the other hand, EATC induced an increase in hepatic phospholipids and free cholesterol of the host which is in agreement with other reports using different types of tumors (45,46). The present study also shows that feeding a diet rich in elaidic acid to tumor-bearing animals resulted in an increase in elaidic acid and decrease in oleic acid concentration in host liver. The increase in hepatic elaidic acid concentration in the presence of the tumor might be due to the increase in liver phospholipids (Table VI), as elaidic acid accumulates predominately in this lipid fraction.

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Distinctive Medium Chain Wax Esters, Triglycerides, and Diacyl Glyceryl Ethers in the Head Fats of the Pacific Beaked Whale, *Berardius bairdi*

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ABSTRACT

Lipids were extracted from the mandibular fat body (jaw), the fatty forehead (melon), and the dorsal blubber of a Pacific beaked whale (*Berardius bairdi*) and separated into lipid classes by preparative thin layer chromatography. The head fats were mixtures of wax esters and triglycerides with a very small amount of diacyl glyceryl ether. The blubber fat contained 97% wax ester and 3% triglyceride. Gas liquid chromatography (GLC) of the intact lipid classes indicated an unusually low C_{26}-C_{30} range for most of the jaw and melon wax esters compared to the more normal C_{32}-C_{40} molecules found in the blubber. Distinctive lower molecular weight C_{24}-C_{40} triglycerides occurred in the head fats vs. the usual C_{44}-C_{58} range in the blubber. Most diacyl glyceryl ethers were in the C_{35}-C_{46} range, below the molecular weight of hexadecyldipalmitoyl glyceryl ether (C_{48}). GLC of the derived fatty acid methyl esters showed that the lower molecular weight neutral lipids in the head fats were due to high levels of iso-10:0, n-10:0, iso-11:0, iso-12:0, n-12:0, and iso-13:0 acids. The wax ester fatty alcohols and the alkoxy chains of the glyceryl ethers were mostly the C_{14}-C_{20} chain lengths commonly observed in marine organisms. The distinctive medium chain neutral lipids in the jaw and melon fats of this whale may be related to the postulated acoustical role of these tissues in echolocation.

INTRODUCTION

Dolphins, porpoises, and toothed whales (the cetacean suborder Odontoceti) are known to use reflected ultrasonic waves for navigational purposes and to locate food (1). The sound generator for this process lies in the nasal passages, directly in front of the skull and right behind the large fatty "melon" in the forehead of the animal. The melon has been postulated to serve as an acoustic lens for concentrating the energy of the emitted sound beam (1). It has also been suggested that the unique fat-filled lower mandibles found in the Odontoceti function in the reception of reflected sound waves by the animal (1).

Our recent survey (2-4) has shown that most odontocete head fats differ markedly from the blubber fats found in the same animal. Possibly this distinctive composition reflects their acoustical function (5). Moreover, lipids of these fatty head tissues differ among the six families of animals within the suborder Odontoceti. In the same survey, we noted that beaked whale head fats produced broad, diffuse triglyceride bands during thin layer chromatography (TLC) and showed a noticeably lower ratio of hydrocarbon to ester absorption in their infrared spectra. We have now conducted a detailed study of the molecular species of neutral lipids found in the head and blubber fats of the Pacific beaked whale, *Berardius bairdi* (family Ziphiidae). We report here on the unusual medium chain wax esters, triglycerides, and diacyl glyceryl ethers found in the fatty head tissues of this animal.

EXPERIMENTAL PROCEDURES

Materials

B. bairdi mandibular fat body (inside lower right jawbone), melon, and dorsal blubber tissues were obtained from a 9.9 m immature male caught near Chyoshi, Japan, in August, 1972. Samples were frozen for air transport to our laboratory where the lipids were extracted with 2:1 chloroform-methanol. Lipid contents of the tissues were: jaw 91%, melon 71%, and blubber 85%. A portion of each lipid sample was hydrogenated in dioxane using a PtO_2 catalyst (6).

Synthetic C_{24}-C_{40} wax esters, C_{15}-C_{60} triglycerides, and C_{48}-C_{52} diacyl glyceryl ethers for identification of gas liquid chromatography (GLC) peaks were purchased from Applied Science Laboratories (State College, PA), Nu-Check-Prep (Elysian, MN), Analabs (North Haven, CT), and Baker Chemical Co. (Phillipsburg, NJ). A mixture of iso-14:0|iso-14:0[1], iso-14:0|n-14:0, n-14:o|iso-14:0, and

[1]The usual shorthand nomenclature for fatty acid and fatty alcohol chains has been extended to wax esters using the convention *alcohol|acid*.

n-14:0|n-14:0 wax esters was synthesized as previously described (7). C_{40} and C_{42} diacyl glyceryl ethers were prepared by acylating 1-hexadecyl and 1-octadecyl glyceryl ethers with lauric anhydride (8,9).

Methods

Lipid class compositions were determined by preparative TLC of the hydrogenated samples on 1.0 mm layers of silicic acid developed in 90:10 petroleum ether-diethyl ether. The major (>1%) bands were individually recovered and quantitated by infrared spectroscopy using calibration curves for the appropriate lipid class and carbon number (2). For example, *B. bairdi* jaw fat triglycerides, which have an average carbon number of 33.0, were quantitated using an interpolated C_{33} calibration curve based on C_{30} (tridecanoin) and C_{36} (trilaurin) absorption data.

Carbon number distributions of the hydrogenated wax ester, triglyceride, and diacyl glyceryl ether bands isolated by TLC were determined by GLC of the intact molecules (7,10-12). Samples were run on a 610 x 2.4 mm ID stainless steel column packed with 3% JXR silicone on 100/120 mesh Gas-Chrom Q (Applied Science Laboratories, State College, PA). Column temperature was linearly programmed from 125 C to 350 C at 4 C min. On-column injection at 320-340 C and a carrier gas flow of 100 ml/min of helium were employed. Peaks were identified by co-chromatography of each sample with known standards. Peak areas measured with an electronic integrator were used to calculate carbon number distributions in mole percent based upon molar calibration factors determined in the usual manner (10).

Unhydrogenated lipids were also fractionated by preparative TLC (as above) for detailed analysis of the constituent acyl and alcohol chains. Triglycerides were converted to methyl esters using 2N methanolic KOH (13). Wax esters were saponified by refluxing 2.5 hr in 20 ml ethanol plus 1.5 ml 50% KOH. The fatty alcohols were then extracted with diethyl ether and were converted to acetates using acetic anhydride. Diacyl glyceryl ethers were similarly saponified and extracted, and the recovered glyceryl ethers were converted to trimethylsilyl (TMS) ethers for GLC (12). Fatty acids were recovered from the aqueous phase of the saponification products by acidulation and extraction; these were reacted with BF_3/CH_3-OH to produce methyl esters (14).

GLC analyses of fatty acid methyl ester, alcohol acetate, and TMS ether samples were carried out on a Perkin-Elmer 900 gas chromatograph equipped with a flame ionization detector using a 46 m x 0.25 mm ID open tubular column coated with butanediolsuccinate polyester and operated at 150 C or 170 C and 50 psig He. Peak identification and quantitation procedures have been described previously (12).

A separate procedure was employed for iso-5:0 analysis. Methyl esters were prepared by KOH-catalyzed methanolysis (15) of the lipid sample using a minimum volume of hexane for methyl ester extraction. The unevaporated extract was immediately injected onto a 1.21 m x 2.4 mm ID stainless steel column packed with 15% OV-101 on 60/80 mesh Gas-Chrom Q (Applied Science Laboratories) at 55 C. After elution of the iso-5:0 peak, the column temperature was raised to 105 C until n-10:0 eluted and then to 200 C to clear all remaining material from the column. The area ratio of the iso-5:0 and n-10:0 peaks was measured and compared with a calibration run on a known mixture of triisovalerin and tridecanoin analyzed in the same manner. The iso-5:0/n-10:0 weight ratio so calculated was then integrated into the total fatty acid analysis from the open tubular column run.

TABLE I

Lipid Class Composition (wt %) of *Berardius bairdi* Jaw, Melon, and Blubber Fats

Lipid class	Jaw	Melon	Blubber
Wax esters	40	26	97
Triglycerides	58	72	3
Diacyl glyceryl ethers	2	2	---

RESULTS

Lipid class analyses (Table I) indicated that *B. bairdi* jaw and melon fats were primarily mixtures of wax esters and triglycerides with a very small amount (2%) of diacyl glyceryl ether. Such compositions are typical of the acoustical fatty head tissues of the Odontoceti (2,3). The blubber fat, however, was composed almost entirely (97%) of wax esters, an unusual phenomenon in mammals apparently found only in the Ziphiidae family of beaked whales (16).

GLC analyses of the intact, hydrogenated *B. bairdi* wax esters isolated by TLC (Table II) revealed that the wax ester compositions of the jaw and melon fats were quite similar to each other but differed greatly from the blubber wax esters. Average carbon numbers were 27.4 in the jaw and 28.0 in the melon, both unusually low values when compared to the 35.5 average

TABLE II

Carbon Number and Isomer Distribution (mole %) of Intact Wax Esters in *Berardius bairdi* Jaw, Melon, and Blubber Fats

Carbon number	Isomer	Jaw	Melon	Blubber
24	iso | n[a]	0.6	1.0	---
	n | n	0.5]	1.3]	---
25	iso | iso	1.3]		---
	iso | n	1.8	1.5	---
26	iso | iso	8.4	3.6	---
	iso | n	12.8	10.1	---
	n | n]	13.0	10.5	---
27	iso | iso]			---
	iso | n	5.7	5.6	---
28	iso | iso	19.7	10.2	---
	iso | n	22.8	21.6	---
	n | n]	4.0	15.0	0.8
29	iso | iso]			---
	iso | n	0.9	1.1	---
30	iso | iso	2.8	2.6	---
	iso | n	3.4	5.0	---
	n | n]	0.7	4.2	3.4
31	iso | iso]			---
	iso | n	---	0.6	---
32	iso | n	0.9	1.9	---
	n | n	0.7	1.8	12.4
33	n | n	---	---	0.6
34	iso | n	---	0.7	---
	n | n	---	0.7	25.1
35	n | n	---	---	0.7
36	n | n	---	0.8	27.8
38	n | n	---	0.2	19.3
40	n | n	---	---	9.1
42	n | n	---	---	0.8

[a]The term iso|n here refers to the combined iso|n and n|iso wax esters, since these two isomers cannot be resolved with the GLC method used (7). Iso actually includes both iso and anteiso chains, but very few of the latter are present (Tables IV & V).

in the blubber (hexadecyl laurate = 28.0; hexadecyl icosenoate = 36.0). Resolution of iso|iso, iso|n and n|n structures in our chromatograms (Fig. 1) indicated a high proportion of branched wax ester structures in the jaw and melon (7). These were not apparent in the blubber wax esters.

GLC analyses of the intact, hydrogenated triglycerides isolated by TLC (Table III) showed a similar difference between the head and blubber fats. Average carbon numbers were 33.0 and 35.2 for the jaw and melon triglycerides vs. 50.5 for the blubber (decanoyldilauroyl glycerol = 34.0; oleoyldipalmitoyl glycerol = 50.0). The complexity of shoulders on peaks in the jaw and melon triglyceride chromatograms again pointed to the presence of high levels of branched fatty acid chains (17).

Carbon number distributions of the diacyl glyceryl ethers in the jaw and melon fats (Table III) were about six carbon numbers higher than those of the corresponding triglycerides. Average carbon numbers calculated for the diacyl glyceryl ethers were 39.2 in the jaw and 41.3 in the melon (hexadecyldilauroyl glyceryl ether = 40.0). C_{38}-C_{42} species were the most prominent in *B. bairdi;* these molecules are much smaller than the C_{48}-C_{54} diacyl glyceryl ethers commonly encountered in marine organisms.

Detailed GLC analyses of the constituent fatty acid chains in the jaw, melon, and blubber lipids are presented in Table IV. They clearly show that the unusually low molecular weight wax ester, triglycerides, and diacyl glyceryl ethers found in *B. bairdi* head fats were due to high levels of C_{10}-C_{13} fatty acids. Iso-10:0, n-10:0, iso-11:0, iso-12:0, n-12:0, and iso-13:0 were prominent in all jaw and melon lipids with the higher levels occurring in the jaw fat. Small amounts of isovaleric acid were also present in the jaw and melon triglycerides, which accounted for the C_{24}-C_{28} molecules reported in Table III. The head lipid fatty acids were mainly saturated (77.0-97.2%). The remaining chains were monoenoic, with 12:1 unexpectedly the most prominent chain length (2.8-6.9%). Polyunsaturates were absent.

In contrast to the jaw and melon lipids, the

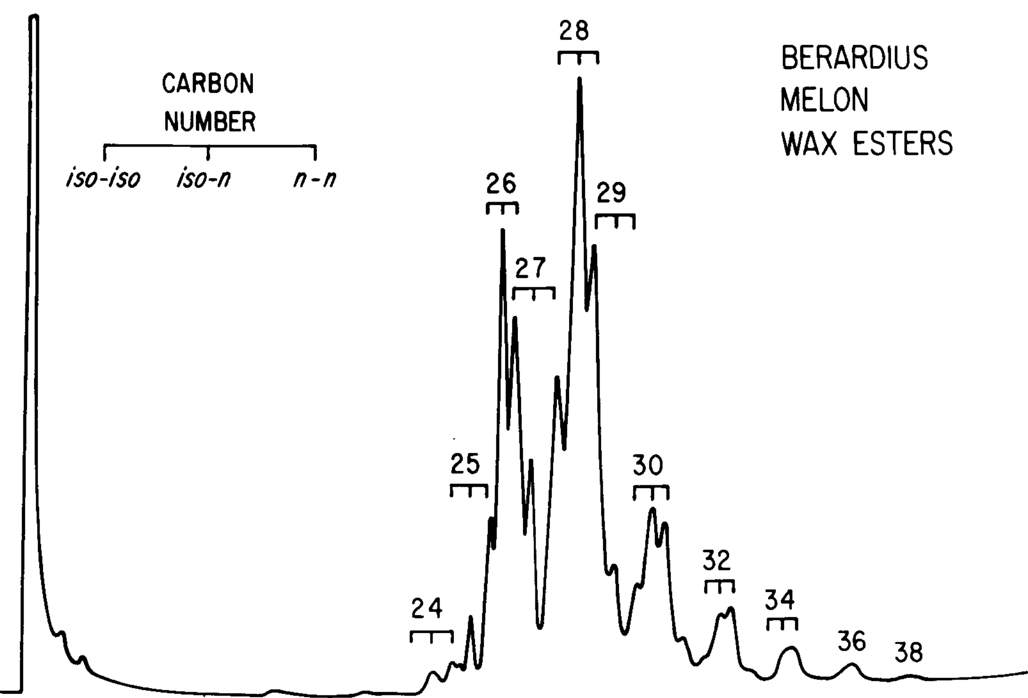

FIG. 1. Gas chromatogram of intact, hydrogenated *Berardius bairdi* melon wax esters on a short, packed JXR silicone column. Note resolution of iso|iso, iso|n, and n|n isomers of the same carbon number. *Operating Conditions:* 610 x 2.4 mm ID stainless steel column packed with 3% JXR silicone on 100/120 mesh Gas-Chrom Q; 100 ml/min. He carrier gas; column programmed 125 C → 350 C at 4 C/min; on-column injection at 325 C; flame ionization detector.

TABLE III

Carbon Number Distribution (mole %) of Intact Triglycerides and Diacyl Glyceryl Ethers in *Berardius bairdi* Jaw, Melon, and Blubber Fats

	Triglycerides			Diacyl glyceryl ethers	
Carbon number	Jaw	Melon	Blubber	Jaw	Melon
24	0.8	0.7	---	---	---
25	1.2	0.5	---	---	---
26	2.2	0.8	---	---	---
27	2.2	0.8	---	---	---
28	2.0	0.9	---	---	---
29 + 30	7.9	3.8	---	---	---
31 + 32	25.6	15.9	---	1.2	---
33 + 34	31.7	26.9	---	3.0	---
35 + 36	15.5	21.6	---	10.9	5.3
38	5.8	10.4	---	38.3	20.8
40	3.2	7.3	1.6	29.0	31.8
42	1.3	4.4	3.1	7.7	17.2
44	0.6	3.2	7.2	3.6	9.4
46	---	1.9	10.2	3.1	6.8
48	---	0.9	14.0	2.6	4.3
50	---	---	16.5	0.6	1.8
52	---	---	17.2	---	1.3
54	---	---	13.7	---	0.8
56	---	---	9.0	---	0.5
58	---	---	5.4	---	---
60	---	---	2.1	---	---

TABLE IV

Fatty Acid Composition (mole % of Lipid Classes in *Berardius bairdi* Jaw, Melon, and Blubber Fats

Fatty acid	Wax esters			Triglycerides			Diacyl glyceryl ethers	
	Jaw	Melon	Blubber	Jaw	Melon	Blubber	Jaw	Melon
iso-5:0	---	---	---	2.8[a]	1.2[a]	---	---	---
iso-10:0	6.3	2.0	---	17.3	6.9	---	8.1	1.1
n-10:0	7.7	5.4	—	15.6	12.2	---	9.4	2.4
iso-11:0	8.4	3.3	---	14.9	7.9	—	8.9	2.0
anteiso-11:0	---	---	---	0.8	—	---	0.9	---
n-11:0	1.2	0.6	—	0.8	0.9	---	1.0	---
iso-12:0	29.3	19.3	---	27.5	25.5	---	31.9	14.7
n-12:0	12.1	19.9	0.7	10.1	17.9	0.5	14.2	18.1
iso-13:0	6.0	4.4	—	4.1	4.3	---	6.6	5.7
anteiso-13:0	0.6	0.7	---	---	---	---	0.8	0.6
iso-14:0	2.2	5.7	---	2.6	5.7	---	6.2	12.3
n-14:0	6.1	5.7	6.9	0.7	3.1	5.5	2.0	7.5
iso-15:0	0.9	1.3	---	---	0.8	---	0.7	2.0
iso-16:0	0.5	1.3	---	---	1.1	---	0.6	2.7
n-16:0	5.1	8.6	6.3	---	2.6	14.0	2.2	6.7
n-18:0	0.7	2.2	1.5	---	0.9	1.3	0.6	1.2
Total saturated	87.1	80.4	15.4	97.2	91.0	21.3	94.1	77.0
12:1ω7	5.2	5.9	---	2.8	3.8	---	4.1	5.5
12:1ω5	0.6	1.0	---	—	0.9	—	---	0.6
14:1ω9	—	1.0	---	---	0.7	—	---	1.6
14:1ω7	---	1.2	---	—	0.7	---	---	1.4
14:1ω5	—	—	1.7	---	---	1.0	—	---
16:1ω11	---	—	1.1	---	---	0.8	---	0.5
16:1ω9	---	1.7	1.3	---	0.9	2.4	0.5	2.3
16:1ω7	1.2	1.8	18.2	—	0.6	18.5	---	2.0
18:1ω11	0.7	0.5	2.9	—	0.5	5.3	---	1.9
18:1ω9	1.6	3.6	27.4	---	0.9	19.9	0.7	4.0
18:1ω7	—	0.6	4.5	---	---	2.9	—	0.7
20:1ω11	0.5	0.6	16.2	---	---	14.9	--	1.3
20:1ω9	1.7	0.9	5.5	---	---	3.1	0.6	0.7
22:1ω13+11	1.4	0.8	4.7	---	---	9.9	---	0.5
22:1ω9	—	—	0.6	—	---	---	—	
Total monoene	12.9	19.6	84.1	2.8	9.0	78.7	5.9	23.0
18:2ω6	—	---	0.5	---	---	---	---	---

[a]Traces of iso-8:0, n-8:0, iso-9:0, and n-9:0 were also present in this sample.

blubber wax esters and triglycerides contained mostly unbranched acyl chains of conventional length (Table IV). The most prominent species were n-14:0, n-16:0, 16:1, 18:1, 20:1, and 22:1 with the monoenoic acids predominating. Curiously, polyunsaturated fatty acids were practically absent (≤0.5%) from the blubber lipids, a phenomenon also observed in the sperm whale (18) and the Amazon River dolphin (12).

Examination of the fatty alcohols from the wax esters and the alkoxy chains from the diacyl glyceryl ethers (Table V) revealed a different spectrum of hydrocarbon chains from that found in the fatty acids. The major alcohol and alkoxy chains were iso-16:0, n-16:0, and 18:1. Saturated chains predominated except in the blubber wax esters, and polyunsaturates were virtually absent. Levels of branched chains were unusually high in the jaw (70.0%) and melon (53.6%) wax esters, moderately high in the jaw (34.1%) and melon (21.9%) diacyl glyceryl ethers, and nearly absent (0.5%) from the blubber wax esters. The C_{10}-C_{13} chain lengths so prominent in the jaw and melon fatty acids were very minor (<1.3%) in the fatty alcohols and glyceryl ethers.

DISCUSSION

The presence of unusually high levels of C_{10} and C_{12} fatty acids in the head fat of the Pacific beaked whale was first reported in 1953 by Saiki (19). His analyses employed fractional distillation techniques, which did not distinguish branched and odd carbon chains. Nevertheless, his findings of 21.2 wt % 10:0 and 31.9% 12:0 for total *B. bairdi* head fat (i.e.,

TABLE V

Fatty Alcohol Composition (mole %) of Wax Esters
and Alkoxy Chain Composition of Diacyl Glyceryl Ethers in
Berardius bairdi Jaw, Melon, and Blubber Fats

Alkyl chain	Wax esters			Diacyl glyceryl ethers	
	Jaw	Melon	Blubber	Jaw	Melon
n-12:0	---	---	---	1.3	1.1
n-13:0	0.8	---	---	---	---
n-14:0	2.5	4.1	4.1	2.8	1.8
iso-15:0	9.6	6.4	---	2.6	1.1
n-15:0	2.6	1.7	0.5	2.3	1.0
iso-16:0	58.4	45.6	0.5	29.7	18.7
n-16:0	22.2	32.7	22.1	46.8	43.2
iso-17:0	2.0	1.6	---	1.8	2.1
n-17:0	---	---	---	1.0	0.6
n-18:0	0.7	1.3	4.3	3.7	2.9
Total saturated	98.8	93.4	31.5	92.0	72.5
16:1ω11	---	0.6	---	---	---
16:1ω9	---	0.5	0.7	0.9	---
16:1ω7	---	1.0	6.3	0.7	---
18:1ω11	---	---	44.0 (18:1ω11 + 18:1ω9 + 18:1ω7 bracketed)	0.6	7.3
18:1ω9	1.2	3.5		5.8	20.2
18:1ω7	---	1.0	8.9	---	---
18:1ω5	---	---	1.1	---	---
20:1ω11	---	---	3.9	---	---
20:1ω9	---	---	3.6	---	---
Total monoene	1.2	6.6	68.5	8.0	27.5

jaw + melon) are reasonably similar to the GLC data reported in Table IV. Saiki's distillations also detected a small amount of isovaleric acid (2.1 wt %) as we do, but he did not analyze the lipid class composition of his sample.

Saiki went on to study the fatty acid (20) and fatty alcohol (21) compositions of *B. bairdi* blubber fat by fractional distillation techniques. His results are roughly comparable to our findings. However, he reported substantially higher levels of C_{20}+C_{22} acids (51.6%) than we found, as well as more polyunsaturation. Saiki's fatty alcohol analyses are not comparable to our results. He performed a urea fractionation on the unsaponifiables prior to distillation, which would have removed many of the branched chain alcohols we detect by GLC. His additional study (22) on the visceral organs revealed $<1\%$ C_{10}-C_{12} fatty acids in the fat obtained by boiling the liver and intestines of the animal. This finding implies that the C_{10}-C_{13} fatty acids found in *B. bairdi* are of endogenous origin.

Fatty acid biosynthesis in the adipose head tissues of the Pacific beaked whale apparently exhibits the same specificities found in other Odontoceti, but the system is controlled to produce a different balance of products. The high levels of iso chains $\geqslant C_{10}$ (37.3-66.4%) in the jaw and melon lipids indicate an extremely active elongation of iso-4:0 and iso-5:0 precursors (from amino acids) permitting little direct esterification of isobutyric and isovaleric acids in the neutral lipids as occurs in the bottlenosed dolphin *Tursiops truncatus* (7,23), the beluga whale *Delphinapterus leucas* (24), and the porpoise *Phocoena phocoena* (25). Chain termination at the C_{10}-C_{13} level is certainly more prominent in *B. bairdi* than in any non-Ziphiidae species. On the other hand, fatty alcohol biosynthesis follows the same specificity characteristics found in most other Odontoceti (7,12,18); i.e., branched chain acids are readily reduced, but chain lengths $<C_{14}$ and polyunsaturates are not.

The distinctive medium chain wax esters, triglycerides, and diacyl glyceryl ethers found in the melon and jaw fats of the Pacific beaked whale may well be related to the postulated role of these fatty tissues in odontocete echolocation (1). In all odontocete genera examined to date (2-5,12,16,18), the melon and jaw fats contain lower molecular weight lipids than the blubber. For an homologous series of fatty compounds, ultrasonic velocity decreases as the molecular weight becomes smaller (26). Major variations in lipid composition have been reported within the melon tissues of several Odontoceti (27-30) which would cause the refraction of sound waves

passing through the tissue. Acoustical consideration of these results suggests that the melon lipid topography is designed to refract and concentrate the ultrasonic pulses generated by the animal for more effective echolocation (28,31). Considerable further study of the anatomy and of the lipid topography of the *B. bairdi* melon is required before we will know if the melon of this ziphiid also functions as a sound refraction device. However, the presence of high levels of low molecular weight neutral lipids makes this possibility seem likely.

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Incidence and Severity of Experimental Allergic Encephalomyelitis and Cerebral Prostaglandin Synthesis in Essential Fatty Acid Deficient and Aspirin-Treated Rats[1]

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ABSTRACT

Experimental allergic encephalomyelitis (EAE) was induced in rats of the Lewis strain fed diets adequate or deficient in essential fatty acids (EFA). After induction of the disease, the diets were supplemented with aspirin (3.75 g/kg diet), and the effects of the drug on the course of EAE and on the synthesis of prostaglandin F (PGF) by brain slices from diseased animals and their Freund controls were examined. Aspirin supplementation delayed the onset of EAE in both dietary groups. EFA-deficient rats experienced an incidence and severity of the disease similar to that of aspirin-free, EFA-deficient rats, while the EFA-adequate group showed a greater severity but not an increased incidence, compared to aspirin-free controls. Aspirin treatment led to an increased PGF production by brain slices from rats on either diet and not subjected to an immunochallenge. When the diet was deficient in EFA, challenge with antigen plus adjuvant or adjuvant alone tended to decrease PGF synthesis by brain slices, and when the diet was adequate in EFA, immunochallenge caused a marked depression on PGF synthesis. It was concluded that the PG synthetase inhibitor aspirin can alter the course of EAE in the rat, providing further evidence that PGs or related metabolites may be involved in the immune response in this disease.

The inhibition of prostaglandin (PG) synthetase by aspirin and indomethacin in several in vitro systems was reported by Ferreira et al. (1), Smith and Willis (2) and Vane (3). Since then, the list of agents that inhibit PG synthesis has been extended to include many nonsteroidal antiinflammatory drugs (4,5). There are other reports that the PG synthetase from different tissues may possess differential sensitivities toward the aspirin-like drugs (6-9). It has also been reported that responses to the nonsteroidal, antiinflammatory drugs in vivo may be different from the responses in vitro (7,10,11). Pretreatment of rats with indomethacin apparently does not influence the ability of brain homogenates to synthesize PGs (12), while exposure of brain slices to indomethacin in the incubation medium depresses PG synthesis (13). Aspirin has been shown to inhibit PG synthetase in brain homogenate when added to the incubation medium (6). That aspirin depressed PG synthesis in brain in vivo is indicated by the reduction in aspirin-treated cats of endotoxin-induced fever, which is believed to be mediated by PGs (14).

Early work by Good et al. (15) indicated that sodium salicylate inhibited the development of experimental allergic encephalomyelitis (EAE) in guinea pigs, by delaying onset of symptoms or preventing them, and by reducing mortality. Kolb et al. (16) were unable to repeat these findings in guinea pigs when they used acetylsalicylic acid as the preventive agent. Sodium salicylate is of uncertain value as a PG synthetase inhibitor (7,17,18). The course of EAE in guinea pigs is different from that seen in rats, and it has been suggested that there might be important differences in immunologic mechanisms responsible for EAE from one species to another, and that work with one might not apply to others (19). Studies on EAE in the rat have shown that rats raised on a fat-free diet are more susceptible to EAE than rats fed a diet adequate in essential fatty acids (EFA) (20,21). We have previously shown (22) that PG synthesis by brain slices of EFA-deficient rats is depressed from that of rats fed a diet adequate in EFA. Moreover, we also showed (22) that the synthesis of PGs by cerebral cortex slices from rats suffering an immunochallenge, including EAE, is also depressed from that seen in unchallenged animals. One aim of this present study was to determine if pretreatment of rats with aspirin would change the ability of brain slices to synthesize PGs. Additionally, in view of the reports on the modification of EAE by salicylates, and because of the involvement of salicylates in inhibition of PG synthesis, a study of the effects of aspirin on the course of EAE in the Lewis rat and on the activity of PG synthetase in brain slices from these rats was

[1] Part of a thesis submitted by Patricia G. Weston to the University of Illinois in partial fulfillment of requirements for the Ph.D degree.

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included.

MATERIALS AND METHODS

Dietary Treatment

Male weanling rats, inbred Lewis/Mai F strain (Microbiological Associates, Walkersville, MD), were divided into two dietary groups. Groups 1 and 2 were reared on diets adequate and deficient in EFA (22). The animals were housed individually in polypropylene "shoebox" cages with Sanicel bedding (Paxton Processing Company, Inc., Paxton, IL). A diurnal light cycle of twelve hours was maintained. Food and water were available *ad libitum* and food intake was noted. On the day of induction of EAE, the rats were moved to new cages with fresh bedding, and their previous diets were supplemented with aspirin (Pure Pac Pharmaceutical Company, Elizabeth, NJ) at the level of 3.75 g/Kg diet.

Induction of EAE

At 70-80 days of age, each dietary group was divided into two groups that received one of the following treatments: (1) a single injection of 0.25 ml into each hind foot pad of Freund's adjuvant (Difco Laboratories, Detroit, MI) containing *Mycobacterium tuberculosis H37Ra*, homogenized with an equal volume of a saline suspension of 35 mg of fresh adult guinea pig spinal cord (EAE), or (2) a single injection of 0.25 ml into each hind foot pad of Freund's adjuvant containing *M. tuberculosis H37Ra* homogenized with an equal volume of 0.9% saline (FC). Several animals from each diet were used as controls (no treatment). After injection, each animal was transferred to the aspirin-supplemented diet, weighed daily, and examined daily for signs of EAE. From the first incidence of clinical symptons, two observers graded each rat, using the following scoring system: weight loss of at least 10 g, 1; fecal impaction, 1; urinary incontinence, 1; paresis, 2; paralysis, 4; paralysis plus lethargy, 5.

Determination of PGF Synthesis by Brain Slices

Preparation and incubation of cerebral cortex slices were carried out as previously described (22). Paralyzed animals were killed in the acute phase of the disease. Those animals whose only sumptom was weight loss were killed when weight loss had ceased and weight gain was again apparent. Animals free of clinical symptoms were killed 28 days postinduction. Freund's control animals were killed at the same time as a weight-matched paralyzed rat, and the unchallenged animals were killed after 7 days on the aspirin-supplemented diet. Radioimmunoassay for PGF was carried out as previously described (22).

Salicylate Assay

Blood was collected with a heparinized syringe from the abdominal aorta of the rats, and the plasma was separated from the cells in heparinized tubes by centrifugation at 600 x g. Salicylate levels in the plasma were determined by the method of Trinder (23), and modified for ultramicro determination by Hanok (24). An aliquot of 0.15 ml of plasma was added to 3.0 ml of Trinder's reagent (40 g $HgCl_2$, 62 g Fe $(NO_3)_3 . 9H_2O$, 120 ml 1N HCl/liter soln.). The tube was shaken vigorously, allowed to stand for 5 min, centrifuged for 5 min to remove the precipitated protein, and then allowed to stand for 20 min. The absorbance of the supernatant was determined on a Spectronic 20 Spectrophotometer (Bausch and Lomb, Rochester, NY) at a wavelength of 546 nm. The salicylate concentration was calculated from a standard curve, constructed by using sodium salicylate levels ranging from 10 μg to 150 μg.

RESULTS

An initial weight loss immediately after the injections, in both EAE and FC rats, was followed by slow growth again until the first clinical signs of EAE were observed, usually an abrupt weight loss. Paralysis, when it occurred, was usually observed two to three days after the first signs of weight loss. There was one incidence of adjuvant-induced arthritis in the FC group, in an EFA-adequate rat. This disease was distinguishable from the clinical symptoms of EAE by the swelling of the hind limbs and the maintenance of weight and appetite in the arthritic animal. The days postinduction when symptoms were first observed and the incidence and severity of EAE are shown in Table I for rats on EFA-adequate and EFA-deficient diets. An aspirin-free group is included for comparison. The aspirin-supplemented rats on both diets showed a significant delay in onset of the disease, when compared to the rats on the aspirin-free diets. The incidence of the disease was not markedly different between EFA-adequate and EFA-deficient groups, or between the aspirin-free and the aspirin-supplemented groups. However, the severity, as measured by the presence of total caudal paralysis or by the scoring system, was more marked in the aspirin-supplemented EFA-adequate rats, compared to all other groups.

Salicylate levels in the plasma of all groups of rats on aspirin-supplemented diets were

TABLE I

Incidence and severity of experimental allergic encephalomyelitis in rats fed diets adequate or deficient in essential fatty acids (EFA-A or EFA-D), and/or supplemented with aspirin, and challenged with guinea pig spinal cord in adjuvant containing *Mycobacterium tuberculosis H37Ra*, at 70-80 days of age

Diet	Aspirin-free diet[a]		Aspirin-supplemented diet	
	EFA-A	EFA-D	EFA-A	EFA-D
Incidence	11/11 (100%)	25/25 (100%)	22/25 (88%)	25/26 (96%)
Total caudal paralysis	5/11 (46%)	13/25 (52%)	19/25 (76%)	15/26 (58%)
Score	3.82	4.48	5.40	4.57
Onset (days)	13 ± 1	12 ± 1	16 ± 2	17 ± 2
range	(12-16)	(9-14)	(14-21)	(15-21)
Weight at induction (g)	331 ± 27	286 ± 17	281 ± 20	258 ± 22

t test for day of onset
Aspirin-free diet: EFA-A vs. EFA-D, $p = 0.009$.
Aspirin-supplemented diet: EFA-A vs. EFA-D, $p = 0.09$.
EFA-A: aspirin-free vs. aspirin-supplemented, $p = 0.0001$; EFA-D: aspirin-free vs. aspirin-supplemented, $p < 0.00005$.

t test for weight at induction
Aspirin-free: EFA-A vs. EFA-D, $p = 0.0001$.
Aspirin-supplemented: EFA-A vs. EFA-D, $p = 0.0003$.
EFA-A: aspirin-free vs. aspirin-supplemented, $p < 0.00005$.
EFA-D: aspirin-free vs. aspirin-supplemented, $p < 0.00005$.

[a]Data from Reference 22.

TABLE II

Serum salicylate levels of rats fed diets adequate or deficient in essential fatty acids (EFA-A or EFA-D), supplemented with aspirin and challenged with guinea pig spinal cord in adjuvant (EAE), challenged with adjuvant only (FC), or unchallenged, at 70-80 days of age

Diet	Serum salicylate mg/100 ml	
	EFA-A	EFA-D
Unchallenged	26.0 ± 2.8(5)[a]	25.2 ± 5.6(4)
EAE	15.0 ± 4.9(25)	18.6 ± 7.9(26)
FC	23.8 ± 7.1(9)	26.8 ± 5.9(10)
Paralyzed	14.0 ± 5.1(19)	15.4 ± 6.5(15)
Nonparalyzed	18.2 ± 2.3(6)	23.0 ± 7.6(11)
Rats fed aspirin-free diet	4.5 ± 3.5(5)	

Student's t test for salicylate levels:
EFA-A: unchallenged vs. EAE, $p < 0.0001$; FC vs. EAE, $p = 0.0003$.
EFA-D: EAE vs. FC, $p = 0.005$.
All others not significant.

[a]Mean value ± SD for number of determinations indicated in parentheses.

significantly elevated over the levels from rats on aspirin-free diets (4.5 ± 3.5 mg/100 ml). The depressed levels of salicylate in diseased rats on both diets were significantly different from levels in both Freund controls and unchallenged animals in the case of the EFA-adequate diet, but significantly different from levels in Freund controls only for rats on the EFA-deficient diet (Table II).

The PGF synthesis by brain slices from unchallenged, aspirin-treated rats from both dietary groups is shown in Table III. The levels of PGF are higher than those we previously reported for unchallenged rats fed the same diets without the aspirin (22). Thus, for EFA-adequate rats not fed aspirin, the PGF level was 7.16 ± 1.72 ($n = 8$) vs. 13.47 ± 2.96 for aspirin-fed rats (Table III). This difference is highly significant ($p = 0.0001$). The increase in PGF synthesis in the EFA-deficient rats receiving aspirin (8.14 ± 4.81) is not significantly different from the same dietary groups not fed aspirin (5.01 ± 2.47; $p = 0.08$). In accord with our previous findings (22), brain slices from

TABLE III

Biosynthesis of prostaglandin F in brain slices of rats fed diets adequate or deficient in essential fatty acids (EFA-A or EFA-D), supplemented with aspirin, and challenged with guinea pig spinal cord in adjuvant (EAE), challenged with adjuvant only (FC), or unchallenged, at 70-80 days of age

Diet	Nanograms PGF/100 mg wet weight	
	EFA-A	EFA-D
Unchallenged	13.47 ± 2.96(8)[a]	8.14 ± 4.81(10)
EAE	5.19 ± 2.70(44)	4.56 ± 2.11(44)
FC	4.83 ± 2.75(18)	5.52 ± 1.98(18)
Paralyzed	5.58 ± 2.81(34)	4.36 ± 2.04(25)
Nonparalyzed	3.90 ± 1.87(10)	4.83 ± 2.23(19)

Student's t test for prostaglandin levels:
EFA-A: unchallenged vs. EAE, $p < 0.00005$; unchallenged vs. FC. $p < 0.00005$.
EFA-D: unchallenged vs. EAE, $p = 0.05$; unchallenged vs. FC, N.S.
Unchallenged: EFA-A vs. EFA-D, $p = 0.01$.
All others not significant.

[a]Mean value ± SD for number of determinations indicated in parentheses.

unchallenged animals on the EFA-adequate diet produced more PGF than did slices from rats on the EFA-deficient diet, and this difference is significant (Table III).

When the diet was adequate in EFA, a challenge with antigen plus adjuvant or with adjuvant alone caused a significant depression in PGF synthesis by brain slices (Table III). On the EFA-deficient diet, a challenge with antigen plus adjuvant led to a marginally significant decrease in PGF synthesis, but the apparent depression in PGF synthesis caused by a challenge with adjuvant alone was not significantly different from the PGF level of the unchallenged animals (Table III). The immunological challenge alone appeared to decrease PGF synthesis, since in no case was there a significant difference between the synthesizing ability of brain slices from diseased animals compared to Freund controls. Whether or not rats were paralyzed had no effect on the PGF synthesis of the brain slices from either dietary group (Table III).

DISCUSSION

The detection of PGF levels in brain slices of aspirin-supplemented EFA-adequate rats, which are elevated over the levels produced by rats on aspirin-free, EFA-adequate diets, is apparently contradictory to previous reports which have shown aspirin to be a PG synthetase inhibitor (4,5). Aspirin has been shown to depress PG synthesis when added to the incubation medium of brain slices (25) and of brain homogenate (6). Prostaglandin synthesis is also apparently depressed in in vivo situations after aspirin has been ingested. Milton (14) reported a reduction in aspirin-treated cats of endotoxin fever, which is believed to be mediated by PGs. Higgs et al. (18) reported the reduction of PG levels in inflammatory exudates in rats which had ingested aspirin for 24 hr following implantation of carrageenin-treated sponges. Ingestion of aspirin by human volunteers markedly reduced PG synthesis by N-ethylmaleimide-stimulated platelets (26). This in vitro incubation is similar to the incubations carried out in the present work; however, the platelet samples were collected 2 hr after the ingestion of the aspirin. This type of sampling, and the samplings in the other reports in which the aspirin treatment was short term, cannot be compared directly to the situation in which the rats had ingested aspirin in their food for a period of seven days. It is possible that the enzyme sustems had adjusted to the constant presence of the inhibitor and that more PG synthesizing enzyme had been induced. Another possibility is that when the drug is ingested the brain enzyme responds to aspirin differently from other tissues. For example, Dembinska et al. (12) reported that pretreatment of rats with indomethacin apparently did not influence the ability of brain homogenates to synthesize PGs, while in homogenates of lungs, kidneys and seminal vesicles, the synthetase was strongly depressed. However, indomethacin in the incubation medium depressed PG synthesis by rat brain slices (13) and rabbit brain homogenate (6).

When the PG levels from brain slices of aspirin-supplemented and aspirin-free rats (22) were compared, the same trends towards depressed PGF synthesis in EFA-deficiency and in immunochallenge were apparent. As in the case with aspirin-free rats, the trend towards decreased PGF synthesis in immunochallenge was

consistently significant only when the diet was adequate in EFA. In EFA-deficient rats, there appeared to be a tendency for decreased PG syntheiss on immunochallenge, but because the PGF synthesis was already depressed by the deficiency, further depression to levels approximately the same as those seen in the EFA-adequate group did not prove to be statistically significant.

Because diseased rats suffered from anorexia for several days before they were killed, their plasma salicylate levels at death were lower than those of unchallenged or Freund controls. However, their daily weight gains and food intakes indicated that until the abrupt weight loss signaling the onset of disease, these rats also had intakes of aspirin similar to those of the controls and challenged rats which showed no symptoms. It is thus reasonable to assume that the aspirin levels were similar in all groups during the induction phase of the disease.

The delay in the onset of EAE seen in the aspirin-supplemented rats as compared to the aspirin-free rats, on both EFA-adequate and EFA-deficient diets, could be due to the lymphocyte response reported by Dewse (27). This investigator reported that aspirin inhibited DNA synthesis is stimulated lymphocytes in vitro. Such inhibition would be expected to be immunosuppressive in that depression of DNA synthesis would lead to a reduction in lymphocyte proliferation. However, Snider and Parker (11) reported that when aspirin was ingested by human volunteers, there was a decrease in the resting cyclic AMP levels of their lymphocytes sampled 30 min later, and a partial inhibition of the rise in cyclic AMP induced by PGF_1, suggesting that aspirin in vivo could abolish the immunosuppressive effects of PGE_1.

The observed delay agreed with the report of Good et al. (15) who found that sodium salicylate administration caused a delay of onset of EAE in guinea pigs. However, in spite of the delay observed in the present work, the course of the disease in the aspirin-supplemented rats eventually became as severe as the disease in the aspirin-free controls in the case of the EFA-deficient rats, and it became even more severe for the EFA-adequate group, as measured by both the score and the presence of total caudal paralysis. These observations contrasted sharply with those of Good et al. (15), who reported a reduction in symptoms and mortality in sodium salicylate-treated guinea pigs, but they confirmed the findings of Kolb et al. (16), who reported that aspirin could not be considered an effective preventive agent for EAE in guinea pigs. Explanation of this discrepancy in results from all three groups could rest on the differences in efficacy between aspirin and sodium salicylate as a preventive agent and on the differences in the course of the disease in guinea pigs and Lewis rats. Moreover, Good et al. (15) used an adjuvant containing *Mycobacterium butyricum*, an agent of lower potentiating ability than the adjuvant containing killed tubercle bacilli used by Kolb et al. (16) and the Freund's containing *M. tuberculosis H37Ra* used in this report (28). The less powerful adjuvant used by Good et al. (15) may have been responsible for the ability of the salicylate to overcome the disease, whereas this effect was not seen with the greater immunopotentiation offered by the tubercle bacilli. In spite of differences between species and adjuvant composition, it is clear that PG synthetase inhibitors such as aspirin can alter the course of EAE providing further evidence that PGs or related metabolites may be involved in the immune responses in this desease (22).

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Esterification of Palmitic Acid in Swine Aortic Homogenates

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ABSTRACT

The incorporation of [1-^{14}C]palmitic acid into tissue lipids of the medial and intimal layers of swine aortic homogenates was investigated. The homogenates obtained were metabolically active as indicated by their ready incorporation of labeled palmitic acid into phospholipids, diglycerides and triglycerides in the presence of α-glycerophosphate in the incubation medium. Predominantly, labeling of phospholipids and especially of phosphatidylcholine was found when α-glycerophosphate or lysolecithin served as the fatty acid acceptor. Glycerol and monoolein did not serve as fatty acid acceptors. More than 98% of the radioactivity was recovered as the phosphatidylcholine fraction at the level of 0.64 μmoles/ml of lysolecithin in the incubation medium.

INTRODUCTION

Stein and Stein (1) reported that aortic slices from rat, dog, rabbit and baboon could incorporate labeled linoleic acid into tissue lipids. Previous work with normal-fed pigs in vitro showed that most of the oleic acid taken up by the aorta was incorporated into phospholipids and triglycerides with little incorporation into cholesterol ester (2). Stein et al. (3) later demonstrated that in rabbit and dog aortic homogenates, labeled linoleate was incorporated into phospholipids and neutral lipids, with diglycerides and triglycerides being the major radioactive components in the neutral lipid fraction. It was suggested that, except for the α-glycerophosphate pathway for lecithin synthesis in rabbit aorta, there appeared to be an alternative pathway in which lysolecithin served as an intermediate in the synthesis (3). However, Portman (4) reported that the cell-free preparation of rat aorta was markedly stimulated by lysolecithin but not by α-glycerophosphate. Incorporation of labeled fatty acid into lipid fractions from aortic homogenates (3,4) and adipose tissue homogenates (5) was shown to be dependent upon the presence of ATP, coenzyme A and α-glycerophosphate.

The present studies were designed to investigate the incorporation of labeled palmitate into aortic lipid constituents. The effect of fatty acid acceptors (i.e., α-glycerophosphate and lysolecithin) or in the presence of alternate precursors (glucose, glycerol and monoolein) on the formation of tissue lipids in swine aortic homogenate system was investigated. The incorporation of labeled palmitate into individual phospholipids under different conditions was also studied.

MATERIALS AND METHODS

Chemicals

[1-^{14}C]Palmitic acid, obtained from New England Nuclear (Boston, MA.) was purified by thin layer chromatography (TLC). Nonradioactive potassium palmitate was obtained from ICN Pharmaceuticals, Inc. (Plainview, NY). Bovine serum albumin (fatty acid free), CoA, ATP-Na, egg lysolecithin, α-glycerophosphate, and monoolein were purchased from Sigma (St. Louis, MO).

Animals and Diets

Crossbred (Yorkshire/Hampshire) 6-month-old swine weighing 100-110 kg were used. The swine were fed a grain diet which contained 87.25% ground yellow corn, 10% solvent-extracted defatted soybean meal, and 2.75% premixed multiple vitamins and minerals (6). Food and water were available ad libitum.

Preparation of Tissue Homogenization

Within 30 min of slaughter, the aortas were removed and kept in ice-cold physiological saline solution. The adventitia together with the outer third of the media from the thoracic aorta was removed; only the intima and the inner two thirds of the media were used. The tissue was finely minced with scissors and homogenized with 6 vol of KCl-Tris buffer [0.15 M KCl - 0.5 M Tris (pH 7.4), 19:1 and 0,1 mM EDTA-Na_2] in a polytron homogenizer (Brinkmann Instrument, Westbury, NY) at a rheostat setting of 5 for 20-40 sec. The homogenate was centrifuged as 600 x g for 15 min in a refrigerated Beckman J-21 B centrifuge, and the supernatant was made up to volume with KCl-Tris to bring the protein concentration to 3-6 mg/ml. This suspension was designated "aortic homogenate" and was used as the esterifying enzyme source. All operations were carried out at 0-4 C.

Incubations

All incubations were carried out in a Gyrotory water bath shaker at 37 C for 60 min

TABLE I

Incorporation of [1-^{14}C]Palmitic Acid into Lipids of Swine Aortic Homogenates[a]

Experiment number	Fatty acid incorporated nmoles/mg protein			
	Phospholipids	Diglycerides	Triglycerides	Cholesteryl ester
1	8.3	2.5	2.9	0.08
2	7.5	1. 1.9	2.4	0.05
3	8.3	2.3	2.7	0.07
4	8.6	2.2	1.2	0.10
Mean ± SEM	8.2 ± 0.2	2.2 ± 0.1	2.3 ± 0.2	0.08 ± 0.01

[a]The incubation medium consisted of 0.25 μmole [1-^{14}C]palmitic acid with sp. act. of 4 μCi/μmole, 10 μmoles Na-ATP, 0.5 μmole CoA, 20 μmoles K-phosphate buffer (pH 7.4), 10 μmoles $MgCl_2$, 10 μmoles Na-α-glycerophosphate, 20 μmoles KF, 0.03 μmoles fatty acid-free bovine serum albumin and 2 ml of aortic homogenate containing 3-6 mg protein. Final vol., 3.0 ml, incubated in air at 37 C with shaking for 60 min.

under air in a final volume of 3 ml of potassium phosphate buffer (0.02 M, pH 7.4), 0.25 μmole [1-^{14}C]palmitic acid with specific activity of 4 μCi/μmole, 0.03 μmoles fatty acid-free bovine serum albumin, 10 μmoles Na-ATP, 0.5 μmole CoA, 20 μmoles KF, and various concentrations of α-glycerophosphate, glucose, glycerol, mono-olein and lysolecithin, and aortic homogenates containing 3-6 mg protein were used in these studies.

Extraction and Lipid Fractionation

After 1 hr of incubation, the reaction was stopped by 12 ml of chloroform-methanol (2:1, v/v). The mixture was mixed thoroughly and allowed to separate into two layers. The chloroform phase was filtered into 4 ml vials and evaporated to dryness under a stream of nitrogen, and the residue was redissolved in a small amount of chloroform-methanol (2:1) solution.

Separation of the lipids into phospholipids, diglycerides, free fatty acids, triglycerides and cholesteryl esters was performed by TLC (instant TLC, polysilicic acid gel impregnated glass fiber sheets, Gelman Instrument Co., Ann Arbor, MI) with petroleum ether (skelly B)-ethyl ether-acetic acid (85:15:1, v/v/v) as solvent system. After development, the chromatogram was dried in the air and lipid spots, the individual spot corresponding to the known standard was circled with pencil, cut with scissors, and placed in a scintillation vial. Individual phospholipids were separated by two dimensional TLC using a modification of the method of Parsons and Patton (7). Silica Gel H plates were developed in the first dimension with chloroform-methanol-15 M ammonium hydroxide-water (130:70:10:5) and in the second dimension with chloroform-acetone-methanol-glacial acetic acid-water (100:40:20:20:10). Following TLC on phospholipid plates, the appropriate spots were visualized with iodine vapor and scraped directly into counting vials.

Scintillation Counting

The vials were filled with 10 ml of Bio-Solv scintillation cocktail (5 g PPO, 300 mg POPOP, 130 ml methanol and 100 ml Bio Solv and diluted to 1000 ml with toluene) and assayed for radioactivity in a Packard scintillation-counter. Quenching was corrected by the use of an external standard.

Protein Measurement

Protein concentrations were determined by the method of Schaterle and Dallack (8) with bovine serum albumin as standard.

RESULTS

Swine aortic homogenates, incubated with [1-^{14}C]palmitic acid, were found to incorporate the labeled fatty acid into tissue lipids (Table I). It is evident that [1-^{14}C]palmitic acid was incorporated to a larger extent into phospholipids than into diglycerides, triglycerides and cholesteryl ester. The incorporation of palmitic acid into cholesteryl ester is extremely small in the aortic homogenates, comprising only 0.4-0.8% of the total label that was incorporated into the lipids evaluated.

Various concentrations of α-glycerophosphate and alternate precursors were used as the fatty acid acceptors, as shown in Table II. The incorporation of fatty acid into lipid fractions was minimal when α-glycerophosphate was omitted from the incubation medium. With increasing concentrations of α-glycerophosphate, increasing amounts of [1-^{14}C]palmitic acid were incorporated into phospholipids and glycerides. When glucose was added to the incubation medium, it stimulated the esterification of labeled palmitic acid into phospholipids and

TABLE II

Incorporation of (1-^{14}C)-Palmitic Acid into Lipids of Swine Aortic Homogenates at Various Concentrations of Precursors[a]

Precursor added (μmoles/ml)	Fatty acid incorporated nmoles/mg protein			
	Phospholipids	Diglycerides	Triglycerides	Cholesteryl ester
None	1.02	0.23	0.13	0.05
α-Glycero-phosphate				
0.5	5.1	1.4	1.6	0.05
1.0	6.0	1.9	2.0	0.05
2.0	7.4	2.6	2.4	0.06
5.0	8.2	2.6	2.5	0.05
10.0	55.5	1.6	1.2	0.06
Glucose				
1.7	2.1	0.26	0.29	0.06
3.0	2.2	0.69	0.40	0.08
10.0	2.1	0.78	0.34	0.08
Glycerol				
3.0	1.01	0.44	0.20	0.10
10.0	1.02	0.43	0.20	0.09
Monoolein				
3.0	0.93	0.42	0.25	0.06
10.0	0.62	0.50	0.23	0.04

[a]Conditions of incubation as in Table I.

TABLE III

Effect of Varying Concentrations of Lysolecithin on Lipid Synthesis in Swine Aortic Homogenates[a]

Lysolecithin added (nmoles/ml)	Fatty acid incorporated (nmoles/mg protein)			
	Phospholipids	Diglycerides	Triglycerides	Cholesteryl ester
None	1.0	0.23	0.13	0.06
2.7	1.5	0.25	0.11	0.06
8	2.6	0.15	0.09	0.05
27	3.4	0.11	0.08	0.05
53	3.8	0.12	0.08	0.06
107	4.7	0.15	0.08	0.05
320	7.1	0.11	0.07	0.03
640	9.8	0.10	0.04	0.03
1280	8.4	0.15	0.05	0.04
2560	6.0	0.05	0.04	0.03

[a]Conditions of incubation as in Table I; α-glycerophosphate was replaced by lysolecithin.

glycerides. Addition of glycerol or monoolein to the incubation medium did not show any significant effect on the incorporation of [1-^{14}C] palmitic acid into tissue lipids.

The role of lysolecithin as a fatty acid acceptor was further investigated. Lysolecithin acts as a substrate for the incorporation of [1-^{14}C] palmitic acid into phospholipids (Table III). Lysolecithin addition had no effect on the esterification of labeled palmitic acid into neutral lipids. Under these experimental conditions, the incorporation of palmitic acid into phospholipids increased with the concentration of lysolecithin up to 640 nmoles/ml. When this concentration of lysolecithin was exceeded, inactivation of the enzyme systems took place.

The incorporation of [1-^{14}C] palmitic acid into individual phospholipids with α-glycerophosphate or with lysolecithin as the acyl acceptor, or in the presence of alternate precursors is shown in Table IV. Phosphatidylcholine was found to be the major labeled component under these conditions. Some radioactivity was also found in other phospholipid fractions when α-glycerophosphate was used as a precursor. There was no apparent difference in the presence or absence of α-glycerophosphate, glucose, glycerol or monoolein with respect to distribution between the individual phospholipids. When lysolecithin was used as

TABLE IV

Separation of Phospholipids Synthesized in Swine Aortic Homogenates at Various Concentrations of α-Glycerophosphate and Lysolecithin and in the Presence of Alternate Precursors[a]

Precorsor added (μmoles/ml)	Percentage of radioactivity incorporated into				
	LL	SP	PC	PS + PI	PE
None	5	4	77	5	9
α-Glycerophosphate					
1.0	4	1	81	4	10
3.3	3	4	83	3	7
5.0	4	2	82	3	9
Glucose					
1.7	2	2	82	2	12
Glycerol					
4.0	2	2	85	4	7
Monoolein					
3.0	2	2	82	6	8
Lysolecithin					
0.0027	1	5	86	3	4
0.027	1	1	93	1	4
0.107	1	1	96	1	2
0.32	0.5	0.2	97.8	0.5	1
0.64	0.4	0.3	98.2	0.9	0.2

[a]LL = lysolecithin; SP = sphingomyelin; PC = phosphatidyl choline; PS + PI = phosphatidyl serine + phosphatidyl inositol; PE = phosphatidyl ethanolamine.

the fatty acid acceptor, most of the radioactivity was recovered in the phosphatidylcholine fraction.

DISCUSSION

The present study has shown that in swine aortic homogenates most of the labeled palmitic acid was incorporated into the phospholipid fraction. Incorporation of radioactive palmitic acid into diglycerides and triglycerides was very similar under the conditions of this study. It has been reported that the major part of a labeled linoleic acid was found in the phospholipid fraction in the homogenates of dog and rabbit aorta (3). Very small amounts of fatty acid were incorporated into cholesteryl ester in this normal aortic homogenates. However, it was reported that cholesterol esterification was stimulated in the atherosclerotic lesion of rabbit and pigeon aorta (9,10) and human femoral arteries (11). It has been demonstrated that this stimulation was due to an increased concentration of microsomal cholesterol and not due to an increase in the concentration of the enzyme, fatty acyl CoA cholesterol acyltransferase (12).

There is an absolute requirement for both ATP and CoA for both the esterification of fatty acid in the aortic homogenates (3-5). It has been noted in our laboratory that Mg^{+2} and F^- is necessary for maximum fatty acid esterification in the swine aortic homogenates (unpublished data). Stimulation of fatty acid esterification by fluoride would possibly be due to either to the inhibition of an ATPase or of a lipase (13). Under this study, potassium fluoride and magnesium chloride were added to the complete incubation medium for the incorporation of labeled palmitate in the swine aortic homogenates.

The results showed stimulation of the incorporation of labeled palmitate into phospholipids by the addition of α-glycerophosphate or lysolecithin. The data suggests that both de novo synthesis from α-glycerophosphate and fatty acyl CoA:lysolecithin fatty acyl transferase activity are active in swine aortic homogenates. However, it was reported that α-glycerophosphate did not stimulate the incorporation of labeled linoleic acid into phosphatidylcholine in the cell-free preparation of rat aorta (4). Stein et al. (3) have demonstrated the presence of both de novo synthesis from α-glycerophosphate and transferase system from lysolecithin for phosphatidylcholine synthesis by the homogenates of dog and rabbit aorta. Glucose was stimulatory for the synthesis of phospholipids in tissue slices of rabbit and dog aorta (1), and a net increase of lipid phosphorus in rabbit homogenates was observed after 2 hr incubation (14), suggesting de novo synthesis of phospholipids. It has also been reported that the incorporation of linoleate into phosphatidylcholine was nearly equally distributed in both the number-1 and number-2 position of molecule when α-glycerophosphate served as precursor, whereas lysolecithin stimu-

lated the incorporation of linoleate specifically into the 2-position of phosphatidylcholine by homogenates of dog and rabbit aorta (3).

As compared to α-glycerophosphate, monoolein was a very poor acceptor of palmitic acid under the experimental conditions (Table II). This observation suggests that the monoglyceride pathway for glyceride and phospholipid synthesis is not active in swine aortic tissue. The results differ somewhat from those reported by Stein et al. (3), who found monoolein served as a precursor of glycerides in the rabbit aortic homogenate system. Similar to the findings in rabbit aorta (3), glycerol did not serve as a fatty acid acceptor. Activity of glycerokinase was not detectable in this swine aortic homogenates.

In the present study, labeled palmitic acid was incorporated predominantly into phosphatidylcholine. However, it has been shown that most of the [^{32}p]phosphate is incorporated into phosphatidylinositol in the normal intima of rabbit (1) and pig aortas (2,15), while in the atheroscerlotic aorta of rabbit, phosphatidylcholine is synthesized to the greatest extent (16,17). The present study indicated that the synthesis of phosphatidylcholine from lysolecithin was stimulated by increased concentrations of lysolecithin. The enzyme involved for this synthesis is fatty acyl CoA:lysophosphatidylcholine fatty acyltransferase. It has been demonstrated that the activity of this enzeme is increased in atherosclerotic tissue (18). This increase is not due to an increase in the amount of enzyme, but rather to an increased concentration of one of its substrates, lysolecithin (4,18). The concentration of endogenous lysolecithin seems to be an important determinant for the synthesis of phospholipids from lysolecithin in aortic tissue. The increased concentration of lysolecithin in the atheroscerotic tissue is probably derived from the circulating plasma (18,19). Another source of aortic lysolecithin for acylation could originate as a product of degradation of phosphatidylcholine, since phospholipase A_2 was demonstrated in aortic tissue (20-23).

The results showed that only minimal synthesis of diglycerides and triglycerides occurred when α-glycerophosphate and lysolecithin were omitted from the incubation medium; however, some phospholipid synthesis was noted. Under these conditions, it is possible that phospholipid synthesis was at least in part through the acylation of endogenous lysolecithin as suggested from positional analysis of labeled linoleic acid in phosphatidylcholine (3). Higher concentrations of lysolecithin inhibited the incorporation of labeled palmitic acid into phosphatidylcholine. Similar results were reported by Stein et al. (3) and Portman (4). The optimal concentrations of lysolecithin in swine aortic homogenate system were much higher than that reported in rabbit aortic system (3). It is possible that this difference of optimal concentrations of lysolecithin for fatty acid esterification may be due to either a difference in the incubation medium attributable to the addition of bovine serum albumin in this study, or due to the differences in species.

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Selective Effects of Fatty Acids Upon Cell Growth and Metabolic Regulation

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ABSTRACT

Positional isomers of *cis*-methyleneoctadecanoic acid differed greatly in their efficiency for growth of an unsaturated fatty acid auxotroph of *Escherichia coli* upon glucose as a carbon source. The 8, 9, and 11 isomers were more efficient in producing cells (60-70 cells/fmole) than the others (0-7 cells/fmole), although all isomers were found esterified to a similar extent into cellular lipid. With *Saccharomyces cerevisiae* mutants, all isomers between 6 and 12 supported some growth of the eukaryotic cells, and the 7 and 9 isomers were slightly more efficient than the 8-isomer. When *E. coli* were grown with glycerol, all isomers from 5 to 14 supported growth, and those with the substituent near the center of the acyl chain had the greatest efficiency (70 cells/fmole). With the glycerol medium, the pattern of efficiencies for the various *cis*-methylene acyl chains resembled the broad selectivity reported earlier for the *cis*-ethylenic isomers in glucose medium, which agreed closely with predictions based upon the physical property of their phospholipid derivatives. Thus, metabolism of glycerol appeared to allow the cyclopropane acyl chains to support cell functions to the limits expected for bulk phase chain-chain fluidity considerations. This broad specificity was also obtained when cells were grown on glucose with cyclic AMP added to the culture. Therefore, the selective inadequacies of the 5, 6, 7, 10, 12 and 13 isomers in supporting cell growth on glucose may occur through an interaction modified by cAMP and dependent upon reduced cellular levels of cyclic AMP. The highly selective pattern of efficiency of the *cis*-methylene acids for *E. coli* growth on glucose resembles that with the acetylenic acids, but was shifted one carbon atom toward the methyl terminus. This observed selectivity pattern seems due to interactions of the individual acyl chains with cellular protein(s) rather than to chain-chain interactions in a bulk phase. The ability of certain positional isomers to support cell function equally well in both nutrient conditions suggests that the role of those acyl chain isomers may be independent of metabolite flux or cyclic nucleotide contents of the cell, whereas the actions of other isomeric fatty acids seem closely related to the metabolic status of the cell. A highly selective role for different fatty acids in modulating cellular function seems possible on the basis of the current evidence.

INTRODUCTION

The acyl chains of cellular lipids are commonly assigned a significant role in maintaining the integrity of the membrane bilayers (1). Combinations of saturated and unsaturated acyl chains presumably can provide favorable degrees of expansion, plasticity or fluidity that facilitate membrane-mediated processes. An essential role for unsaturated fatty acids in cell physiology is evident in studies with auxotrophic microbial mutants that cannot synthesize the unsaturated bond (2,3) and thereby cannot grow without supplemental nutrient unsaturated fatty acids. Such organisms provide useful model systems for determining the manner by which acyl chains play their vital roles.

A wide variety of acyl chains occur in nature, and the more complex eukaryotic organisms have evolved a series of dehydrogenases which introduce *cis*-ethylenic bonds into preformed acyl chains to give many different acids in the cellular lipids (4). The eukaryotic acyl-CoA:phospholipid acyltransferases have a selectivity (5) that ensures rapid incorporation (and thereby persistent retention) of long-chain polyunsaturated acids in tissue phospholipids. An important role in mammalian cells for some of these acids may be as a precursor of the very potent autacoids, prostaglandins and thromboxanes (6). The need for such autacoids to mediate and modulate complex, integrated eukaryotic cell functions may explain the recognized need by mammals (7) for the (n-6) class of "essential" fatty acids. For example, esterified linoleate [18:2(n-6)] could be regarded as an esterified pool of precursor for forming eicosatrienoate [20:3(n-6)] and arachidonate [20:4(n-6)] which can in turn form PGE_1 and PGE_2, respecitively. However, many additional fatty acids occur for which a distinct physiologic role has yet to be assigned, and the consequence(s) of their occurrence in a cell are uncertain. In addition, we still do not know the degree to which arachidonate (or other (n-6) acids) are aiding cells by forming prostaglandins in comparison to contributing to membrane fluidity. Apparently, an octadecenoate with only one *cis*-ethylenic bond can provide ade-

quate plasticity or fluidity for growth of the microbial mutants studied (8,9,10). The value of having the variety of elongated and desaturated acyl chains that are commonly seen in the cellular lipids of eukaryotic organisms is not clearly evident.

We have developed evidence to show that an unsaturated fatty acid may influence cellular functions in a manner other than that expected from contributing to membrane fluidity or from forming prostaglandins and related autacoids. Our previous studies (11-13) indicated that some fatty acids exhibit effects that do not fit the additive, nonselective properties attributable to chain-chain interactions that are associated with fluidity. The current report illustrates that cyclopropane fatty acids can interact with cells in a highly selective manner that affects cell proliferation and that depends upon other aspects of cellular metabolism.

MATERIALS AND METHODS

The *Escherichia coli* mutant 30Eβox⁻ (14) was obtained from Drs. C.D. Linden and C.F. Fox (Dept. of Bacteriology, UCLA). It was derived from a β-glucoside-fermenting derivative of K-12 strain MO,F⁻ str^r (15) by nitrosoguanidine mutagenesis and penicillin selection to obtain strain 30⁻, an unsaturated fatty acid auxotroph lacking the β-ketoacyl acyl carrier protein synthetase (fab B), but not lacking the β-hydroxydecanoyl thioester dehydrase (fab A) (16,17). Subsequently, an elaidate-utilizing mutant (30E) was derived from 30⁻ (18) as described by Schairer and Overath (19), and mutagenized by nitrosoguanidine to give, following penicillin selection, the 30Eβox⁻ strain (14) used in these studies. The cells showed a 100-fold reduction in release of $^{14}CO_2$ from carboxy labelled fatty acid when compared to the parent 30E, and showed no β-oxidation products derived from exogenous fatty acids upon gas chromatographic analysis of the cellular phospholipids (14). The unsaturated fatty acid requirement was routinely validated by the lack of growth on replicate plates without oleic acid, and the absence of functional β-oxidation was shown by a lack of growth on plates containing oleic acid as the only carbon source.

The medium used, as in the previous studies (11,12), was Medium A supplemented with 0.5% casamino acids (Difco, Detroit, MI) and either 1% glucose or 0.5% glycerol as the energy source. Nutrient unsaturated fatty acids were synthesized as described in an earlier publication (20), and added as the ammonium soaps in ethanol. The solid medium used was medium A with 0.4% sodium succinate plus 0.1% glucose as the energy source, 1.5% agar, 0.5% Tween-40 (Sigma, St. Louis, MO) and when desired, 0.15% oleic acid. The lack of casamino acids in the solid medium also provided conditions to prevent appearance of spontaneous amino acid auxotrophs.

All growth studies were performed at 37 C. Culture tubes were inoculated at 10^7 cells per ml with log phase cells, and growth was monitored turbidimetrically at 660 nm with a Bausch & Lomb Spectronic 20. Microscopic counts were made concurrently with A660 readings during growth and at the harvest of the culture. From these data, a computer-fitted polynomial regression showed cells/ml = $-.004 + 4.47A_{660} + 34.56A^2_{660} - 67.05A^3_{660} + 42.46 A^4_{660}$. Periodic checks of this relationship confirmed the validity of the more convenient turbidimetric readings. In addition to monitoring cell number, aliquots of the cultures were plated on control plates described above at various times during the growth and at stationary phase to check for the presence of possible contaminants or revertants. No contaminants or revertants were observed.

Yeast mutants, *Saccharomyces cerevisiae* KD-46 and KD-115, were obtained from Dr. A. Keith (Department of Biophysics, Pennsylvania State University). KD-46 is a respiratory deficient mutant (21) that cannot synthesize the porphyrin (22) needed for unsaturated fatty acid synthesis, and KD-115 has respiratory activity, but is deficient in the desaturase (23). The KD-115-PR respiratory-deficient strain is a petite mutant isolated from the KD-115 (Graff and Lands, unpublished). The yeast cultures were maintained with the media and conditions described previously (24,25).

RESULTS

Increasing amounts of the cylopropane fatty acids gave increased cell yields of both types of cells used. With *E. coli*, only the 8, 9, and 11 isomers produced net cell yields greater than 10^9 cells/ml (Fig. 1). The effectiveness (ε) of each acyl chain in supporting bacterial cell growth was determined from the slope of the cell yield response curves between 10 and 30 μM nutrient, and averaged values for the different positional isomers are compared in Figure 2. The results indicate that isomers 2 to 7 and 12-17 were very ineffective in supporting growth on glucose. Also, the 10-isomer differed greatly from the adjacent isomers (9 and 11). All isomers were found esterified in cellular phospholipids (Table I), and the amount at stationary phase was similar for most isomers whether expressed as mole percent of total

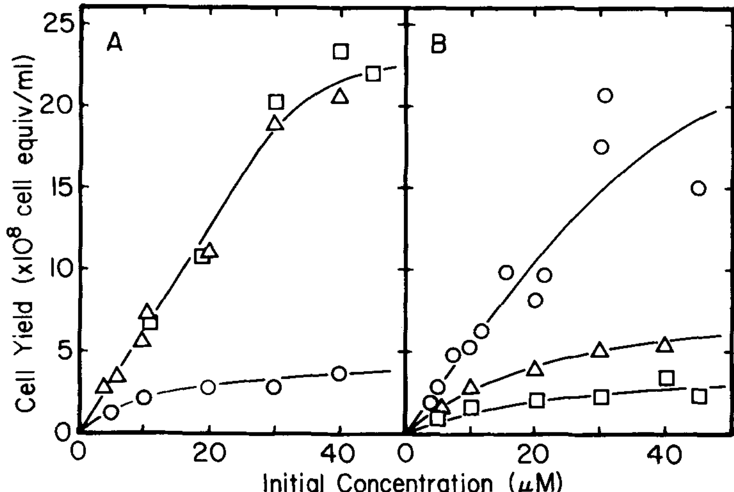

FIG. 1. Cyclopropane acids support growth on glucose. The effect of increasing concentration of *cis*-methyleneoctadecanoic acid isomers on the net cell yields of *E. coli* 30Eβox⁻ was measured at 37 C with 1% glucose as the energy source. Values for the odd-numbered isomers (panel A) are noted: 7, 0; 9, □; and 11, △. The even-numbered isomers (panel B) are noted: 8, 0; 10, □; and 12, △. Each data point represents the average of several determinations at any one concentration of nutrient acid. Inocula were prepared as previously described (12).

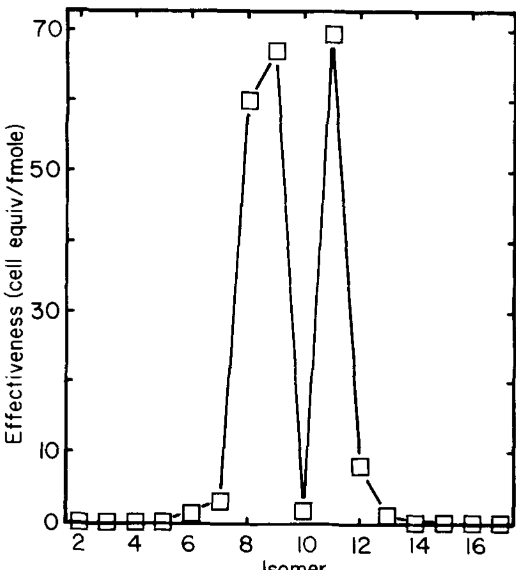

FIG. 2. Effectiveness of *cis*-methyleneoctadecanoic acid isomers in supporting growth. *E. coli* 30Eβox⁻ was grown at 37 C with 1% glucose as the energy source. The ordinate value for each nutrient isomer represents the magnitude of the slope of the linear region of plots of net cell yield vs. initial fatty acid concentration such as those shown in Figure 1.

esters or as amoles of nutrient acid esterified per cell. The values of these contents were greater for the isomers with the substituent near the methyl end of the acyl chain.

Mutants of the yeast *S. cerevisiae* were also able to use cycloproppropane fatty acids to accommodate a nutritional requirement for unsaturated fatty acids. From graphs similar to Fig. 1 (results not shown), the linear regions of increase in cell yield with increased nutrient concentration gave efficiency values (ϵ) shown in Fig. 3. With all three yeast mutants tested, isomers with the substituent near the center of the chain were the more effective ones. The pattern of selectivity with the respiratory incompetent mutant, KD-46, resembled that with the respiratory competent, KD-115, although the latter mutant formed about 3-fold more cells per fmole of nutrient acid and also grew more effectively with the 12 to 15 isomers. One unexpected difference between the KD-115 (Fig. 3A) and its derived petite mutant (Fig. 3B) was the greater number of cells per fmole produced by the parent strain (KD-115) with the 8 to 10 isomers.

Growth of the bacteria on glycerol provided linearly increased cell yields over a wide range of nutrient fatty acid concentration as shown by the examples in Fig. 4A. The growth effectiveness (ϵ) of the 8, 9 and 11 isomers was equal to that seen for growth on glucose, whereas isomers 5, 6, 7, 10, 12, 13 and 14 supported much more growth on glycerol than on glucose (Fig. 4B). The pattern of the ϵ values for the various isomers was a fairly smooth curve with an optimum for the 10-

isomer and with none of the sharp irregularities observed for growth on glucose (dashed line).

To test for the involvement of cAMP in the growth response with different carbon sources, cells were grown on glucose plus 1 mM cAMP. Fatty acid isomers observed to be ineffective for growth on glucose alone supported linear cell yields with 10-fold higher efficiencies in the presence of added cAMP (Fig. 5A). The pattern of ϵ values in the presence of the cyclic nucleotide (Fig. 5B) differed greatly from the pattern on glucose alone, but was almost identical to that shown in Fig. 4B for growth on glycerol. The pattern for growth efficiency on glucose with added cAMP also closely resembled the "bell-shaped" curve reported earlier (12) for growth with the various *cis*-octadecenoate isomers on glucose with one exception being the inability of the 6-*cis*-octadecenoate isomer to support growth on glucose. We found that when cAMP was added to the culture medium, the effectiveness of the 6-isomer became nearly 10-fold greater (Fig. 6), whereas that for many other *cis*-octadecenoate isomers was unchanged.

Analysis of the lipids from cells grown to stationary phase with cyclopropane acids and glucose plus added cAMP produced results (Table II) very similar to those obtained in the absence of added nucleotide. The amoles of total esterified acid per cell ranged from 101 to 190 (compared to 134-198 in unsupplemented), and the amoles of nutrient acid per cell was 7 to 36 (compared to 5-33 in unsupplemented). In both experimental conditions, the positional isomers with the methylene bridge near the center of the chain were present at lower amounts per cell than were those isomers with the substituent nearer the ends of the chain.

TABLE I

Incorporation of the *cis*-Methyleneoctadecanoic Acid Isomers into Cellular Lipids of *E. coli* 30Eβox$^-$ When Grown on Glucose[a]

	Acids in Cellular Phospholipids		
Isomer	Total amoles/cell	Mole % nutrient	Nutrient amoles/cell
4	140 (2)	3.5	5.0
5	134 (2)	7.0	9.4
6	150 (2)	6.7	10.0
7	175 (4)	8.0	14.0
8	163 (4)	8.7	14.3
9	158 (4)	8.0	12.7
10	175 (4)	7.3	12.7
11	182 (4)	8.2	14.9
12	198 (4)	11.4	22.7
13	164 (2)	15.9	26.1
14	148 (2)	22.0	32.6
15	158 (2)	11.4	18.0

[a]The content of nutrient acid in the total phospholipid was determined by GLC on 10% diethyleneglycol succinate at 180 C utilizing a Varian 2740 Gas Chromotograph. Cultures were supplemented with concentrations (10 – 20 μM) of the indicated isomer and harvested and extracted as described earlier (12). Values in parentheses indicate the number of determinations made.

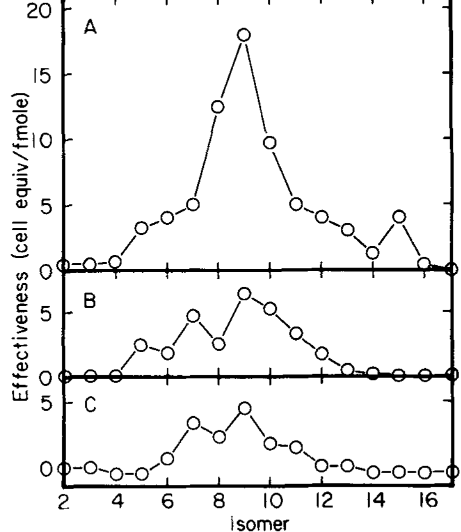

FIG. 3. Effectivenss of *cis*-methyleneoctadecanoic acid isomers in supporting growth of *Saccharomyces cerevisiae*. The ordinate value for the efficiency of each isomer was determined as described in Figure 2. Preparation of inocula and growth conditions have been described elsewhere (19). For each mutant, the energy source was glucose. KD-115 (panel A), KD-115 PR (panel B), and KD-46 (panel C).

DISCUSSION

The possibility that fatty acids may exert selective effects beyond their accepted nonselective role in fluidity led us to develop quantitative estimates of the contribution (functionality factor; ref. 25) of the esterified acyl chains to cellular function. We reported a close quantitative agreement between the functionality factor calculated for isomeric *cis*-octadecenoic acyl chains esterified in cellular membranes and the degree of expansion (or fluidity) of their synthetic phospholipid derivatives (12). The functionality factors calculated for the various *cis*-ethylenic isomers formed a rather smooth curve with an optimum for the 9- and 10-isomers (12). The nutritional effectiveness (ϵ) of the different *cis*-octadecenoates gave a pattern similar to that for the functionality factors with one exception: the 6-isomer. Thus, the *cis*-6-octadecenoate seemed to be involved in some selective interaction that was

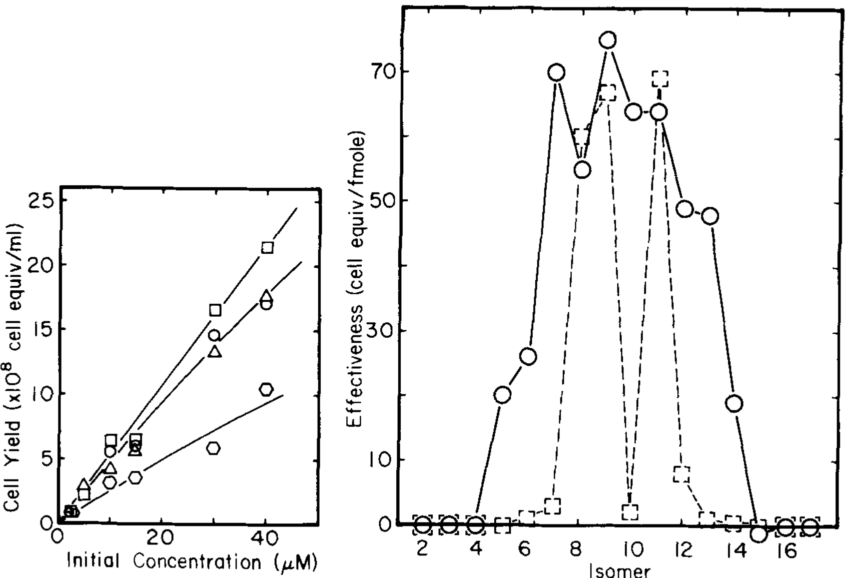

FIG. 4. Cyclopropane acids support growth on glycerol. A. The effect of increased initial fatty acid concentration of the even-numbered isomers of *cis*-methyleneoctadecanoic acid on the net cell yield with 0.5% glycerol as the energy source for the *E. coli* mutant at 37 C. Data were obtained with inocula and growth conditions (except carbon sources) as described in Figure 1. Symbols: 6,⬡; 8, O; 10, □; and 12, △. B. Comparison of the efficiencies of the *cis*-methyleneoctadecanoic acid isomers for supporting growth of the *E. coli* mutant with either 0.5% glycerol (O-O) or 1.0% glucose (□ - □) as the energy source.

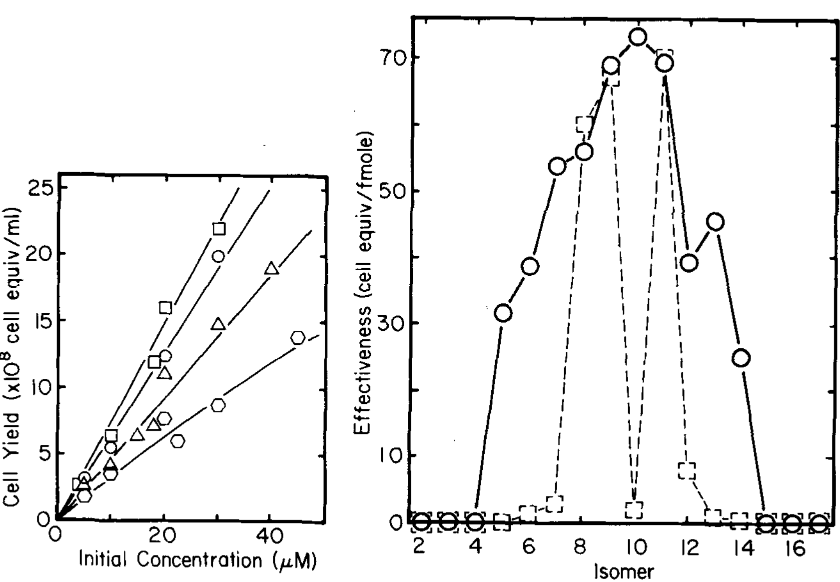

FIG. 5. Modification of acyl chain effectiveness by added cAMP. A. The effect of added cAMP (1 mM) on the net cell yields of the *E. coli* mutant at 37 C with selected even-numbered *cis*-methyleneoctadecanoic acid isomers and 1% glucose as the energy source. Symbols: 6,⬡ ; 8, O; 10, □; and 12, △. B. The efficiencies of the *cis*-methyleneoctadecanoic acid isomers to support growth of the *E. coli* mutant when cAMP (1 mM) was added to cultures with 1% glucose as the energy source (O-O) is compared to the corresponding efficiencies obtained for growth with glucose alone (□- □).

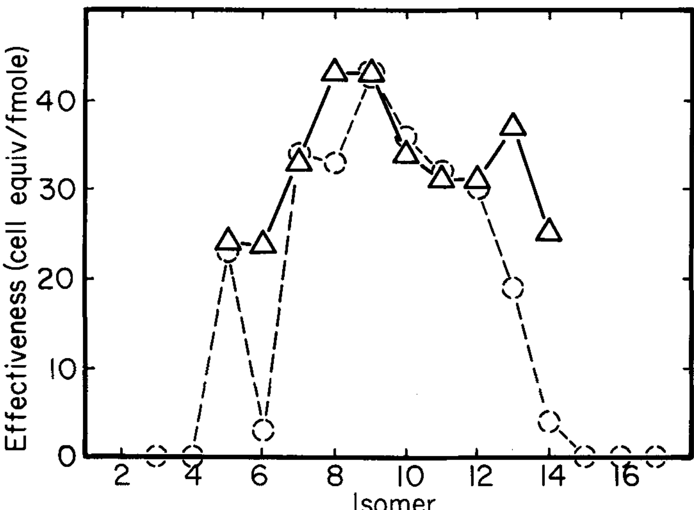

FIG. 6. Modification of selective growth responses by added cAMP. Comparison of the effectiveness of selected *cis*-octadecenoic acid isomers for supporting growth of the *E. coli* mutant at 37 C with 1% glucose when supplemented with 1 mM cAMP, △ - △; and glucose alone, 0-0.

restraining cell growth in a way not expressed by the other *cis*-octadecenoate isomers. Selective effects are manifested in two ways: (a) a lower than normal ratio of the nutrient effectiveness (ϵ) to the calculated functionality factor (f), and (b.) a low maximum cell yield that becomes independent of increased nutrient acid concentration while still below that obtained with normal fatty acids (as shown in Fig. 1). Our studies were designed to quantitatively compare acids under conditions where the cells have just ceased growth as a result of insufficient nutrient fatty acid. Under these conditions, an acid that is highly effective in supporting growth will produce many cells per fmole and therefore be present at a small number of amoles per cell (25). Thus, a low concentration of nutrient acid at stationary phase may not indicate that the acid is an ineffective nutrient, but that it is actually a very effective one that can support further cell division when present at a concentration below that where other fatty acids may fail. The higher content (mole % and amoles nutrient per cell) observed for isomers with the functional group near either end of the acyl chain (particularly the methyl end) is consistent with the recognized lower contribution to fluidity in bulk phase of the corresponding *cis*-ethylenic analogs (12,26), and reaffirms the presumed importance of fluidity in supporting cell replication. Those isomers (at positions, 6, 7, 10, and 12) with low ratios of ϵ/f provide evidence for selective growth-limiting events that are due to processes other than fluidity.

TABLE II

Incorporation of *cis*-Methyleneoctadecanoic Acid Isomers Into Cellular Phospholipids of *E. coli* 30Eβox⁻ when Growth on Glucose in the Presence of Added cAMP[a]

	Acids in Cellular Phospholipids		
Isomer	Total amoles/cell	Mole % nutrient	Nutrient amoles/cell
4	112 (2)	6.1	6.8
5	172 (2)	15.6	26.8
6	185 (2)	13.7	25.3
7	154 (2)	7.0	10.8
8	161 (2)	8.5	13.8
9	153 (1)	8.0	12.2
10	171 (2)	8.5	14.6
11	101 (1)	4.4	4.4
12	190 (2)	6.1	11.7
13	160 (2)	7.1	11.4
14	154 (2)	30.1	46.4
15	128 (2)	23.0	29.5

[a]Conditions and procedures were the same as in Table I with 1 m*M* cAMP added to the medium.

Conformational Aspects

Many acetylenic analogs exhibted a highly selective inability to support bacterial growth (11) that was not predicted on the basis of general physical-chemical considerations (which tend to be nonselective and additive). Figure 7 illustrates the striking similarity of the growth efficiencies for the acetylenic and cyclopropane fatty acids. The patterns for the two types of fatty acid isomers are almost superimposable with the exception of a shift of one carbon atom. We first described the "frame-shift"

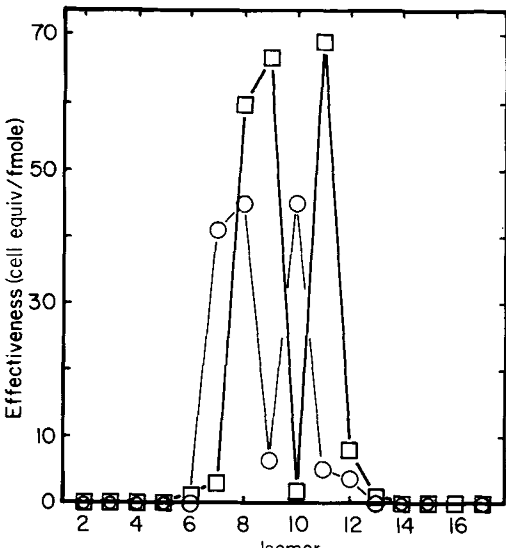

FIG. 7. Comparison of cyclopropane acids with acetylenic acids. Efficiencies of the *cis*-methylen-octadecanoic acid isomers (□ - □) compared to those obtained previously (11) for the octadecynoic acid isomers (O-O) in supporting growth of the *E. coli* mutants at 37 C with 1% glucose as the energy source.

phenomenon when examining acyl-CoA: phospholipid acyltransferases (27). In that work, the 9-ynoic acid was metabolically equivalent to and could be oriented in a manner that is spatially equivalent to the 10-isomer of *cis*-octadecenoate. The enzyme(s) appeared to interact in a highly selective manner with a *cis*-rotamer (rotational conformer) of the individual, solvated acetylenic acyl-CoA derivatives. Space-filling models readily demonstrated that the *cis*rotamers of the 7, 8 and 10 acetylene analogs are spatially equivalent to the corresponding 8, 9 and 11 cyclopropane isomers to which they are also nutritionally equivalent (Fig. 7). The appearance of a highly selective isomeric difference in the present study leads us to suggest that the irregular pattern of nutrient effectiveness obtained with cells in the glucose medium probably reflects some specific configurational acyl chain-protein interactions. At the present time, all studies of fatty acid transport, activation, esterification and hydrolysis in *E. coli* have failed to exhibit a specificity that accounts for the highly selective interactions reported here.

Since many of the *cis*-ethylenic acids were more effective nutrients in glucose than their spatially-equivalent cyclopropane analogs, some feature of the *cis*-ethylenic bond appears to allow them to be more consistently effective. If the required feature was merely the presence of π-bonds, as described earlier for 1-acyl-GPC:acyl-CoA acyltransferase activity (28), we might expect the acetylenic isomers to serve equally well as the *cis*-ethylenic analogs. The fact that they do not indicates that both spatial and electronic aspects are important features. Prior studies by Silbert et al. (29) noted that some cyclopropane acids were suitable nutrients for auxotrophic cultures, although Cronan et al. later concluded (30) that fatty acids of this type appear to impede exponential growth. Speculations on the impact of the cyclopropane acids on cell physiology were reviewed recently (31) without a definite role being demonstrated. Our results clearly indicate that different positional isomers of these acids can have highly selective effects, and that the presence or absence of π-bonds may indeed influence a cell's response to an acyl chain.

The use of a series of positional isomers has provided a range of cellular responses to a given type of acyl chain substituent. Not all cyclopropane acids are defective growth promotors, and some acetylenic acids support growth even though they have high melting points. General conclusions regarding the effect that a type of acid (e.g., cyclopropane or *trans*) may have upon cell physiology are not reliable when based on observations of only one or two members of a series. Furthermore, although all fatty acids will influence the membrane fluidity, effects other than fluidity can occur.

Acyl Chain Adequacy in Different Media

Although the growth on glucose was low with the 5, 6, 7, 10, 12 and 13 isomers, the high growth observed with added cAMP or with glycerol indicates that there was nothing about the chemistry of those acyl chains *per se* that prevented their phospholipid derivatives from supporting cell functions. In these later media, the cyclopropane acids had ϵ values comparable to their corresponding *cis*-ethylenic analogs, which correlated well with the relative contribution that each isomeric acid can make to phospholipid fluidity (12). The close agreement with these two types of structural analogs indicates that the expected similarity in physical properties of *cis*-ethylene and *cis*-methylene prevails in the membrane lipids even though a different nutrient effectiveness is observed. In addition, the mole % values for the cyclopropane acids in the presence of cAMP are similar to those in the absence of cAMP. This result leads to similar estimated functionality factors in both media. Such a result suggests that the cyclic nucleotide enhanced cell growth without appreciably altering the acyl chain's proportional contribution to membrane fluidity.

Regular "bell-shaped" responses to different positional isomers were observed more consistently with the eukaryote, *S. cerevisiae*, than with the prokaryote, *E. coli*. For this reason, we conclude that the plasticity or degree of expansion of the membrane lipids, which follows the same "bell-shaped" pattern, is a principal factor limiting yeast cell growth with these acids. This pattern of results was also obtained for *cis*-octadecenoate isomers with KD-115 grown with either glucose (repressed) or glycerol (depressed) as the carbon source (24). Thus, the eukaryotic yeast cells have fewer growth-limiting selective interactions with nutrient fatty acids than do the bacteria studied, and they seem more resistant than *E. coli* to this aspect of glucose repression. We have reported, however, that the yeast mutant KD-46 showed highly selective inhibitory effects of isomeric *trans*-octadecenoates, whereas the bacteria appeared unresponsibe to the positional differences among the *trans* fatty acids (13). Thus, highly selective interactions of acyl chains are also clearly evident in yeast.

Modulation of Catabolite Repression

The ability of some fatty acids to support greater bacterial growth with glycerol than with glucose is an indication that catabolite repression may be involved. Repression of cellular functions by glucose (32) may occur by reduction of the cellular content of cAMP (33) although an alternative accumulation of a repressive factor has been reported (34). The well-recognized action of cAMP in combination with catabolite activator protein (35) in stimulating transcription of some genes is paralleled by an ability of unadenylyated glutamine synthetase to stimulate transcription in the case of the enzymes of the histidine utilizing (*hut*) system (36). In this case, both glucose and ammonia can repress the formation of the *hut* enzymes, and reduction of the ammonium concentration allows release of the system from the glucose (catabolite) represssion (37). A related example is the synthesis of tyramine oxidase which is also relieved from catabolite repression under conditions of nitrogen limitation (38) perhaps involving a factor other than glutamine synthetase. Our present results suggest that general metabolic regulation by carbohydrates (energy sources) and ammonia and amino acids (nitrogen sources) may be complemented by effects of lipids. Fatty acids or their intracellular derivatives appear to modulate or mediate a form of catabolite repression in which cell growth is not possible with certain types of fatty acids and is fully competent with others.

The failure of cells to grow indicates that some fatty acids can selectively create a serious inbalance in the regulatory systems of the cell. A pleiotropic effect on transport systems of *E. coli* K-1061 was described (39) for cells grown in glucose with high levels of *cis*-11-octadecenoate or *cis*-9-methylenehexadecanoate. The manner in which the fatty acids were involved in the response of catabolite repression was not clear, and oleate (*c*9-18:1), palmitoleate (*c*9-16:1) and *cis*-7-hexadecenoate gave normal transport activity (39). In those experiments, no reduction in the growth of the cells was observed in contrast to the present report. An earlier report (19) noted that an *E. coli* auxotroph could grow in glycerol on 9-*cis*, 9-*trans*, 9-acetylenic and 11-cyclopropane acids, but only on 9-*cis* and 11-cyclo in glucose. Subsequent experiments focused attention on the physical properties of the different chemical types of acid and have used mutant variants that may not have the regulatory features of the parent mutant. Our results suggest that a wide variety of variants permitting growth on certain fatty acids could occur by spontaneous mutations in the pleiotropic cAMP regulatory system.

Our current study documents a highly selective effect and indicates the conformation of isomer and chemical type of acyl chain capable of creating the impaired growth condition. Further work is needed to identify the enzyme activities that are directly affected by this specific regulatory response to fatty acids or their intracellular derivatives. The current findings are of particular importance in regard to how different acyl chains may influence cell physiology since they show that cellular cyclic nucleotides can alter, and may mediate, markedly different effects produced by closely related fatty acids. These different regulatory effects of fatty acids can be expected to occur in other cells to varied degrees and to be more pronounced under conditions of catabolite repression.

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The Role of Dietary Fat and Hepatic Triglyceride Secretion in Cancer-Induced Hypertriglyceridemia[1,2]

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ABSTRACT

Growth of Ehrlich ascites carcinoma induces hyperlipemia in mice. In the present study using male Swiss-Webster mice, we examined whether the usual elevations of plasma triglyceride levels in cancerous mice would occur in the absence of dietary fat. Hypertriglyceridemia developed at a similar rate and to a comparable degree in tumorous mice eating a fat-free (58% glucose) diet and in those fed Purina chow. Maximal hyperlipidemia was observed on day 6 or day 8 in tumorous mice fed either diet. To determine whether the endogenous cancer-induced hyperlipidemia was due to hypersecretion of triglycerides by the liver, triglyceride secretion rates were studied 0, 2, 4, 6, 8, 10, and 12 days after tumor inoculation using Triton WR-1339. The secretory rates did not increase prior to or during the development of hypertriglyceridemia in tumorous mice and were not significantly different from those of control mice. On days 10 and 12, triglyceride secretion actually decreased in tumorous mice. Other possible causes for hypertriglyceridemia are discussed in light of the present findings of undetectable differences in triglyceride secretion rates accompanying growth of Ehrlich ascites carcinoma in mice.

INTRODUCTION

The occurrence of hyperlipemia during cancer growth is a well-documented phenomenon in experimental animals (1-3), but the cause of the hypertriglyceridemia has not been established. Brenneman et al. have shown that hypertriglyceridemia in cancerous CBA mice is due to the accumulation of VLDL-triglycerides and have stated that hyperlipemia is manifest in the absence of dietary fat (4). However, the latter conclusion was based upon an observation that we could not confirm. Whereas they reported exclusion of dietary fat by fasting does not reduce the cancer-induced hyperlipemia (4), we find that it does (5,6).

In the present study, we have attempted to see whether nonfasted, cancerous mice when fed a fat-free diet will develop hypertriglyceridemia. We have also tried to ascertain whether hypersecretion of VLDL particles by the liver occurs in tumor-bearing mice prior to and during the development of hypertriglyceridemia.

EXPERIMENTAL PROCEDURES

Mice and Tumor

Male, Swiss-Webster mice (Hilltop Lab Animals, Inc., Chatsworth, CA) 6-10 weeks or 18-20 weeks of age, were used. In one set of experiments to test the role of dietary fat in the development of hypertriglyceridemia, control and tumorous mice (see below) maintained on two different diets, namely Purina chow and 58% glucose, fat-free diet (7), were used. The composition of the two diets was as follows: the major constituents of Purina chow as given by Ralston Purina Company are 23% protein, 4.5% fat, 6% fiber, and 57% carbohydrate. The fat-free diet contained 58% glucose, 22% casein, 6% Hawk-Oser salt mixture, 11.8% nonnutritive cellulose, 2% defatted liver VioBin and 0.2% vitamin mixture. Mice from control and tumor groups were placed on a 58% glucose, fat-free diet two days prior to or on the day of tumor inoculation. The tumor inoculum was 15 x 10^6 cells per mouse, using tumor obtained from donor animals bearing 7 to 10-day-old Ehrlich-Lettre hyperdiploid carcinoma (5). The tumor was originally obtained from Dr. R. McKee (Biological Chemistry Department, UCLA School of Medicine). Body weights of the control and tumorous mice were recorded on alternate days during tumor growth.

In some experiments, triglyceride levels were determined on days 0, 2, 4, 6, 8, 10, and 12 of tumor growth in mice maintained on either Purina chow or 58% glucose diet. Blood was obtained from the opthalmic sinus (8) in heparinized capillaries around 9 a.m. to 11 a.m. The blood was immediately centrifuged at 1,000 RPM for 2 min at 4 C. Tumor samples were obtained by puncturing the abdomen of the tumor-bearing animals with a needle. The needle was removed from the peritoneal cavity, and ca. 0.1-0.2 ml of the tumor was allowed to

[1]A preliminary report of some of the present data was presented at the 1976 meeting of the American Society of Biological Chemists.

[2]Supported in part by VA Medical Research and NIH, USPHS Grant No. CA 15813.

TABLE I

Development of Hypertriglyceridemia in the Absence of Dietary Fat in Mice Bearing Ehrlich Ascites Carcinoma

Experiment	Age of mice (weeks)	Age of tumor (days)	Secretion study with triton	Blood plasma and ECF triglycerides (mg/dl)			
				Diet	Control blood[a]	Tumor, blood[a]	Tumor, ECF[a]
1	18-20	8	---	Purina	110 ± 10 (6)	192 ± 50 (3)	27 ± 3 (3)
				58% glucose	68 ± 6 (6)	192 ± 55 (3)	30 ± 7 (3)
2	6-7	6	+	Purina	221 ± 39 (6)	355 ± 35 (6)	n.d.[b]
				58% glucose	190 ± 16 (6)	344 ± 50 (6)	n.d.
3	8-10	8	+	58% glucose	62 ± 9 (11)	310 ± 48 (12)	n.d.
4	6-8	8	+	58% glucose	44 ± 7 (4)	371 ± 95 (3)	n.d.

[a]Mean ± S.E. Number of animals is given in parentheses.
[b]N.D., not determined.

drip into heparinized capillaries by gently squeezing the abdomen. Extracellular fluid was separated from the tumor cells by centrifugation as described above. In some experiments, the volume of the tumor was measured by opening the peritoneal cavity and collecting the tumor in graduated centrifuge tubes.

Triglyceride Secretion Rates

Triglyceride secretion rates (TGSR) were determined using Triton WR-1339 (9). A 10% solution of Triton WR-1339 in 0.9% saline was used for this purpose. The detergent (0.5 mg/g body weight) was injected i.v. into control and tumorous, *ad libitum*-fed mice (10). In the first experiment, using mice with 8-day old tumors, plasma triglyceride concentrations were determined at t_0 and 60, 120, and 180 min after Triton injection. A separate group of four mice was used for each time point. TGSR was determined using the formula $[(TG_t\text{-}TG_0)/t] \times PV$, where TG_t represents the triglyceride level at time t, TG_0 the initial concentration, and PV, the plasma volume. In a separate experiment to determine plasma volume, using ^{131}I-albumin, no significant difference was found between control mice and mice bearing Ehrlich ascites carcinoma; the plasma volume in both groups of mice was 5.2 ± 0.3 (mean ± S.E., n=5) % of body weight. This value agrees closely with earlier observations (11,12).

For determining TGSR in control and tumorous mice at different stages of tumor growth, only two time points (0 and 60 min) were obtained in all experiments. The number of mice used were 6/group (days 0-8) and 4/group (days 10 and 12).

A separate group of three mice was injected with 0.9% saline instead of Triton, and, as in the experimental animals, blood was collected at 60, 120, and 180 min for triglyceride analysis. No significant difference was found between presaline and postsaline triglyceride levels at any time in both control and tumorous mice.

Triglycerides of blood plasma and extracellular fluid were determined using the method of Galetti (13) with slight modifications. The aliquot volumes of isopropyl ether extracts for triglyceride analysis were increased from 0.5 to 1.0 ml for blood plasma and to 2.0 ml for tumor extracellular fluid samples.

RESULTS

Development of Hypertriglyceridemia in the Absence of Dietary Fat in Tumorous Mice

Triglyceride levels in blood plasma and tumor extracellular fluid were determined in

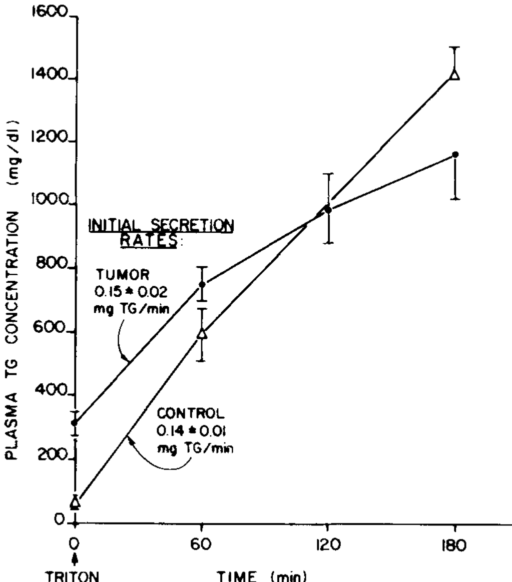

FIG. 1. Time response curve of triglyceride accumulation after i.v. injection of Triton WR-1339 (0.5 mg/g BW) into control mice and mice bearing 8 day old E. ascites carcinoma; mice were maintained on a 58% glucose, fat-free diet. Mean ± S.E. (n=4) for each time point.

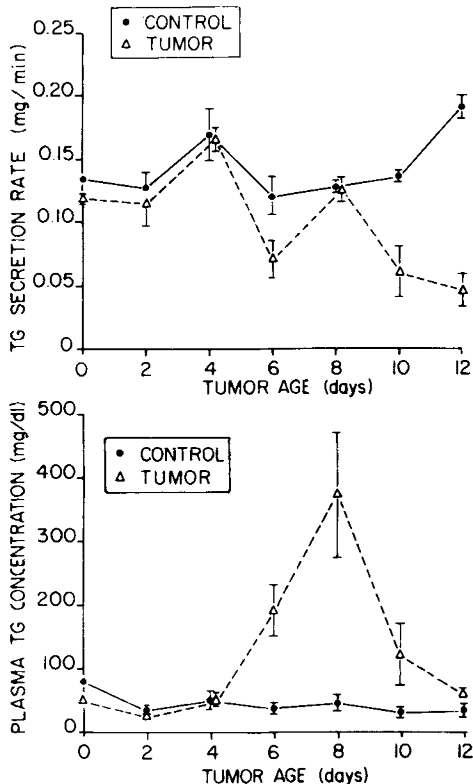

FIG. 2. Triglyceride secretion rates (mg/min, top) and blood plasma triglyceride levels (mg/dl, bottom) in control mice and mice bearing E. ascites carcinoma. Control and tumorous mice were maintained on a 58% glucose, fat-free diet from the day of i.p. inoculation of tumor cells (15 x 10^6 cells/mouse) into the tumorous group. Mean ± S.E. from 6 mice/group except for days 10 and 12 (n=4).

two groups of mice, one fed a fat-containing Purina chow diet and the other a fat-free, glucose diet. Results of four experiments are listed in Table I. Triglyceride determinations were made on day 6 or day 8 of tumor growth when the peak of hyperlipidemia was known to occur from previous studies (5,6). Withholding fat from the diet did not prevent the development of hypertriglyceridemia in tumorous mice in any of the experiments. The tumor extracellular fluid triglyceride concentrations also did not differ in mice fed the 58% glucose diet compared with those fed Purina chow. The absolute triglyceride levels in control mice varied considerably from experiment to experiment. Such variation could reflect differences in the exact dietary states of groups of mice, as shown in our earlier studies (5,14). In experiments 3 and 4, all the mice were kept on fat-free diets (only) in order to study rates of hepatic triglyceride secretion. Data from these studies are included in the present table to illustrate that the triglyceride levels in tumorous animals on a fat-free diet were always equal to or higher than the corresponding Purina chow animals studied in experiments 1 and 2.

Rates of Triglyceride Secretion in Control and Tumorous Mice in the Absence of Dietary Fat

The rates of triglyceride secretion were studied using Triton WR-1339 in groups of control mice and mice bearing 8-day old carcinoma. As shown in Figure 1, the initial secretion rates averaged 0.14 ± 0.01 mg/min in control mice and 0.15 ± 0.02 mg/min in tumorous mice. In two out of three additional experiments, no significant difference was found between the control rates (0.135 ± 0.004 mg/min, n=17, mean ± S.D.) and those of tumorous mice (0.12 ± 0.008 mg/min, n=17, mean ± S.D.). In the third additional study, TGSR in tumorous mice (0,07 ± 0.014 mg/min, n=3) was actually lower than that in control mice (0.12 ± 0.014 mg/min, n=4).

As shown in Figure 2, the tumorous mice developed marked hypertriglyceridemia, the intensity and time-course of which were identical to those reported previously in tumorous mice fed Purina chow which contains dietary fat. The triglyceride secretion rate in these mice was never greater than that of

controls throughout the period of tumor development. The TGSR actually began to drop after day 8, and fell to values that were significantly lower than the controls. The decrease in TGSR in tumorous mice with large tumors could have resulted from chronic decreased food intake. Although dietary intakes were not actually measured in these studies, a decreased caloric intake during later stages of tumor growth is a well-established phenomenon that we have documented in other studies using Swiss-Webster mice. For example, the food intake of other tumorous mice on both diets averaged 50 ± 10% of that of the controls during the 5th and 8th day of tumor growth (unpublished observations).

DISCUSSION

Mice in which transplantable tumors are growing provide an interesting and convenient model for studying mechanisms by which hyperlipemia may develop (4-6). In the case of mice inoculated with Ehrlich ascites carcinoma, there have been several studies describing the time course of hyperlipemia, variations in degree of lipemia depending upon mouse-strain differences, characterization of the plasma lipoproteins, and studies of fasting effects on the hyperlipidemia once it develops (3-6). However, the cause of the hypertriglyceridemia has not been established. Prior to the present study, there have been a couple of studies showing that dietary fats contributed to or were required for hyperlipemia development in cancerous rats (15,16). However, we have found a pronounced decrease in plasma triglyceride levels of cancerous mice during fasting (5,6); therefore, the question of whether exogenous FA are required for hyperlipemia to develop in our cancerous mice remained moot. We have now carried out several studies in cancerous mice in the fed-state, but with dietary fat excluded, to see whether the usual hypertriglyceridemia would develop. Our results show clearly that both the time course and degree of hypertriglyceridemia were independent of dietary fat, provided other caloric sources were available in the diet. This is not to say that the TGFA that accumulated were all synthesized do novo from carbohydrates and amino acids. Some of the TGFA must have come from the depot fat which was, to some extent, surely derived from dietary fat ingested before our experiment began. Indeed, this may explain the observations of a recent study by Mathur and Spector who showed a significant contribution of dietary fatty acids to the accumulation of VLDL-TGFA, but not to chylomicrons, in the tumor fluid (17).

Since our observations support the earlier conclusion of Brenneman et al. (4) that the cancer-induced hypertriglyceridemia is independent of exogenous fat, hypertriglyceridemia in the tumorous mice must result from either the hypersecretion of triglycerides by the liver or a decreased rate of triglyceride removal from the circulation (or both). In the present study, we hypothesized that hypersecretion of triglycerides by the livers of the fed, cancerous mice was the cause of the cancer-induced hyperlipemia. To test this hypothesis, we have carried out a study of endogenous triglyceride secretion in control and cancerous mice that were eating a fat-free diet. We used Triton WR-1339, which has been employed for this purpose in both rats (3,18) and mice (10), and obtained triglyceride secretory rates that were in fair agreement with earlier studies of the disappearance and replacement of intravenously injected labeled VLDL-TG in lean mice (12). During the period in which hyperlipemia was developing, the rates of triglyceride secretion did not increase in tumorous as compared to control mice. In one experiment, we actually observed a decreased rather than an increased secretory rate in the tumorous mice. Therefore, we can only conclude that the hypersecretion of triglycerides was either not the cause of the endogenous, cancer-induced hyperlipemia or that the hypersecretion, if it occurs, is so subtle that our techniques could not detect it. In this regard, it is important to stress that a prolonged increase in the secretory rate too small for us to detect could produce dramatic changes in plasma triglyceride levels in a 24-hr period. Thus, an increase of 5% in the secretory rate, even if accompanied by a slight increase in the removal rate so that the net increment were, say, 0.005 mg/min greater than the control value of 0.14 mg/min, would lead to an accumulation of 7 mg triglycerides in the circulation in one day. This would raise the plasma concentration from 100 mg/dl to 450 mg/dl in a 40 g cancerous mouse (2 ml plasma volume) in that period.

Several other possible explanations for the development of hypertriglyceridemia in cancerous mice remain. There is always the possibility that the agent used here, Triton WR-1339, through some unkown mechanism could negate an effect of the cancer, in which case our data would be invalid. There is also the possibility that the hyperlipemia depends upon a continuing normal rate of triglyceride secretion by the liver, but that the defect lies in the clearance of this triglyceride from the circulation. Such a defective clearance was proposed

by Begg and Lotz who found lower lipoprotein lipase activity in tumor-bearing rats than in controls (19,20). Tracer experiments using labeled VLDL-triglycerides to test the possibility of imparied triglyceride clearance in mice bearing Ehrlich ascites tumor are in progress.

ACKNOWLEDGMENTS

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Effects of Essential Fatty Acid Deficiency on Prostaglandin Synthesis and Fatty Acid Composition in Rat Renal Medulla

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ABSTRACT

Studies are reported on the capacity of isolated rat renal papilla (inner medulla) to synthesize and release prostaglandin (PG) E from endogenous and exogenous precursor(s) during development of an essential fatty acid (EFA) deficiency in the rat. Weanling (21-day-old) male Sprague-Dawley rats were fed a fat-free diet supplemented with either 5% hydrogenated coconut oil (HCO) or 5% safflower oil (SO). At approximately 3, 6 and 7 weeks (6, 9 and 10 weeks of age), groups of animals fed each diet were killed for studies of PGE synthesis in the renal papillae. Differences in the fatty acid composition of the papillae lipids of the animals of each group were also determined. The in vitro production of PGE from endogenous precursor(s) was significantly reduced in the papillae from the 6-week-old rats fed the HCO diet compared to the control (SO) rats, and appeared to be near maximally depressed in the 10-week-old animals compared to that of animals fed an EFA deficient diet for over a year in an accessory experiment. Analyses of the fatty acids of the papillae lipids of the HCO groups showed that the levels of 18:2 and 20:4 were markedly reduced, and those of 16:1, 18:1 and 20:3 were elevated compared to the controls even in the 6-week-old animals, typical of an EFA deficiency. The papillae lipids of the animals fed the HCO diet were also depleted of their stores of 22:4ω6. A fatty acid believed to be derived by chain elongation of 20:3ω9, 22:3, was found in large concentrations in the papillae triglycerides of the EFA deficient rats. Incubations of exogenous arachidonic acid (20:4) in homogenates and tissue slices of the papillae of the HCO dietary groups showed that the PG synthetase was not impaired by an EFA deficiency. The rate of PGE synthesis in the papillae of the EFA deficient animals was generally enhanced when exogenous 20:4 was added, indicating that the concentration of available precursor(s) is a primary factor in the control of PGE synthesis in the papilla of the rat.

INTRODUCTION

Since the discovery that the kidney has a relatively high capacity to synthesize prostaglandins (PGs), there has been considerable interest in the role of renal PGs in kidney function and their possible involvement in blood pressure regulation (1). Several investigators have attempted to alter the course of experimental hypertension in rats (2,3) by chronic administration of nonsteroidal anti-inflammatory drugs such as indomethacin which block PG synthesis (4). However, due to the diverse actions (1) of these compounds and their toxicity to the gastrointestinal tract of rats, the conclusions of such studies might be open to question. Alternatively, essential fatty acid (EFA) deficiency, which has been shown to reduce PG synthesis in rabbit kidney (5), might serve as a useful model for studying potential roles of PGs in renal function. Rosenthal and coworkers (6) noted that hypertension was induced by high salt intake in rats fed an EFA deficient diet, but the degree of EFA deficiency and its effect on renal PG synthesis was not reported.

As recently summarized (7, and refs. therein), EFA deficiency has variable effects on the degree of depletion of PG precursors and PG synthesis depending on the tissue examined. For example, brain levels of arachidonic acid (20:4) and synthesis of $PGF_{2\alpha}$ by brain slices were only marginally affected by EFA deficiency in rats (7). Rat lung, small intestine (8) and kidney medulla (9) of rats fed an EFA deficient diet have been reported to give enhanced synthesis of PGs when supplied with exogenous 20:4; however, the decrease in endogenous PG synthesis relative to fatty acid composition of the tissues was not reported.

Although previous studies have shown that renal medullary lipid droplets (10,11) and medullary tissue (12) contain large amounts of triglycerides which are rich in polyunsaturated fatty acid PG precursors, the temporal relationship of the expected depletion of these fatty acids by EFA deficiency to renal PG synthesis has not been reported. In the present study, the synthesis of PGE in the renal papillae of rats fed a semisynthetic diet devoid of EFA was examined relative to changes in fatty acid composition.

MATERIALS AND METHODS

Animals

Littermate male Sprague-Dawley rats were

obtained (BioLab Corp., St. Paul, MN) at 21 days of age, divided into two groups and fed a basal fat-free diet supplemented with either 5% by weight safflower oil (SO) or 5% hydrogenated coconut oil (HCO). The composition of the basal fat-free diet was based on that described previously (13) with the exception that Williams-Briggs modified mineral mix (Teklad, Madison, WI) was employed rather than Wesson mineral mix. The complete diet was prepared in small fresh lots by adding HCO or SO (Teklad), vitamins A and E (ICN Pharmaceutical, Inc. Cleveland, OH) and vitamin D (Nutritional Biochemicals Corp. Cleveland, OH) in ether to the dry basal diet (Teklad test diet 76244). After placing the rats on the test diets, animals were killed at weekly intervals to determine the capacity for renal PGE synthesis from both endogenous and exogenous substrate. The kidneys were removed from anesthetized (sodium pentobarbital, 50 mg/kg) rats and rapidly chilled in ice-cold saline. The inner medulla (papilla only) was excised, weighed and incubated under different conditions as described below. In all experiments the papillae were preincubated in physiologic buffered saline medium (PBS) at 4 C for 5 min, and this medium discarded prior to 37 C incubations.

For comparison with the above experiments, animals fed similar diets for 51-63 weeks in another experiment were also used in this study.

Incubation of Renal Papillae

Intact papillae. Each papilla was halved lengthwise and incubated with gentle vortexing (Buchler Evapo-Mix) in 1 ml of preoxygenated PBS (Tyrodes) at 37 C for 15 min after which the PBS was removed and acidified (ca. pH 3) with formic acid.

Tissue homogenates. Upon completion of the above incubation, the tissue was homogenized in a glass microhomogenizer for 1 min with cold preoxygenated PBS (1 ml), and an aliquot incubated as such at 37 C for 15 min as above. Another aliquot was similarly incubated following addition of sodium arachidonate (Nu-Chek Prep, Elysian, MN, $>$ 99% pure). These incubations were terminated by addition of 7 vol. of chloroform-methanol (1:1).

Tissue slices. Papillae were sliced (1 mm) with a H. Mickle chopper, preincubated as described above, followed by 5 successive 30 min incubations in 2 ml PBS (Krebs) medium in an atmosphere of 95% O_2–5% CO_2 with gentle shaking in a metabolic shaker. In these incubations, the medium was removed after each 30 min period and replaced with fresh prewarmed and oxygenated PBS. Sodium arachidonate was added at the beginning of the fifth incubation.

Extraction and Measurement of PGE

The acidified PBS from the intact papilla incubations was extracted with petroleum ether (3 x 2 vol, 30-60 C bp) after which acidic lipids were extracted into ethyl acetate. The chloroform-methanol extract of homogenates was evaporated to dryness, dissolved in PBS (pH 7.5) and extracted with petroleum ether. The aqueous phase was then acidified with formic acid and extracted with petroleum ether and ethyl acetate as above. Prostaglandin E in the extracted lipid was measured following its base-catalyzed conversion to PGB (14) by a high performance liquid chromatographic (LC) method. The development of the LC method and its verification by independent measurements of PGE in renal tissue has been described previously (15). Briefly, PGB is separated from other PGs and lipids on a silicic acid LC column and simultaneously quantitated by UV absorbance at 280 nm employing a LDC model 1280 UV photometer with an 8 μl flow cell. In the present study, PGs were separated with a 25 cm, 10 μ Varian SI60 column eluted at 0.4 ml/min (ISCO model 384 pump) with chloroform-acetonitrile-formic acid (87.3:12:0.7; v/v/v). Peak heights were measured for quantitation of the extracted PGB relative to a PGB_2 standard prepared from authentic PGE_2 (Upjohn, Kalamazoo, MI) as previously described (15). Inasmuch as this method does not distinguish between PGE_1 and PGE_2, the PG measured is referred to as PGE. Prostaglandin A, if present in the extracts, would also be converted to PGB and be measured by the LC method; however, this PG was not found in amounts exceeding 10% of the PGE synthesized by rat papillae (15).

Fatty Acid Analysis

The fatty acid composition of the triglyceride and polar lipid fractions isolated by thin layer chromatography (TLC) (16) from papillae lipid extracted with chloroform-methanol (2:1, v/v) was determined by gas liquid chromatography (GLC) of methyl esters prepared by transesterification with methanol using HCl as a catalyst (17). GLC was carried out with a Barber Coleman gas chromatograph equipped with a flame ionization detector and a 6' x 1/4" glass column packed with 15% FGS on gas chrom P, 100-200 mesh (Applied Science Lab, Inc., State College, PA), at 180 C. Nitrogen was used as the carrier gas at a flow rate of 30 ml/min and the percentage composition was calculated from the proportionalities of the peak areas by an automatic digital integrator

TABLE I

Effect of EFA Depletion on PGE Synthesis by Isolated Rat Renal Papillae (nanograms/mg tissue *wet wt.*/15 *min*; M ± S.E.M.)

Age (weeks)	Diet[a]	Endogenous Substrate				Exogenous Substrate	
		Intact tissue[b]		Tissue homogenate[b]		Tissue homogenate + 20:4	
6	HCO	(10)[c]	2.0 ± 0.2	(10)	3.6 ± 0.7	(10)	17.9 ± 2.2
	SO	(9)	6.0 ± 0.7	(9)	11.4 ± 2.5	(9)	24.1 ± 2.8
9	HCO	(11)	1.1 ± 0.1	(10)	2.5 ± 0.5	(10)	31.3 ± 4.8
	SO	(8)	7.8 ± 1.0	(8)	11.5 ± 1.7	(7)	25.6 ± 3.6
10	HCO	(8)	0.8 ± 0.1	(9)	1.6 ± 0.2	(9)	27.5 ± 3.9
	SO	(7)	6.2 ± 0.9	(7)	13.0 ± 1.7	(7)	19.4 ± 1.9
	HCO/SO	(12)	4.7 ± 0.5	(11)	8.2 ± 0.8	(12)	22.0 ± 3.8
51-63	HCO		-------	(3)	1.0 ± 0.1	(3)	18.4 ± 1.8
	SO		-------	(3)	5.0 ± 0.3	(3)	16.2 ± 2.5

[a]HCO = hydrogenated coconut oil supplementation; SO = safflower oil supplementation; animals were fed each diet *ad libitum* from weaning (21 days); HCO/SO = 9 week old rats switched from HCO to SO diet for one week.

[b]See text for method of incubation.

[c]Number of rats. Each number represents separate incubations and separate PGE analyses.

(Model CS 1-208, Columbia Scientific Industries, Austin, TX). The peaks in the chromatogram were identified by comparison of retention times with known standards and by chemical ionization mass spectrometry (18).

RESULTS

Reduced Renal PGE Synthesis from Endogenous Substrate in EFA Deficient Rats

A significant ($P < .001$) depression of renal papillary PGE synthesis and release from intact papillae (66% decrease from controls) was found after 3 weeks feeding of the HCO diet (Table I). The small quantity of PGE released into the PBS from the papillae of the animals of the HCO group (2.0 ± 0.2 mμg/mg tissue wet weight/15 min; M ± S.E.M.) relative to the papillae of the controls (6.0 ± 0.7 mμg/mg/15 min) was not due to a defect in "release" of PG rather than reduced *biosynthesis* from endogenous substrate, since a similar reduction of PG synthesis (68%, $P < .01$) was found in broken-cell preparations of papillae from the HCO group of animals. Although the HCO rats were still actively growing at this age (6 weeks), their weights (134 ± 20 g; M ± S.D.) already lagged behind those of the control rats (160 ± 18 g).

After an additional 3 weeks feeding of the HCO diet (9 week-old rats), renal PGE synthesis and release in the intact papillae was reduced further ($P < .01$ compared to 6 week old HCO rats) to about 14% that of the control rats (Table I). The level of PG synthesis from endogenous substrate, which was ca. 10-20% that of the control rats at 9 weeks, appeared to be near-maximally depressed inasmuch as similar results were found in studies of papillae from 10 week old rats (7 weeks on the HCO diet) and in rats fed an HCO diet for ca. 1 year in an accessory experiment (Table I).

It was also found that the near-maximal reduction of renal PGE synthesis observed in the 9-week-old HCO groups could be rapidly reversed when PG precursors were supplied by diet. Switching the HCO group of animals to the SO diet for one week returned PGE synthesis (subsequently measured in vitro) to near-normal levels (Table I).

The weights of the 9-week-old rats of the HCO group (218 ± 28 g) were also significantly less than those of the controls (288 ± 13 g), but they appeared healthy and were still actively growing. At this age, the kidneys of HCO rats were slightly larger, as a percent of body weight, than those of the control rats, and papillae wet weights as a percent of kidney weights averaged 50% more than those of the control rats.

The year old animals exhibited severe dermal symptoms of an EFA deficiency, and their weights were substantially lower than the control group (320 ± 13 vs 605 ± 60 g).

Effect of Added Substrate on Renal PGE Synthesis in EFA Deficient Rats

Despite their reduced PGE synthesis from endogenous substrate, papillae from the HCO rats retained an active PG synthetase system as demonstrated by essentially normal or enhanced production of PGE when exogenous arachidonic acid was provided. When arachi-

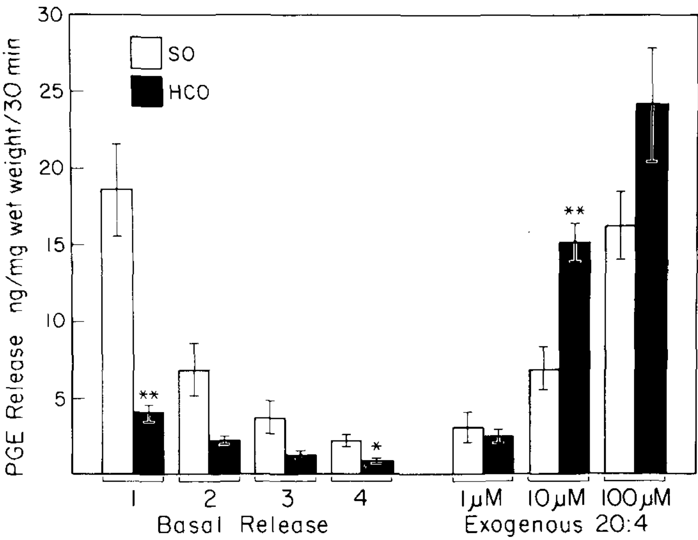

FIG. 1. Comparison of the effectiveness of exogenous arachidonic acid to increase PGE synthesis and release by renal papillae slices from SO- and HCO-fed rats, following four (1-4) consecutive 30 min periods of basal release. Column heights and brackets indicate the mean ± S.E.M. PGE release determined in experiments on tissue from 3-8 separate rats. Asterisks indicate conditions where PGE release from HCO papillae was statistically different than the corresponding SO papillae (*, $P < .05$;**, $P < .01$). See text for method of incubation.

donic acid was added (180 μM) to papillae which were homogenized after the initial 15 min incubation, PGE synthesis was increased by about 2-fold in papillae from the SO rats and by 6- to 20-fold in the HCO rats (Table I). Since these results could have been influenced by the levels of endogenous substrate remaining in the papillae, the high concentration of 20:4 employed, and/or effects of potentially inhibitory fatty acids released by the homogenization, an additional series of experiments was conducted to examine the concentration-dependency of the apparent enhanced PG synthesis in papillae of the HCO groups when exogenous 20:4 was provided. In these experiments, sliced papillae were incubated for 4 x 30 min periods in an effort to reduce PGE synthesis in the papillae prior to addition of 20:4. The results are depicted in Figure 1. After 2 hr incubation (four 30 min incubations), PGE synthesis and release by the papillae of the control rats was reduced to a level near, although still higher than, that of the papillae of the HCO group. Addition of 20:4 (1-100 μM) enhanced PGE synthesis by the papillae of the HCO groups to a greater degree, particularly at lower 20:4 concentrations, than it did in the papillae of the control rats as illustrated in Figure 1. For further verification that the material measured in the final assay (PGB measurement by LC) in the experiments in which exogenous 20:4 was supplied was indeed produced by PG synthetase, the ability of the PG synthetase inhibitor, indomethacin, to reduce the amount of PGB measured was examined. In two experiments, indomethacin (1 or 5 μM) was added to the incubation media in the 4th 30 min incubation period and in the 5th period (when 20:4, 10 μM, was added). Indomethacin reduced the enhanced PGE generation effected by 20:4 in papillae of the animals of the HCO dietary group by 60% (1 μM indomethacin) and by 93% (5 μM indomethacin) in these experiments.

Fatty Acid Composition of Papilla Lipids

The fatty acid composition of the triglyceride fraction of the papillae lipids of the 6, 9 and 60-week-old animals (Table II) showed that near maximal changes occurred after feeding the HCO diet for 3 weeks. The 18:2 and 20:4 were markedly reduced, and the 18:1 and 20:3 (ω9) were highly elevated, typical of an essential fatty acid deficiency. The analyses in Table II show, in accord with the studies of others(10-12) on kidney lipid and particularly the lipid droplets of the papillae, that the triglycerides are rich in polyunsaturated fatty acids, particularly 20:4 and 22:4. These fatty acids which were concentrated in the triglycerides also were depleted from the papillae of the EFA deficient animals (Table II). In addition to the typical pattern of fatty acids of an EFA deficiency, a fatty acid with a retention time

TABLE II

Fatty Acid Analyses of Renal Papillae Lipids[a] (% wt)

Age	6 weeks				9 weeks				60 weeks			
Diet[b]	HCO		SO		HCO		SO		HCO		SO	
	TG[c]	PL[c]	TG	PL	TG	PL	TG	PL	TG	PL	TG	PL
16:0	13.7 ± 5.4[d]	15.8 ± 1.8	11.9 ± 1.1	19.2 ± 2.9	25.8 ± 5.9	23.9 ± 1.3	14.8 ± 1.7	22.5 ± 1.4	17.8 ± 0.6	21.9 ± 1.7	12.7 ± 0.8	27.6 ± 2.5
16:1	2.2 ± 1.1	2.8 ± 0.9	1.3 ± 0.4	1.0 ± 0.1	2.6 ± 1.1	1.8 ± 0.6	1.8 ± 0.8	2.4 ± 1.0	2.3 ± 0.9	1.6 ± 0.4	0.8 ± 0.3	0.3 ± 0.1
18:0	11.3 ± 2.2	18.1 ± 0.6	10.5 ± 0.7	22.7 ± 2.7	16.2 ± 5.1	24.7 ± 2.0	16.1 ± 3.2	25.8 ± 2.8	11.5 ± 0.9	24.7 ± 1.4	10.9 ± 0.5	32.1 ± 1.5
18:1	20.3 ± 1.9	24.3 ± 2.1	10.1 ± 1.1	14.1 ± 1.7	20.7 ± 6.2	25.8 ± 0.8	10.9 ± 1.5	15.0 ± 2.7	20.1 ± 0.7	21.4 ± 1.1	8.3 ± 0.7	8.1 ± 0.6
18:2	1.1 ± 0.3	1.1 ± 0.2	14.7 ± 1.8	10.7 ± 1.5	2.1 ± 1.5	0.5 ± 0.1	10.6 ± 0.8	7.6 ± 1.3	4.6 ± 0.9	0.6 ± 0.2	18.7 ± 1.2	8.6 ± 0.4
20:3ω-6	----	----	2.3 ± 1.5	2.9 ± 0.1	----	----	1.8 ± 0.6	0.7 ± 0.4	----	----	2.1 ± 0.5	0.9 ± 0.7
20:3ω-9	22.0 ± 0.9	19.2 ± 1.1	----	----	19.0 ± 2.6	16.7 ± 1.0	----	----	12.3 ± 1.1	19.7 ± 2.7	----	----
20:4	1.5 ± 0.4	7.7 ± 1.1	22.3 ± 1.7	23.4 ± 3.4	----	4.9 ± 1.5	18.8 ± 2.6	18.7 ± 3.9	0.9 ± 0.4	4.3 ± 1.2	21.3 ± 0.9	17.6 ± 1.5
22:3[e]	20.6 ± 3.5	7.4 ± 0.1	----	----	10.9 ± 4.8	----	----	1.3 ± 0.4	23.5 ± 1.8	3.8 ± 0.3	----	----
22:4	0.7 ± 0.8	----	22.5 ± 1.3	3.5 ± 0.2	----	0.4 ± 0.9	19.3 ± 4.2	2.4 ± 0.7	0.5 ± 0.4	----	22.4 ± 2.8	2.0 ± 0.4
Other[f]	6.9	3.6	4.4	2.5	----	1.3	5.9	3.6	6.5	2.0	2.8	2.8

[a]Major components.
[b]HCO, hydrogenated coconut oil; SO, safflower oil diets fed from weaning (21 days).
[c]TG = triglycerides; PL polar lipids separated by TLC.
[d]M ± SD.
[e]Double bond position not determined.
[f]Minor fatty acids by difference from 100%.

corresponding to 22:3 was detected in the triglycerides of the papillae of the EFA deficient animals (Table II). Its identity was confirmed by chemical ionization mass spectral analysis, which with its structural analysis will be reported in a separate communication.

DISCUSSION

This study shows that when fed as the sole source of fat in the diet, HCO produced marked effects on the fatty acid composition of the kidney papilla and on its synthesis of PGE. The changes in the fatty acid composition of the HCO group relative to the controls showed the typical pattern of an EFA deficiency, that is, elevated 16:1, 18:1 and 20:3 ω9 with a corresponding reduction in 18:2 and 20:4. Kidney contains, in addition, 22:4 (10-12) which is also diminished with the feeding of the HCO diet. The 22:3 fatty acid detected in the papillae of EFA deficient animals has not been previously reported. Presumably this fatty acid is derived by chain elongation of 20:3 ω9, but the positions of unsaturation were not determined.

Although the animals were still growing at six weeks of age (three week feeding period) on the HCO diet, and they exhibited no overt symptoms of an EFA deficiency, the triene/tetraene ratio was above that considered as positive evidence of this syndrome (19). The enhanced conversion of substrate by the papillae of the EFA deficient rats is not unexpected inasmuch as several lipid enzymes such as lecithin: cholesterol acyltransferase (16) exhibit greater activity in EFA deficient animals. Enhanced enzyme activity in EFA deficient animals might be explained on the basis of a loss of regulatory mechanisms with the changes induced by a deficiency of EFA.

The renal papillae were not completely depleted of arachidonic acid even in the 1 year old animals, but apparently little of it was available for PG synthesis upon feeding HCO as the sole source of fat, since, as evidenced by the ability of this tissue to synthesize PGE when exogenous 20:4 was provided, the PG synthetase was not impaired by an EFA deficiency. The reduced synthesis of PGE from endogenous substrate in the EFA deficient animals might also be attributed in part to an inhibitory effect of 20:3ω9 (20) because, being in greater concentration than 20:4, it might be released simultaneously and block PG synthesis. In view of the markedly elevated 20:3ω9 levels of the HCO animals, it is possible that an adequate quantity of 20:4 remained for normal production of PG endoperoxide intermediates by the renal papilla, and that the subsequent synthesis

of PGE relative to other PGs was merely reduced. Only PGE was measured in the present study. It has recently been shown that 20:3ω9 inhibits $PGH_2 \rightarrow PGE_2$ isomerase (21). It has also been shown that 18:2 depresses the synthesis of PGs (22).

These studies suggest that a "PG deficiency" can be induced in rat kidney in a relatively short time by feeding weanling rats an EFA deficient diet. Inasmuch as the reduction of in vitro PG synthesis shown here precedes severe growth retardation and development of overt symptoms of EFA deficiency, it would appear that EFA deficiency might be an acceptable alternative to PG synthetase inhibitors as a tool to reduce PG synthesis in chronic studies of the role of PGs in cardiovascular-renal function. Caution should be exercised, however, in extrapolating these results to the in vivo situation. It is well known that in vitro protocols, as in the present study, are potent stimuli for PG synthesis and, thus, reflect the *capacity* for PG synthesis. It is possible that the amount of remaining 20:4 in the renal medulla of the EFA deficient animal is sufficient for basal PG synthesis in the conscious, unstressed animal. Although numerous studies have demonstrated the efficacy of PG synthetase blockers in reducing the elevated PG synthesis associated with stress and trauma (i.e., anesthesia, surgery), recent studies (23,24) indicate it is difficult to appreciably reduce basal PG synthesis and excretion with these compounds. The evidence that EFA deficiency will reduce basal renal PG synthesis in vivo is still outstanding.

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Availability of Lysophosphatidylcholine in Single Bilayer Vesicles for Hydrolysis by Lysophospholipase

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ABSTRACT

Single bilayer vesicles were prepared from total rat liver microsomal lipids to which 5 mol% lysophosphatidylcholine had been added. The availability of lysophosphatidylcholine for enzymatic hydrolysis by lysophospholipase (EC 3.1.1.5) was found to be higher in vesicles prepared by the cholate dispersion technique when compared with sonicated vesicles. Sepharose 4 B chromatography showed that the vesicles prepared by the cholate technique were smaller than those prepared by sonication. This is in contrast to previous observations for egg phosphatidylcholine vesicles. Total rat liver microsomal extracts were found to contain proteolipid, which could be removed by ether precipitation. Cholate vesicles prepared from proteolipid-free extracts were still smaller than sonicated vesicles from this extract. Experiments with [^{14}C] dextran entrapped in the vesicles indicate that there is no loss of the permeability barrier of the vesicles for high molecular weight solutes during vesicle treatment with lysophospholipase. The high availability of lysophosphatidylcholine in cholate vesicles of total rat liver microsomal lipids is discussed in terms of a highly asymmetric distribution of lysophosphatidylcholine over the inner and outer monolayer of the bilayer.

INTRODUCTION

Previous results have shown good agreement between the extent of lysophosphatidylcholine availability in single bilayer lipid vesicles for hydrolysis by lysophospholipase (EC 3.1.1.5) and the amount of lysophosphatidylcholine present in the outer monolayer of such vesicles as deduced from ^{13}C-NMR measurements (1,2). Both techniques have led to the conclusion that lysophosphatidylcholine is distributed asymmetrically over the inner and outer monolayer of the vesicles with enrichment in the outer monolayer. Such an asymmetric localization is in line with the structural dimensions of highly curved phospholipid bilayer membranes. It has recently been reported (3) that the area per headgroup in the outer monolayer of egg phosphatidylcholine vesicles amounts to 74Å^2, with a tapering of the available surface space at the acyl tail end of the molecule to 46 Å^2. The corresponding values for a lipid molecule in the inner monolayer were reported to be 61 Å^2 and 97 Å^2, respectively. Similar values have been obtained also for dipalmitoylphosphatidylcholine vesicles (4). Lysophosphatidylcholine is a wedge-shaped molecule with a narrow acylchain attached via glycerol to a phosphocholine with a relatively larger cross-sectional area. On the basis of geometrical considerations, such tapered molecules can be expected to orient preferentially in the outer monolayer, as was experimentally observed (1,2,5).

This paper reports on the availability of lysophosphatidylcholine in single bilayer vesicles prepared by either a cholate dispersion (6) or sonication procedure from total rat liver microsomal lipid extracts.

EXPERIMENTAL PROCEDURES

Materials

1-[9,10-^{3}H$_2$] Stearoyl-*sn*-glycero-3-phosphocholine, specific activity 1200 dpm/nmol, was prepared as described (7). [1α, 2α(n)-^{3}H$_2$]Cholesteryl oleate, specific activity 60 μCi/μmol, was prepared (8) from [1α, 2α(n)^{3}H$_2$]cholesterol purchased from The Radiochemical Centre (Amersham, U.K.). [Carboxyl-^{14}C]Dextran, mean M.W. 20000, specific activity 2.76 mCi/g, was a product from New England Nuclear (Boston, MA). Ultrogel AcA 44 was obtained from LKB (France). Lysophospholipase II was purified from beef liver as described previously (9).

Methods

Preparation and extraction of rat liver microsomes. Rat liver microsomes were prepared as before (5) and extracted by a modification (10) of the method described by Folch et al. (11). When desired, proteolipid was removed from the extracts by diethyl ether precipitation (12) as described by Cattell et al. (13).

Sonicated and "cholate" vesicles. Single-walled liposomes were made as described (5). The term "cholate" vesicles refers to lipid vesicles prepared by the cholate dispersion method (6). In the study of the intactness of "cholate" vesicles during the enzymatic hydrolysis of lysophosphatidylcholine, lipids were dispersed in buffered [^{14}C]dextran and applied to a Sephadex G-50 column. The void volume fractions were pooled and the vesicles

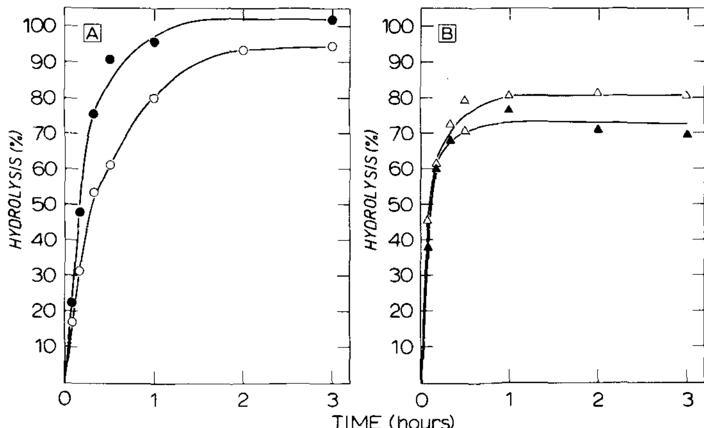

FIG. 1. Time courses of lysolecithin enzymatic hydrolysis in vesicles made of total rat liver microsomal extract and 5 mol% lysolecithin. In A, vesicles were prepared by the cholate method and the incubation mixtures contained: 3.8 μmol lipid phosphorus and 1.8 mg lysophospholipase II (EC 3.1.1.5) in a total volume of 13.5 ml (●) or 288 nmol lipid phosphorus and 105 μg lysophospholipase II in a total volume of 500 μl (○). In B, sonicated vesicles were used and incubation mixtures contained 4.3 μmol lipid phosphorus and 1.8 mg lysophospholipase II in a total volume of 13.5 ml (▲) or 137 nmol lipid phosphorus and 105 μg lysophospholipase II in a total volume of 500 μl (△). All points were corrected for blank hydrolysis.

concentrated at 4 C under nitrogen through a Diaflo XM-100 membrane. After incubations with and without enzyme, the mixtures were applied to Ultrogel AcA 44 columns (42 x 2 cm) operated at 4 C with a constant upwards flow rate of 12 ml/hr. Fractions of 4 ml were collected and 500 μl from each were analyzed for ^{14}C and ^{3}H radioactivity using a dioxane scintillation fluid (5).

Lysophosphatidylcholine enzymatic hydrolysis. Incubations were performed at 37 C, and the hydrolysis was followed by determination of the $^{14}C/^{3}H$ ratio in the heptane layer of modified Dole extracts (14) of incubation mixtures containing vesicles with 1-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine and [^{3}H] palmitic acid as internal standard, essentially as described (15). In the case of the vesicles containing entrapped [^{14}C] dextran, 1-[9,10-$^{3}H_2$] stearoyl-*sn*-glycero-3-phosphocholine was used, and the Dole extraction was performed only to estimate the extent of hydrolysis during the incubation step. For a more accurate determination, the void volume fractions from the Ultrogel AcA 44 columns were pooled, freeze-dried and extracted according to Folch et al. (11). The organic layers were concentrated and applied to a Silica Gel G thin layer plate. After development in chloroform-methanol-water (65:35:4, v/v), free fatty acids and lysolecithin spots were located by iodine vapor and by a radioactivity scan of the plate using a Panax thin layer scanner. The radioactive areas were scraped into counting vials and analyzed for ^{3}H radioactivity after addition of toluene scintillation fluid containing Triton X-100 and water (2:1:0.2, v/v).

Relative size analyses. Two vesicle populations bearing different nonexchangeable, radioactive markers were mixed and then chromatographed through a Sepharose 4 B column operated as described (5). Aliquots from each fraction were analyzed for ^{3}H and ^{14}C radioactivity. For further experimental details, see legend of the respective figures.

Gel electrophoresis. Electrophoresis in 10% acrylamide gels containing 0.1% SDS and fixation were performed as described by Laemmli (16). Gels were kept in methanol-acetic acid-water (5:1:5, v/v) during 24 hr and were then stained for 4 hr with a 0.25% Coomassie Brilliant Blue R 250 solution made up in this solvent mixture. Destaining was done by diffusion in this solution without staining reagent for about 28 hr. Gels were stored in 7% acetic acid.

Analytical procedures. The method of Mokrasch (17) was followed to determine proteolipid in Folch extracts of rat liver microsomes using bovine serum albumin as standard. All other methods including lipid phosphorus determinations were as previously reported (5).

RESULTS

Single bilayer vesicles were prepared from total rat liver microsomal lipids to which 5 mol% of radiolabeled lysophosphatidylcholine had been added. As can be seen in Figure 1, the availability of the lysophosphatidylcholine for hydrolysis by external lysophospholipase

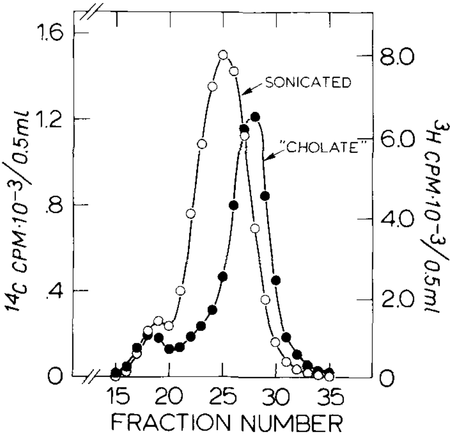

FIG. 2. Sepharose 4 B elution pattern of a mixture of "cholate" and sonicated vesicles made of total rat liver microsomal extract and 5 mol% lysophosphatidylcholine. "Cholate" vesicles were prepared from 28.16 μmol microsomal lipids (expressed as P), 1.499 μmol egg lysophosphatidylcholine and 6 nmol [^{14}C]phosphatidylcholine dispersed in 1.26 ml buffer containing 139.2 μmol sodium cholate. After detergent removal through a Sephadex G-50 column, the vesicle fractions were pooled (50 ml). An aliquot of 9 ml was then combined with 1 ml of sonicated vesicles prepared from a 1.5 ml dispersion containing 16.104 μmol microsomal lipids (expressed as P), 838 nmol egg lysolecithin and a trace (4.6 nmol) of [^{3}H]cholesteryl oleate. Each fraction eluting from the Sepharose 4 B column was analyzed for ^{3}H (o—o) and ^{14}C (●—●) radioactivity by counting aliquots of 0.5 ml.

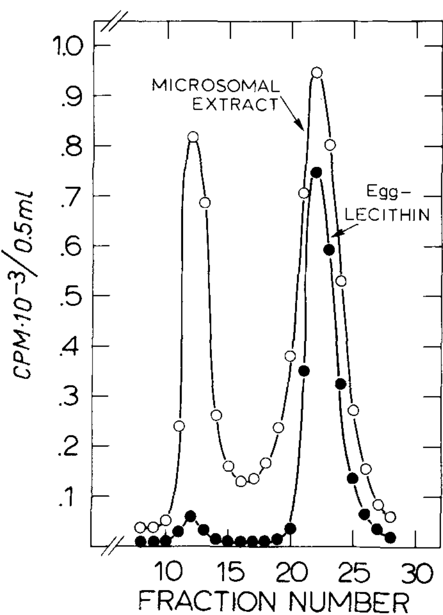

FIG. 3. Sepharose 4 B chromatographic behavior of "cholate" vesicles prepared from total rat liver microsomal extract or from egg phosphatidylcholine (in both cases, 5 mol% lysophosphatidylcholine was added). A dispersion containing 14.070 μmol microsomal extract (as P), 706 nmol egg lysolecithin, 45 nmol [^{3}H]cholesteryl oleate and 69.6 μmol sodium cholate in 1.56 ml buffer was applied to a Sephadex G-50 column. A similar column was loaded with 1.56 ml of a buffered dispersion containing 14.090 μmol egg phosphatidylcholine, 706 nmol egg lysolecithin, 12 nmol [^{14}C]lecithin and 69.2 μmol sodium cholate. The void volume fractions from each column were combined (50 ml per vesicle preparation), and aliquots (6 ml from the microsomal extract vesicles, 3 ml from the egg lecithin vesicles) mixed and applied to a Sepharose 4 B column. Fractions were analyzed for ^{3}H (o—o) and ^{14}C (●—●) radioactivity by counting aliquots of 0.5 ml.

amounted to 94-100% in the vesicles prepared by the cholate method (Fig. 1A). This value is somewhat larger than the 70-80% availability found in vesicles prepared by the classical sonication procedure (Fig. 1B). Since it has previously been shown (5) that both methods of vesicle preparation yielded vesicles with a lipid composition very similar to that of the microsomal extract, the different availability of lysophosphatidylcholine for hydrolysis is unlikely to be attributed to differences in lipid composition. It can be expected from theoretical considerations (3,4,18) that the distribution of phospholipids over inner and outer monolayer of single bilayer vesicles becomes the more asymmetric the higher the curvature of the vesicles. Accordingly, the higher availability of lysophosphatidylcholine in "cholate" vs. sonicated vesicles as observed in Figure 1 could, in theory, be caused by a higher curvature of the "cholate" vesicles. However, for those cases in which a comparison of sizes has been made so far, i.e., for vesicles composed of egg phosphatidylcholine (6) or of a mixture of egg phosphatidylcholine containing 5 mol% lysophosphatidylcholine (5), the vesicles prepared by the cholate method were found to be considerably larger than the corresponding vesicles prepared by sonication. In order to determine the relative sizes of vesicles prepared from total rat liver microsomal lipids by the cholate and sonication method, a mixture of these vesicles containing differently labeled nonexchangeable markers was chromatographed over a Sepharose 4 B column. As can be seen in Figure 2, the average size of the "cholate" vesicles appeared to be smaller than that of their sonicated counterparts. Since this was in contrast with the previous results with egg phosphatidylcholine vesicles (5,6), it became of interest to compare the relative sizes of vesicles with a different lipid composition. When this was done for vesicles prepared by the cholate method from total rat liver microsomal lipids and from egg phosphatidylcholine, they were found to have the same average size (Fig. 3).

TABLE I

Phospholipid Composition and Proteolipid Content of Rat Liver Microsomal Extracts

Lipid extract	Phospholipid composition[a]					Proteolipid[b]
	PC	PE	PI	PS	SPH	
Total	63	20	10	3	4	42
After ether precipitation	66	20	9	2	4	–[c]

[a]Phospholipid composition is expressed as percent of total phosphorus recovered from the thin layer plates. P = phosphatidyl, C = choline, E = ethanolamine, I = inositol, S = serine, SPH = sphingomyelin.

[b]Proteolipid is expressed as μg protein/ μmol lipid phosphorus.

[c]Proteolipid was not assayed in the extract after ether precipitation. The precipitate contained at least 80% of the initial proteolipid. See also Fig. 4 and text.

Upon careful examination of the total microsomal lipid extract, this was found to contain small amounts of chloroform/methanol soluble proteins (Table I, Fig. 4). It appeared possible to remove these proteins by ether precipitation, without changing the lipid composition of the microsomal extract (Table I, Fig. 4). Vesicles prepared from such protein-free extracts by the cholate method were still smaller than sonicated vesicles from the same extracts (Fig. 5). It must, therefore, be concluded from these experiments that the lipid composition determines the relative sizes of vesicles prepared by the cholate and sonication methods. The original conclusion that "cholate" vesicles are larger than their sonicated counterparts holds for phosphatidylcholine vesicles, but cannot be generalized.

Although the greater availability of lysophosphatidylcholine in total microsomal lipid vesicles prepared by the cholate method when compared with the sonicated vesicles of similar composition is in line with the smaller size of the former, it is doubtful whether this can be the whole explanation. As can be seen in Figure 3, egg phosphatidylcholine vesicles have the same size, yet the availability of lysophosphatidylcholine for hydrolysis by lysophospholipase is greater in the vesicles derived from total microsomal lipids (Table II). This suggests that, apart from the size of the vesicles, the lipid composition also contributes to the asymmetric distribution of lysophosphatidylcholine over inner and outer monolyer of the vesicle. In view of the near complete availability of lysophosphatidylcholine in the microsomal lipid vesicles prepared by the cholate method, it was felt necessary to exclude the possibility of vesicle leakiness for the lysophospholipase (M.W. 60000), thus allowing the enzyme to act on the lysophosphatidylcholine in both inner and outer monolayer. Vesicles containing entrapped

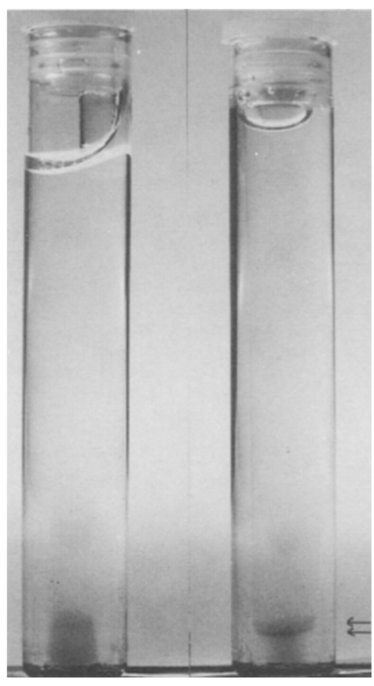

FIG. 4. Polyacrylamide gels after electrophoresis of 1.2 μmol total lipid extract (right) and 0.9 μmol protein-free extract (left). Quantities are expressed as lipid phosphorus. Coomassie Brilliant Blue positive bands are indicated by arrows. Diffuse bands are photographic artefacts of opaque areas in the gels due to the presence of lipids.

[^{14}C]dextran (mean M.W. 20000) were prepared from a mixture of total microsomal lipids and 5 mol% of 1-[9,10-$^3H_2$] stearoyllysophosphatidylcholine as described under Methods. Sepharose 4 B chromatography of the preparation showed that more than 90% of the entrapped [^{14}C]dextran was present in the single bilayer vesicle fraction, and the percentage of multilayered liposomes as deduced from the

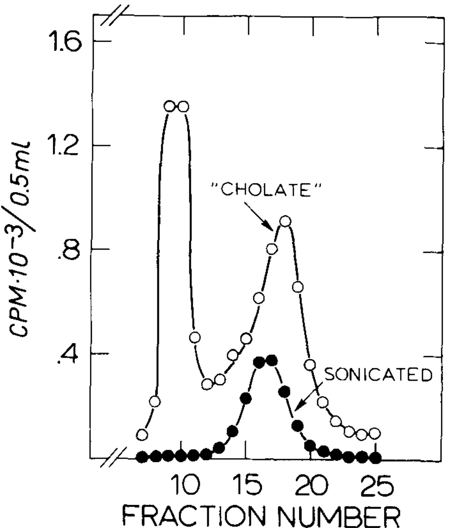

FIG. 5. Sepharose 4 B elution patterns of sonicated and "cholate" vesicles made of protein-free microsomal extract. Cholate vesicles were prepared from a protein-free extract, 5 mol% lysolecithin and a trace of [^{3}H] cholesteryl oleate using the same conditions as described in legend of Figure 3 for the total extract vesicles. The vesicle fractions were pooled and an aliquot of 6.5 ml was mixed with 200 μl taken from a 1.5 ml preparation of sonicated vesicles containing 14.060 μmol protein-free extract (expressed as P), 706 nmol egg lysolecithin and 2 nmol of [^{14}C] lecithin. The combined vesicles were then applied to a Sepharose 4 B column and 0.5 ml from each fraction analyzed for ^{3}H (○—○) and ^{14}C (●—●) radioactivity.

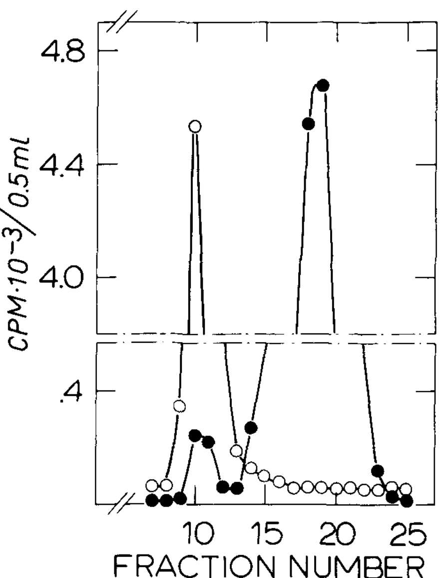

FIG. 6. Ultrogel AcA 44 elution pattern of an incubation mixture after lysophospholipase II action on cholate vesicles made of rat liver microsomal extract. A dispersion containing 28.160 μmol microsomal extract (expressed as P), 838 nmol egg lysolecithin, 644 nmol [^{3}H] lysolecithin and 20 μCi [^{14}C] dextran in 2 ml buffer was chromatographed through a Sephadex G-50 column. The void volume fractions were pooled (ca. 70 ml) and concentrated at 4 C under nitrogen through a Diaflo XM-100 membrane until a volume of ca. 5 ml. No vesicle aggregation tood place during this step, as revealed by control experiments (not shown). Part of this vesicle preparation (2.5 ml) was incubated in a total volume of 2.8 ml at 37 C, during 2 hr, with 2.1 mg lysophospholipase II. The mixture (2.65 ml) was then applied to an Ultrogel AcA 44 column and aliquots of 0.5 ml from each fraction analyzed for ^{3}H (○—○) and ^{14}C (●—●) radioactivity.

[^{3}H] lysophosphatidylcholine distribution amounted to only 4% (data not shown). The latter value is comparable to that found in previous experiments (5).

Following incubation of the vesicle preparation for 2 hr at 37 C with or without lysophospholipase, the mixtures were applied to an Ultrogel AcA 44 column. As shown in Figure 6, a good separation between free and entrapped dextran was achieved. A similar pattern was obtained for the incubation in which lysophospholipase was omitted (not shown). The ^{3}H/^{14}C ratios in the vesicle fraction were 17.1 for enzyme treated and 18.2 for blank incubated vesicles. These data indicate that [^{14}C] dextran did not leak out of the vesicles during enzyme treatment. Analysis of the vesicles after the 2 hr incubation with lysophospholipase showed that 93% of the lysophosphatidylcholine had been hydrolyzed, in agreement with the results presented in Figure 1 A. This extent of hydrolysis cannot be explained by enzyme penetration into the vesicle, and is most likely due to an extremely asymmetric localization of lysophosphatidylcholine in the outer monolayer of these vesicles.

DISCUSSION

The different availabilities of lysophosphatidylcholine in "cholate" and sonicated vesicles prepared from rat liver microsomal lipids are in line with the relative size of these vesicles, i.e., the smaller the vesicles, the higher the availability of lysophosphatidylcholine for hydrolysis by external lysophospholipase. However, in general, size alone cannot explain the different availabilities. This is obvious from a comparison of "cholate" vesicles prepared from either egg phosphatidylcholine or from microsomal lipids. Despite similar sizes (Fig. 3), the maximal extent of lysophosphatidylcholine hydrolysis is higher in the bilayers originating from microsomal lipids. Although the different lipid compositions can be an obvious explanation in the latter case, when taking the results as a whole, it is conceivable that the different mechanisms of vesicle formation in the cholate

TABLE II

Apparent Mean Sizes and Lysophosphatidylcholine Availabilities for Enzymatic Hydrolysis of Vesticles with Different Lipid Composition and Prepared by Two Different Methods

Vesicle composition[a]	Method	Apparent mean size[b]	Availability[c]
Egg PC	Sonication	2.00	85-90
Microsomal lipids	Cholate	1.83	94-100
Egg PC	Cholate	1.85	85-90
Microsomal lipids	Sonication	1.50	70-80

[a]In each case, 5 mol% lysophosphatidyl choline were added. Abbreviations as in Table I.
[b]Apparent mean sizes are expressed as the ratio between the elution and the void volumes after Sepharose 4 B chromatography.
[c]Availabilities are expressed as percent of total lysophosphatidylcholine available for alkaline hydrolysis.

and sonication procedures contribute to the different lysophosphatidylcholine distributions. In this view, the cholate method would seem to result in higher lysophosphatidylcholine asymmetry than the sonication procedure. In the case of egg phosphatidylcholine vesicles, this effect would be exactly counterbalanced by the difference in size. "Cholate" vesicles of egg phosphatidylcholine are larger than their sonicated counterparts, but the lower lysophosphatidylcholine asymmetry expected from this larger size is counterbalanced by a higher asymmetry resulting from the mechanism of vesicle formation, thus leading to similar lysophosphatidylcholine availabilities (Table II). In the case of vesicles from microsomal lipids, the higher asymmetry expected for "cholate" vesicles due to the mechanism of formation is reinforced by the smaller size of those vesicles when compared with their sonicated counterparts, thus leading to higher lysophosphatidylcholine availability in the "cholate" vesicles.

The finding that [^{14}C] dextran is not able to leak out from microsomal lipid vesicles prepared by the cholate method during lysophospholipase treatment makes it very unlikely that the enzyme has access to both monolayers. This interpretation is corroborated by the reported presence of an efficient permeability barrier for ^{22}Na ions in "cholate" vesicles prepared from egg phosphatidylcholine (6) or from a mixture of phospholipids and acetylcholine receptor (19). Then, the extreme lysophosphatidylcholine availability (94-100%) found in "cholate" vesicles prepared from rat liver microsomal lipids is most likely due to the presence of that amount of lysophosphatidylcholine in the outer monolayer. The asymmetric distribution found previously for this compound in phosphatidylcholine vesicles can be extended to artificial bilayers with a lipid composition which is more closely comparable to that of a biomembrane. Lysophosphatidylcholine has been implicated in membrane fusion processes. When extrapolation to biomembranes is allowed, our results would seem to indicate that formation of highly curved areas of biomembranes would result in enrichment of lysophosphatidylcholine in the outer monolayer of the bilayer in these regions, thus perhaps facilitating fusion due to destabilization of the bilayer in these areas.

The alternative explanation that the high availability of lysophosphatidylcholine results from a rapid transbilayer movement of this compound cannot be completely excluded at present. Such high rates for lysophosphatidylcholine translocation have not been detected in phosphatidylcholine vesicles (1,2,5), unless glycophorin was incorporated (20). Even in the latter case, a biphasic behavior was found for lysophosphatidylcholine hydrolysis. Since this is not observed in "cholate" vesicles from microsomal lipids (Fig. 1 A), this implicates that transbilayer movement, if occurring, would have to be faster than the enzymatic hydrolysis. We have considered the possibility that the proteolipid in the microsomal extract, the presence of which has also been noted by others (21,22), would facilitate the transbilayer movement of lysophosphatidylcholine in much the same way as described for glycophorin (20). This idea was abandoned by the finding that only 70-80% of lysophosphatidylcholine was hydrolyzed in the sonicated vesicles from total microsomal lipids. Also, "cholate" vesicles prepared from proteolipid-free extracts showed comparable lysophosphatidylcholine availabilities as vesicles prepared from extracts still containing the proteolipid.

As a note of caution, we would like to emphasize our finding that cholesteryl oleate, often used as nonexchangeable marker in vesicle studies, is only partially incorporated in single-bilayer vesicles prepared by the cholate method (Figs. 3 and 5). This compound can, therefore, only be used as nonexchangeable

marker for "cholate" vesicles when the latter are sized by Sepharose 4 B chromatography.

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LETTERS TO THE EDITOR

The Occurrence of a Furanoid Fatty Acid in *Hevea brasiliensis* Latex

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ABSTRACT

Methyl esters from the triglyceride fraction of the neutral lipids of natural rubber latex were found by gas liquid chromatography to contain about 90% of a furanoid acid. Spectroscopic analysis identified the acid as 10,13-epoxy-11-methyloctadeca-10,12-dienoic acid.

INTRODUCTION

Fatty acids containing a furan ring have been isolated from *Exocarpus cupressiformis* seed oil (1) and from the oils of several species of fish (2-4). The furan ring in the former has no methyl substituents, while the fish oils have methyl or dimethyl substituents on the ring. Here we wish to report the discovery of a furanoid fatty acid containing a methylfuran group, 10,13-epoxy-11-methyloctadeca-10,12-dienoic acid in *Hevea brasiliensis* or natural rubber latex. It is mainly found in the triglyceride fraction of the neutral lipids of natural rubber latex and constitutes about 90% of the total esterified acids. The latter fact indicates that the main triglyceride found in *Hevea* latex contains three furanoid fatty acids and is thus a rare triglyceride known in nature.

EXPERIMENTAL PROCEDURES

Fresh *Hevea* latex from clone RRIM 501 was added with stirring to a large volume of acetone. The extract was dried, concentrated and separated into different classes of lipids by thin layer chromatography (TLC) on silica gel plates, using n-hexane-diethyl ether-acetic acid (80:20:1, v/v) as the developing solvent system (5). The triglyceride band was scraped off from preparative TLC plates and eluted with chloroform. The methyl esters were prepared by refluxing the triglycerides with a 5% solution of acetyl chloride in methanol for 1-2 hr, or with a 0.5 M solution of sodium methoxide in methanol for 15 min (6). The esters were further purified by TLC on silica gel plates developed in n-hexane-diethyl ether-acetic acid (90:10:1, v/v).

Argentation chromatography of the mixed

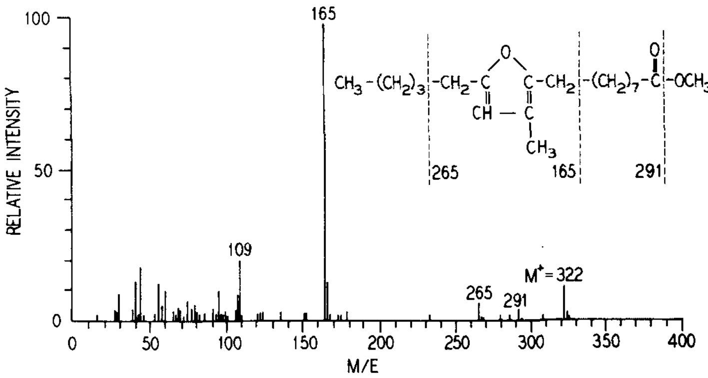

FIG. 1. GC-MS spectrum of the methyl ester of furanoid acid from the triglyceride fraction of *Hevea brasiliensis* latex.

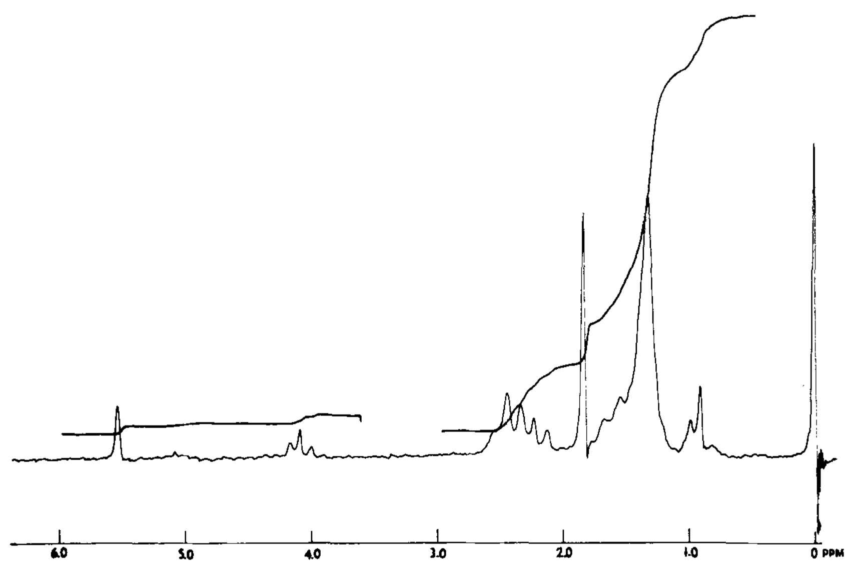

FIG. 2. ^{1}H NMR spectrum of the triglyceride containing three furan fatty acids from *Hevea brasiliensis* latex.

TABLE I

Mass Spectral Data of Methyl 10(13)-hydroxy-11-methyloctadecanoate

Structure	Characteristic Fragments m/e (relative abundance)
$CH_3OC\overset{O}{\overset{\|}{C}}(CH_2)_9\overset{CH_3}{\overset{\|}{C}H}\text{-}CH_2\text{-}\underset{OH}{\underset{\|}{C}H}\text{-}(CH_2)_4CH_3$	310(1.2), 257(17.3), 225(37.7), 213(2.3), 185(15.1), 101(20.8), 74(66.37)
$CH_3OC\overset{O}{\overset{\|}{C}}(CH_2)_8\underset{OH}{\underset{\|}{C}H}=\overset{CH_3}{\overset{\|}{C}H}\text{-}(CH_2)_6CH_3$	201(26.8), 169(100), 74(28.3) 113(0)[a]

[a]Absence of C_8-alkyl fragment indicates branching at C_{11} of the futanoid acid.

esters and of the triglycerides was carried out on TLC plates of Silica Gel G impregnated with 20% silver nitrate, using benzene and chloroform-methanol (99:1, v/v) as the developing solvents, respectively. The separated bands were located under UV light of wave length 354 nm after spraying with an alcoholic solution of 2,7-dichlorofluorescein.

Hydrogenation of the furanoid acid methyl ester was carried out in glacial acetic acid using platinum dioxide as catalyst. The resulting products were fractionated by TLC, and the hydroxy methyl ester band was scraped off and eluted with chloroform.

The methyl esters were analyzed on a Pye Unicam GCD Gas Liquid Chromatograph equipped with a flame ionization detector using 5 ft. glass columns of 10% SE-30 and PEGA. The hydroxy methyl esters were analyzed in a column of 3% SE-30.

The mass spectra of the esters were obtained on a GC/AE 1 MS 3074 instrument attached to a DS-50S mass spectroscopy data system. The nuclear magnetic resonance (^{1}H NMR) spectrum was recorded from a carbon tetrachloride solution on a Hitachi-Perkin Elmer R-20B instrument at 60 MHz using tetramethylsilane as internal reference. The infrared spectrum was obtained from a carbon tetrachloride solution on a Beckmann IR 4250 spectrophotometer and the ultraviolet spectrum from a hexane solution on a varian Tectron spectrophotometer.

RESULTS AND DISCUSSION

Gas chromatographic analysis of the methyl

esters from the triglyceride fraction revealed a major peak constituting about 90% of the total esters. It had a carbon number of 20.93 on a PEGA column and 18.87 on SE-30. (The retention time on the PEGA column at 185 C with a nitrogen flow rate of 48 cm^3 min^{-1} was 55.5 min; stearic acid, 21.5 min). The rest was made up of esters from 18:0, 18:1 and 18:2 acids and traces of 16:0 and 16:1 acids.

The mass spectrum of the major componet (Fig. 1) gave a parent molecular ion (M^+) at m/e = 322, corresponding to a molecular formula of $C_{20}H_{34}O_3$. The characteristic base peak at 165 was similar to that of a furanoid ester (1-3). The presence of a furan ring was also shown by an ultraviolet absorption band at λ = 224 nm. The infrared spectrum showed two C=C stretching bands at 1580 cm^{-1} and 1640 cm^{-1} with weaker ring vibration bands at 800 cm^{-1} and 1025 cm^{-1}. The values reported by Glass etal. (3) for a trisubstituted furan ring compound are 1570, 1630, 785 and 1025 cm^{-1}.

Argentation TLC revealed the presence of the ester close to the saturated ester band. This agrees with the fact that the heteroaromatic character of the ring weakens the interaction of the double bonds with silver ions.

The trisubstituted nature of the furan ring was shown by a singlet proton absorption peak at δ = 5.56 in the ^1H NMR spectrum. A methyl substituent was shown by a singlet peak at δ = 1.84. The NMR spectrum of the triglyceride (Fig. 2) showed absorption peaks in the same positions, thus indicating that the furan ring was present initially and was not generated during the methylation step.

The mass fragmentation pattern with characteristic ions at m/e = 265 (M^+-alkyl), 165 (M^+-alkyl ester) and 109 (furan fragment) corresponds well with the furanoid ester F_2 identified by Glass et al. (3). This suggests that the compound is the methyl ester of 10,13-epoxy-11 (or 12)-methylocatadeca-10,12-dienoic acid.

The position of the ring methyl group was confirmed as follows. Hydrogenation of the methyl ester with PtO_2 in acetic acid yielded the tetrahydro furanoid ester together with the products of the hydrogenolysis of the furan ring, viz. hydroxy methyl esters. The latter, after separation by TLC, appeared as two peaks which were not completely resolved on a SE-30 column. Mass spectrometry revealed that the fragmentation pattern was that expected for methyl 10(13)-hydroxy-11-methyloctadecanoate (Table I). Thus, the methyl group is attached at C-11, and the compound is 10,13-epoxy-11-methyloctadeca-10,12-dienoic acid.

From 100 g of fresh latex, 0.7 g of neutral lipids was obtained. The triglycerides constituted ca. 40% of the neutral lipids. Thus, the furanoid fatty acid was present at a concentration of about 0.25% in latex. This is a relatively large amount for a single component in the non-rubber fraction of *Hevea* latex. The latter is now only the second known plant source of the furanoid acids, and the present finding adds considerable interest to the biosynthetic origin of these acids.

ACKNOWLEDGMENTS

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Separation of Sulfated Bile Acids by High-Performance Liquid Chromatography

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ABSTRACT

In order to establish a new method for simultaneous determination of sulfated bile acids without deconjugation, separation of 3-sulfates of unconjugated, glyco- and tauroconjugated bile acids by high-performance liquid chromatography has been undertaken. The preliminary experiment indicated that reversed phase chromatography on an ODS SC-02 column using ammonium carbonate aqueous solution/acetonitrile as a mobile phase would be promising. The ratio of peak width to retention time was varied depending upon the salt concentration. The content of water in mobile phase exerted an influence on the capacity ratio to a certain extent, but not on the resolution. The use of 0.5% ammonium carbonate/acetonitrile (26:8 and 20:8, v/v) was found to be suitable for complete resolution of sulfated cholate, chenodeoxycholate, deoxycholate, lithocholate and their glyco- and tauro-conjugates.

INTRODUCTION

The commonly used methods for determination of bile acids in biological material involve the hydrolytic cleavage of conjugates under drastic conditions and subsequent chromatographic separation of the hydrolyzate. Such procedures have inevitable disadvantages, such as the lack of reliability and the loss of information about the conjugated forms.

Since the first report on the occurrence of sulfated bile acids in man (1,2), considerable attention has been drawn to the metabolic significance of conjugated bile acids in liver diseases (3). The resolution of 3-sulfates of major bile acids by thin layer chromatography has previously been attempted (4,5), but satisfactory results have not yet been attained. In this paper, we wish to report the separation of 3-sulfated bile acids, their glyco- and tauro-conjugates by high-performance liquid chromatography.

RESULTS AND DISCUSSION

The effect of salt concentration in the eluent on the chromatographic behavior of sulfated bile acids was studied using a Nihon Bunko Model Tri Rotar high-performance liquid chromatograph (Nihon Bunko Co., Tokyo) equipped with a Model UV-100 II ultraviolet detector monitoring absorbance at 210 nm. In the preliminary experiment, reversed phase chromatography on an ODS SC-02 column (Nihon Bunko Co., Tokyo) (25 cm x 4.6 mm i.d.) with ammonium carbonate aqueous solution/acetonitrile as a mobile phase appeared to be promising for efficient separation. The sulfated cholate, chenodeoxycholate and deoxycholate were progressively separated with increasing concentration of ammonium carbonate up to 0.3% as listed in Table I. In addition, the

TABLE I

Effect of Salt Concentration in Mobile Phase on the Relative Capacity Ratio of 3-Sulfated Bile Acids

Salt concentration[a] (%)	Flow rate (ml/min)	Relative k' value[b]		
		C	CDC	DC
0.05	0.8	1.00 (0.49)	1.00 (1.28)	1.00 (1.52)
0.10	1.2	1.96	1.95	2.01
0.30	2.4	4.18	4.37	4.68
0.50	2.5	4.51	4.63	4.98
0.70	2.6	5.00	5.08	5.43
0.90	3.0	5.45	5.67	6.11

[a]Ammonium carbonate aqueous solution/acetonitrile (26:8, v/v) was used as a mobile phase.

[b]Figures in parentheses express the capacity ratio of each bile acid. C: cholate, CDC: chenodeoxycholate, DC: deoxycholate.

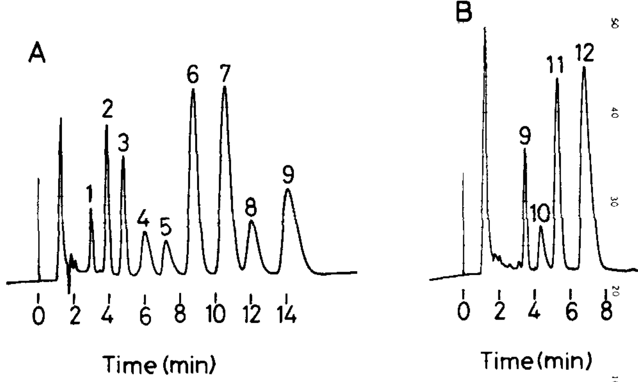

FIG. 1. Separation of a mixture of 3-sulfated bile acids. 1: cholate, 2: glycocholate, 3: taurocholate, 4: chenodeoxycholate, 5: deoxycholate, 6: glycochenodeoxycholate, 7: glycodeoxycholate, 8: taurochenodeoxycholate, 9: taurodeoxycholate, 10: lithocholate, 11: glycolithocholate, 12: taurolithocholate. Conditions: ODS SC-02 column(25 cmx 4.6 mm i.d.), mobile phase (A) 0.5% ammonium charbonate/acetonitrile (26:8, v/v), (B) 0.5% ammonium carbonate/acetonitrile (20:8, v/v), flow rate 2.1 ml/min.

ratio of peak width (w) to retention time (t_R) was variable depending upon the salt concentration. We observed a marked difference in the correlation of w/t_R value to salt concentration between the two closely related bile acids, sulfated chenodeoxycholate and deoxycholate. Chenodeoxycholate exhibited a constant value in the range of 0.1 to 0.7% of salt concentration, whereas deoxycholate showed an optimum at 0.5% concentration. The content of water in the mobile phase exerted an influence on the capacity ratio to a certain extent but not on the resolution.

On the basis of these data, 0.5% ammonium carbonate/acetonitrile (26:8, v/v) was chosen as a suitable mobile phase by which a synthetic mixture of 3-sulfates of unconjugated, glyco- and tauroconjugated bile acids was completely resolved. Among these bile acids, a sulfated lithocholate group was separated much more efficiently when 0.5% ammonium carbonate/acetonitrile (20:8, v/v) was employed. The detection limit of glycochenodeoxycholate 3-sulfate was 50 ng. The typical chromatograms of 3-sulfated cholate, chenodeoxycholate, deoxycholate and lithocholate are illustrated in Figure 1. It is of particular interest that the elution order of 3-sulfated bile acids, their glyco- and tauroconjugates is dependent upon the number of hydroxyl groups on the steroid nucleus (k′: cholate < chenodeoxycholate < deoxycholate < lithocholate) as well as on the structure of the side chain at C-17 (k : -COOH < $-CONHCH_2COOH$ < $-CONHCH_2CH_2SO_3H$).

The availability of a novel method for separation of sulfated bile acids without prior solvolysis and/or hydrolysis which involves high-performance liquid chromatography and preceding separation of the sulfated and unsulfated fractions (6) may provide much more precise information on the metabolic profile of bile acids in patients with various hepatobiliary diseases. The potential application of the present method to clinical specimens will be the subject of a future communication.

ACKNOWLEDGMENTS

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High Density Lipoproteins, 1978 — An Overview

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ABSTRACT

High density lipoproteins (HDL) have come of age. For years it has been fashionable to study HDL as an approach to understanding lipoprotein structure and lipid binding. Available in abundant amounts from normal human plasma, readily separable into its individual lipid and soluble apolipoprotein components, HDL has provided much information for lipoprotein model building. Suddenly it has been thrust center stage clinically by a host of convincing epidemiologic studies that clearly establishes an inverse relationship between HDL levels and coronary vascular events. Biochemists, clinicians, cardiologists and epidemiologists are simultaneously focusing attention on HDL. Familial High Density Lipoprotein Deficiency (Tangier Disease) has been well described but is poorly understood as a clinical syndrome complex. We have suddenly become aware of how little we understand about HDL's normal ultracentrifugal and apoprotein heterogeneity, about its functional role(s) or the determinant(s) of its concentration in plasma. The relative contributions of the two sites of HDL origin, the liver and intestine, are yet to be determined as are the site(s) of degradation. Awareness of a problem and its importance is the first step toward the solution(s) of the problem.

HDL 1978 — AN OVERVIEW

Interest in high density lipoproteins (HDL) has greatly intensified in recent years, stimulated largely by the finding that HDL is inversely related to coronary artery disease. Clinical and epidemiologic observations of a striking, consistent and independent negative association between HDL levels and coronary vascular events have, in turn, generated new interest in the structure, composition and metabolism of this fascinating lipoprotein. A tremendous amount of new data has been amassed over the past decade from studies based in a multiplicity of different disciplines. The breadth of topics on the program of this symposium offers a comprehensive view of the facts and insights collected thus far. It should also leave us with an awareness of how much more remains to be learned.

As isolated in the density range 1.063-1.21 g/ml by ultracentrifugation, HDL is composed by weight of ca. 50% protein, 30% phospholipid, 20% cholesterol and 5% triglyceride. The lecithin to sphingomyelin ratio is 5:1, and the ratio of esterified to free cholesterol is ca. 3:1. HDL is heterogeneous in particle size and content. It is customarily divided into two density classes: HDL_2 (d = 1.063-1.125 g/ml) and HDL_3 (d = 1.125-1.210 g/ml). HDL_2 consists of 60% lipid and 40% protein, while 55% of HDL_3 is attributed to protein. The lecithin/sphingomyelin ratio and the ratio of esterified to free cholesterol are higher in HDL_3 than in HDL_2. Recent studies have reported the presence of at least three different HDL subfractions (isolated by density gradient ultracentrifugation) within the HDL density range.

The existence of heterogeneous lipoprotein particles within each HDL density class has been documented through the use of physical and immunological procedures. Further work is needed to define whether these are real findings or artifacts resulting from isolation techniques such as ultracentrifugation.

Alternative methods for HDL quantitation have been evaluated and demonstrate clearly that the HDL concentration will vary with the component one chooses to measure (e.g., cholesterol or protein), and the isolation technique employed. Apoprotein apoA-I is lost from HDL with each centrifugation. HDL_c (a HDL fraction increased with cholesterol feeding and high in the arginine-rich apoprotein) is precipitated with VLDL and chylomicrons when one uses polyanion or polycations and heavy metals to separate lipoproteins. Standardization of methodologies will be an important priority for future studies.

HDL is available in plentiful amounts from normal human plasma and is readily separable into its lipid and apoprotein components. Because of the complexity of its structure, it has provided a wealth of information for lipoprotein model building. Various HDL models have been proposed on the basis of evidence from chemical, enzymatic and electron microscopic studies, as well as nuclear magnetic resonance spectroscopy. HDL particles appear to be spherical and range in size from ca. 70 A to 120 A in diameter, consisting of an apolar or hydrophobic lipid core with a solubilizing, more polar surface coat of phospholipids and globular A apoproteins, whose apolar regions are in turn usually embedded in the lipid core.

The protein moiety of HDL is hetero-

geneous. The two A apoproteins, apoA-I and apoA-II, constitute about 90% of HDL protein. The ratio of apoA-I to apoA-II is about 3:1. ApoA-I is almost completely absent in patients with Tangier disease, while the amount of apoA-II is reduced to 6% of normal.

Apoprotein C, the main apoprotein of VLDL, constitutes about 5% of HDL protein mass. At least three different peptides belong to the C family of apoproteins: C-I, C-II, and C-III. Apoprotein D, also known as the "thin line peptide," and apoprotein E, or the arginine-rich apoprotein, are other minor constitutents of HDL. The unique features of the HDL protein components and their role in lipid metabolism are the subject of prolific investigation.

The major HDL apoproteins, apoA-I and apoA-II, can be readily isolated from human HDL and are easily separated by various chromatographic techniques. A number of reports at this symposium deal with these methods. Radioimmunoassays have been used to measure the density distribution of apoA-I and apoA-II in the plasma of normal and hyperlipoproteinemic subjects. We are uncertain today about the relative contribution of the liver and intestine to HDL synthesis. It remains to be demonstrated whether the majority of HDL arises as a "nascent" lipid-poor form from the liver or a delipidated chylomicron remnant from the intestine. Similarly, the relative contribution of these organs to plasma A-I and A-II apoprotein levels is unclear. Also unclear is the site(s) of HDL degradation, though liver and kidney lysosomes have been incriminated.

Little is also known about HDL function(s). It has been postulated that HDL may be an important factor in cholesterol efflux from the tissues, thereby reducing the amount of cholesterol deposited there. Alternatively, it has been suggested that HDL may pick up cholesteryl ester and phospholipid during normal VLDL lipolysis in the plasma. Present information does not allow the acceptance or rejection of either theory.

It has also been suggested that HDL may play a role in triglyceride metabolism. The extent to which HDL is involved in triglyceride metabolism beyond its role as a carrier of apoC-II is unknown. HDL levels are markedly decreased in subjects with exogenous hypertriglyceridemia, and HDL apoprotein catabolism is enhanced by the increased triglyceride flux in patients with nephrotic syndrome as well as in normals on high carbohydrate diets (80% of calories). Why this occurs is uncertain.

HDL levels are clearly lower in humans than in animal species relatively resistant to atherosclerosis, like the dog, sheep and rat. In contrast to LDL, which varies widely with dietary excesses (especially saturated fat and cholesterol), HDL levels are relatively insensitive to diet, increasing somewhat with weight reduction in hypertriglyceridemic subjects or with modest increases in dietary alcohol, and decreasing with diets extremely high in carbohydrate. HDL levels are not affected by any of the current hypolipidemic drugs other than nicotinic acid which raises the level primarily of HDL_2 (and HDL cholesterol). Cholestryramine, colestipol, and D-thyroxine all manifest their effects on VLDL and LDL, as does the surgical technique of ileal bypass, but none of these affects HDL concentration.

The revival of interest in HDL has been fueled by repeated epidemiologic evidence from studies in Hawaii, Framingham, Norway and others, documenting an inverse and independent correlation between HDL cholesterol and coronary heart disease (CHD). Mounting findings support the tempting theory that increased levels of HDL may exert a protective effect against the development of vascular disease. The cause-and-effect of HDL's inverse relation to CHD remains unclear at present. Equally unclear are the reasons behind the inverse relationship of HDL with other risk factors such as male sex, cigarette smoking, obesity, and a sedentary life. These questions promise to be the focus of much investigation. In the meantime, cogent evidence continues to accumulate from studies such as Framingham, the Lipid Research Clinics Program, the Bogalusa Heart Study, and the Multiple Risk Factor Intervention Trial, reported at this symposium.

Before we can understand HDL's role in arteriosclerosis, we must clarify some basic questions:

How can we isolate native HDL and keep it intact?

How can we quantify a lipoprotein family (HDL) that is obviously heterogeneous?

How can we quantify HDL when its lipid and apoprotin components vary with time and with the methods of isolation?

What is the primary site of origin of HDL?

Where is it primarily degraded?

What role or roles does HDL play in normal and abnormal lipid transport – specifically in triglyceride clearances and cholesterol transport?

What are the determinants of plasma levels of HDL?

Why is there an inverse relationship of HDL with arteriosclerosis?

Is the *protective* vascular factor correlated best with HDL cholesterol or HDL pro-

tein? If it is with protein, with which protein and why?

Is HDL's effect on the vascular system a primary one or is it secondary to other factors that affect both HDL and the vessel wall?

The scope and diversity of the papers on the program for this symposium attest both to how much we know and how much more we need to learn. It is a wonder that, after years of study of lipid transport and ready access to abundant amounts of HDL and its apoproteins, there are still so many unanswered questions.

All of us at this symposium have every reason for optimism, however, since the first step in answering a question or problem is the recognition of the question (in this case questions) and the great potential importance of their answers to human health.

[Received July 15, 1978]

The Evidence for the Antiatherogenicity of High Density Lipoprotein in Man

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ABSTRACT

It has long been recognized that patients with clinical coronary heart disease (CHD) have, on average, higher concentrations of plasma very low density and low density lipoproteins than do healthy subjects. The same studies clearly demonstrated that coronary victims tend also to have low plasma concentrations of high density lipoprotein (HDL). It is only recently, however, that the possible significance of this second observation has been examined. Direct evidence for an inverse relationship between HDL cholesterol concentration and the prevalence of clinical CHD, independent of other plasma lipoproteins, has been provided by the Honolulu Heart and Cooperative Lipoprotein Phenotyping Studies. The Tromsø Heart and Framingham Studies subsequently demonstrated that this relationship precedes the clinical manifestation of coronary disease. More recently, angiographic studies have confirmed that the severity of existing coronary atherosclerosis is inversely related to HDL cholesterol concentration. Other investigations have shown that coronary victims also have low mean concentrations of apolipoproteins AI and AII (the major protein components of HDL), although the reduction of apoAI concentration may be less marked than that of HDL cholesterol, and preliminary findings from Tromsø have suggested that apolipoprotein AI may be less powerful than HDL cholesterol as a predictor of CHD. Such observations have supported the proposal that HDL may exert a protective effect against coronary atherosclerosis. Final confirmation (or otherwise) of this hypothesis, however, must await the results of carefully controlled animal experiments and of regression studies in patients with angiographically defined atherosclerosis.

One of the principal features of atherosclerosis is the focal deposition of cholesteryl ester in the inner layers of the artery wall. For this reason, much research into the pathogenesis of atherosclerosis has concerned the metabolism and transport of cholesterol. Particular attention in this regard has been directed towards the plasma total cholesterol concentration. This followed the proposal by Virchow in 1856 (1) on the basis of animal experiments that atherogenesis is largely determined by the rate of influx of cholesterol from plasma into the artery wall. This concept of atherogenesis was subsequently fostered by the frequent finding of hypercholesterolaemia in patients with clinical coronary heart disease (CHD) and by later demonstrations that coronary risk increases with increasing plasma total cholesterol concentration (2-4).

The application of lipoprotein fractionation techniques to clinical investigation in the early 1950s demonstrated at once that the hypercholesterolaemia commonly present in coronary victims reflected an increase in the plasma concentrations of very low density (VLDL) and/or low density (LDL) lipoproteins (5-7). The same studies clearly demonstrated that such patients also tended to have low HDL cholesterol concentrations. In later studies with the analytical ultracentrifuge, Gofman and coworkers noted that the reduction of HDL concentration in coronary prone subjects was particularly marked in the relatively cholesterol-rich HDL_2 subfraction (8). However, for reasons which have been discussed elsewhere (9), these findings failed to arouse interest, and the attention of epidemiologists, clinicians and experimental pathologists continued to be focused on the roles of VLDL and LDL in atherogenesis.

The possible significance of HDL in relation to CHD was not seriously examined until 1975, when Miller and Miller (10,11) proposed that the rate of progression of atherosclerosis might be an inverse function of the plasma HDL cholesterol concentration by virtue of a role of the lipoprotein in facilitating the egress of cholesterol out of the artery wall. In essence, this hypothesis represented a synthesis of earlier in vitro data, most notably those of Bailey (12) and of Glomset (13), which had demonstrated the ability of HDL to accept cellular cholesterol, and the results of epidemiological and clinical studies which had suggested: firstly, that many coronary risk factors are associated with a low HDL cholesterol concentration; secondly, that the reduction of HDL cholesterol in coronary victims is independent of other lipoprotein concentrations, age and body weight (based on a reanalysis of earlier data of Nikkila (5); thirdly, that population groups with high average HDL cholesterol concentrations tend also to have a low incidence of CHD; and fourthly, that body cholesterol pool size increases with decreasing HDL cholesterol concentration (14), while being apparently less

TABLE I

Situations in Which a Low Mean Plasma HDL Cholesterol Concentration is Associated with a High Incidence of Coronary Heart Disease

Coronary risk factors	Disease states	Ethnic group
Hypertriglyceridaemia (17-26)	Diabetes mellitus (22,38-40)	New Zealand Maoris (46)
Obesity (18,20,22,27)	Uraemia (41,42)	
Physical inactivity (25,28-32)	Nephrotic syndrome (43,44)	
Cigarette smoking (32-34)	Chronic cholestasis (44,45)	
Family history of CHD (35,36)		
Male sex (22-24,37)		

TABLE II

Situations in Which a High Mean Plasma HDL Cholesterol Concentration is Associated with a Low Incidence of Coronary Heart Disease

	Ethnic groups
Female sex (22-24,37)	Greenland Eskimos (53)
Athletes (29,30,32)	Black Americans (21,54)
Alcohol consumption (47-49)	Jamaican hill-farmers (25)
Familial hyperalphalipoproteinaemia (50,51)	Turks and Caicos Islanders (49)
Octogenarians (52)	

sensitive to changes in LDL concentration (15,16).

During the three years since the publication of this hypothesis, a variety of situations has been identified in which a low mean HDL cholesterol concentration is associated with a high incidence of clinical CHD, and vice versa. These are included in Tables I and II. Such observations, although consistent with the HDL hypothesis, and suggesting factors which might have an important influence on HDL metabolism, do not in themselves provide certain evidence of a direct relationship between clinical CHD and HDL cholesterol concentration. Such evidence can be provided only by studies in which other relevant covariates are either controlled or are allowed for by the use of appropriate multivariate statistical techniques.

The relationship of CHD prevalence to the plasma HDL cholesterol concentration, independent of other lipoproteins, has been the subject of two recent reports. In the Cooperative Lipoprotein Phenotyping Study (24), data were analyzed from 6859 men and women, aged 40 years or more, living in five centers of the United States: Albany, Evans County, Framingham, Honolulu and San Francisco. In the combined data, CHD prevalence increased from 8 to 18 per 100 as HDL cholesterol decreased from ≥45 to ≤25 mg/dl. This association was independent of the plasma triglyceride and LDL cholesterol concentrations. Similar trends were observed in each center, in both sexes, in all age groups, in different ethnic groups, and for both myocardial infarction and angina pectoris, considered separately. In the Honolulu Heart Study (20), 2019 men of Japanese descent aged 50-72 years, including 264 cases of CHD, were examined. CHD prevalence again showed more than a two-fold rise between the highest (≥53 mg/dl) and lowest (≤36 mg/dl) quartiles of HDL cholesterol, which again was independent of the plasma triglyceride and LDL cholesterol concentrations.

These two studies established that patients with clinically manifest CHD tend to have a low HDL cholesterol independent of other lipoproteins, but did not, of course, exclude the possibility that this might be secondary to the disease process itself or to consequent changes in diet or living habits. Although an earlier prospective study of 10,000 Israeli men (55) had already provided evidence for a negative correlation between HDL cholesterol and the five-year incidence of first myocardial infarction, the data from this survey had unfortunately been submitted to single variate analysis only. Accordingly, the critical question of the independent contribution of HDL cholesterol concentration to coronary risk had not been addressed, and no significance had been attached to the correlation. Thus, it was not until 1976 that the predictive power of HDL cholesterol concentration for clinical CHD, independent of other lipoproteins and risk factors, was examined as part of the Tromsø Heart Study in Norway (56) and of the Framingham Study in the United States (22,38).

In the Tromsø Heart Study, Miller et al. (56)

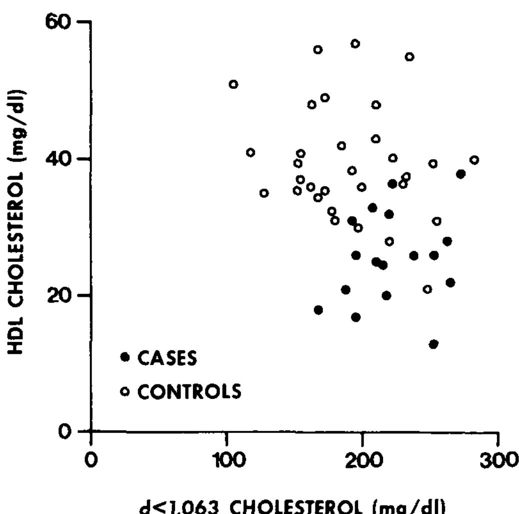

FIG. 1. Plasma concentrations of HDL cholesterol and of cholesterol in lipoproteins of lower density (d<1.063 g/ml) in participants of the Trømso Heart Study who subsequently developed a coronary event during two years of follow-up and in controls matched for sex (all males), ethnic origin, age, area of residence and level of habitual physical activity. There were no statistically significant differences between the two groups in plasma triglyceride (nonfasting), relative body weight, cigarette consumption and blood pressure. Data from Miller et al. (56).

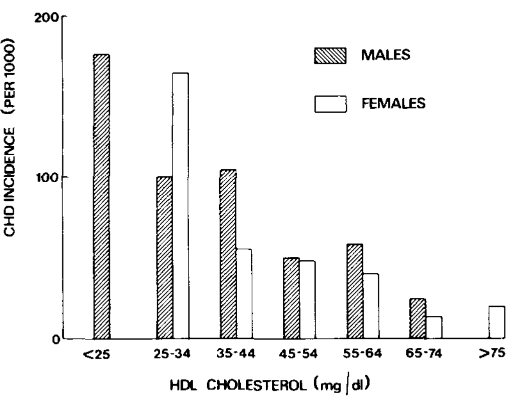

FIG. 2. Incidence of coronary heart disease by plasma HDL cholesterol during 2-8 yr (mean 4 yr) of follow-up in the Framingham Study. Data from Gordon et al. (22).

followed 6595 men aged 20-49 years (representing 83% of the target population) for two years, during which time 21 of the participants developed a new coronary event (myocardial infarction or sudden death). For each of these cases, two control subjects were randomly selected after matching for age, ethnic origin, area of residence and level of habitual physical activity. The mean HDL cholesteerol concentration in the cases (26 mg/dl) was found to be 33% lower (P<0.005) than that in the controls, while the converse was true for the cholesterol concentration in lipoproteins of lower density (d<1.063 g/ml; i.e., VLDL plus LDL) (Fig. 1). Discriminant function analysis showed that the relationships of coronary risk to HDL cholesterol and d<1.063 cholesterol were independent of each other and of plasma triglyceride, blood pressure, cigarette consumption and relative body weight. Furthermore, the contribution of HDL cholesterol to the discrimination between cases and controls was found to be substantially greater than that of density <1.063 g/ml cholesterol.

Essentially identical results were reported by the Framingham group (22) after following for 2-8 yr (mean, 4 yr) 1025 men and 1445 women aged 49-82 yr, all of whom had initially been free of clinical CHD. CHD incidence per 1000 subjects increased from 25 to 105 in men and from 14 to 164 in women as HDL cholesterol decreased from 65-74 to 25-34 mg/dl (Fig. 2). In both sexes, this association was independent of the other measured variables (plasma triglyceride and LDL cholesterol concentrations, blood pressure, glucose tolerance, cigarette consumption, and relative body weight), and applied to each 10-yr age group and to all categories of CHD examined (myocardial infarction, angina pectoris, CHD death). On the basis of the likelihood ratio statistic, the predictive power of HDL cholesterol concentration was again substantially greater than that of LDL cholesterol which had an independent positive effect on coronary risk.

Evidence that the correlation between HDL cholesterol and coronary risk reflects an underlying relationship to the severity of coronary atherosclerosis, as quantified by angiography, has been provided by four recent investigations. Pearson et al. (57) studied angiograms from 189 men and 65 women, most of whom were being investigated for chest pain, without knowledge of the patients' lipoprotein patterns. A coronary artery was classified as diseased when there was 50% or more narrowing of its lumen. On this basis, threee major conclusions were drawn: (a.) that patients with coronary disease had a lower mean HDL cholesterol concentration than did those without; (b.) that within the deseased group, patients with left main artery disease had the lowest HDL cholesterol levels; and (c.) that amongst the remaining subjects, the number of vessels diseased was a negative function of HDL cholesterol concentration. These associations were present in both sexes and in both age groups examined (30-49, 50-70

yrs), whereas the direct relationships of coronary disease to the plasma triglyceride and LDL cholesterol concentrations were less consistent, suggesting that the correlation with HDL cholesterol was direct. This conclusion has since been supported by a multivariate analysis of angiographic and lipoprotein data from a group of similarly selected Australian subjects (58) in which HDL cholesterol emerged as a powerful independent predictor of coronary atherosclerosis. Barboriak et al. (59) also observed a negative correlation (univariate analysis) between HDL cholesterol and the extent of angiographically determined coronary stenosis, expressed as a score ranging from 0 (no stenosis) to 300 (all 3 main arteries occluded), in 228 men and 70 women. Tan et al. (60) concluded that the reduction of HDL cholesterol in subjects with coronary narrowing was most marked in young males.

In all of the investigations so far described in which multivariate statistical procedures have been employed (20,22,24,56,58), any positive relationship of CHD or of coronary atherosclerosis to plasma triglyceride concentration on single variate analysis was found to be dependent upon the well recognized negative correlation between plasma triglyceride and HDL cholesterol, contrasting with the positive relationship of CHD to LDL cholesterol concentration which was uniformly independent of HDL. Disturbed plasma triglyceride transport may thus be the primary factor responsible for the reduction of HDL cholesterol concentration in many coronary prone subjects.

Other studies have indicated that the reduction of HDL cholesterol concentration in coronary prone subjects is accompanied by low concentrations of HDL phospholipid (6) and of the major peptides of HDL, apolipoproteins AI and AII (23,61-64). In one study the reduction of apolipoprotein AI concentration was found to be proportionately less than that of HDL cholesterol (23). This finding was consistent with the early observations of Gofman et al. (8), using the analytical ultracentrifuge, that it is the mass of the relatively cholesterol-rich HDL_2 subfraction which shows the greatest reduction in CHD. Preliminary prospective data from the Tromsø Heart Study have confirmed the presence of low apolipoprotein AI concentrations in subjects who developed a coronary event during the first 2 yr of follow-up, but have suggested also that apolipoprotein AI concentration is a weaker predictor of CHD than is HDL cholesterol (65). Such observations concerning HDL composition and the predictive power of different HDL components have intriguing pathophysiological implications. However, consideration of these is beyond the scope of this article.

Considered together, the foregoing epidemiological findings must be regarded as providing strong support for the postulated antiatherogenic effect of HDL. This is particularly so when considered against the background of metabolic data, reviewed elsewhere (49,66), which have indicated the plausibility of such an effect. Of particular significance in this regard is the very recent in vivo evidence that HDL is indeed the prinicipal acceptor of tissue cholesterol, at least during weight reduction, in man (67), and that HDL cholesterol is used preferentially by the human liver for bile acid synthesis (68) and biliary cholesterol secretion (69). Nevertheless, possible explanations for the epidemiological associations between HDL and CHD other than a direct antiatherogenic effect of the lipoprotein clearly exist, and final acceptance or rejection of the hypothesis must await the results of carefully designed animal experiments, preferably in primates, and of regression studies in patients with angiographically defined atheroscerosis. During such studies, particular attention should be directed towards the HDL_2 subclass, which appears to show the strongest epidemioligical associations.

Finally, no discussion of a possible antiatherogenic effect of HDL can exlude a consideration of Tangier Disease, an inborn error of metabolism characterised by extremely low plasma HDL concentrations and the accumulations of cholesteryl esters in the reticuloendothelial system. Controversy exists as to whether or not susceptibility to CHD is increased in Tangier Disease (70,71), but it does seem clear that atherogenesis is not increased to the extent that would be anticipated on the basis of the epidemiological relationships between HDL cholesterol and CHD. The explanation for this may lie in the very low plasma LDL cholesterol concentrations that have been well documented (but perhaps less well recognized) in this disorder (70). It is possible that the importance of HDL as a determinant of atherogenesis may be considerably less under these circumstances, assuming significance only above a certain threshold of LDL concentration. Such a concept would similarly explain the apparent lack of premature of CHD in familial type I hyperlipoproteinaemia, in which a low HDL cholesterol is agin accompanied by a very low LDL concentration (70).

There are two lines of evidence that plasma LDL concentrations may be abnormally high in the great majority of individuals in western societies. Firstly, essentially all nonwestern communities so far studied have been found to

have very much lower mean plasma LDL concentrations than European and North American populations (e.g., 25,53,72). Secondly, on the basis of their tissue culture studies, Goldstein and Brown (73) have pointed out that the LDL concentrations in the great majority of western subjects are probably sufficient to completely saturate the LDL receptors of peripheral cells, suggesting that these concentrations are inappropriately high. Under these circumstances, cholesterol metabolism in peripheral tissues, including vascular smooth muscle, would be expected to be influenced more by the efficiency of reverse (or centripetal) cholesterol transport than by changes in LDL concentration, since in vitro further increases in LDL concentration above that required to saturate the LDL receptors have relatively little effect on cellular cholesterol content (74). Such a concept might explain why the negative correlations of body cholesterol pool size and of CHD incidence with HDL cholesterol concentration have been stronger than their positive correlations with LDL concentration in clinical and epidemiological studies.

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Ultrastructure of Serum High Density Lipoproteins: Facts and Models

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ABSTRACT

The complexity of the structure of plasma high density lipoproteins (HDL) has invited numerous approaches which have been directed at the study of the intact particles, their apolipoproteins and reassembled complexes. Parameters such as flotation and sedimentation coefficients, size and molecular weight have been determined and in addition, through scattering techniques, an understanding has been obtained on the long range organization between core (cholesteryl esters and triglycerides) and surface components (unesterified cholesterol, phospholipids and apoproteins). In the case of the apolipoproteins, the knowledge of their primary structure has facilitated the study of their physicochemical properties in solution and at the air-water interface and has also permitted realistic predictions of the two dimensional organization, not only of their α-helical segments but also of the β-pleated sheets, random coil and β-turns, all of which have amphipathic properties. When all of the information from the physical and chemical studies is put together, the various HDL can be described as spherical structures having a liquid core of radius, r - 20.2 Å, surrounded by a monolayer of cholesterol and phospholipids with closely packed hydrophobic ends on the surface of the core. The organization of the apoproteins at the lipoprotein interface is comparatively less understood. However, reasonable predictions can be made on secondary structure considerations and on their behavior at the air-water interface. The emerging overall structural information can be translated into a space-filling model that not only provides a useful visual representation of HDL, but, more importantly, a basis for planning future studies on the elucidation of the structure of these particles on a molecular level.

INTRODUCTION

The strucural organization of plasma high density lipoproteins (HDL) has been the subject of intensive studies for a number of years (1-3). Only recently a precise knowledge of the chemical properties of their major apolipoproteins has been achieved. This knowledge, coupled with the information already available on the various lipid constituents and on the physicochemistry of intact and reconstituted particles, has opened the way for the formulation of structural models which, although not faithfully reproducing the molecular details of HDL structure, are potentially capable of generating novel ideas for future experimental approaches. In this brief review, I will discuss the pertinent physico-chemical findings regarding HDL and then examine the various models proposed thus far, including recent work carried out in this laboratory that has resulted in the construction

[1]Operated by The University of Chicago for the United States Department of Energy under contract EY-76-C-02-0069.

TABLE I

Properties of Human HDL_3 Males[a]

$s^{\circ}_{20,w}$(S)	4.65
$D^{\circ}_{20,w}$(cm^2 sec^{-1})	3.93 x 10^{-7}
MW x 10^{-3}	1.77 ± 0.09
Density (g/cm^3)	1.153
$\bar{v}^2$ (ml/g)	0.867
Diameter $(nm)_{ultrac.}$	7.9 ± 0.3
Diameter $(nm)_{electron\ microscopy}$	8.9 ± 0.8
$F^{\circ}_{1.20}$	1.56 ± 0.11
f/f_o	1.126
Percentage of protein	55.4
Phospholipids	23.1
Unesterified cholesterol	2.8
Cholesteryl esters	17.7
Triglycerides	2.0

[a]These data are compiled from references 4 and 5.

TABLE II

Human HDL_3 Number of Lipid Molecules/Mole

Polar lipids	Phosphatidylcholine	38
	Sphingomyelin	5
	Lysophosphatides	4
	Phosphatidylethanolamine	1
	Phosphatidylinositol	1
Nonpolar lipids	Unesterified cholesterol	13
	Cholesteryl esters	32
	Triglycerides	8

TABLE III

Molecular Parameters of HDL_3

Molecular volumes	Cholesteryl esters	1090Å^3
	Triglycerides	1600Å^3
	Phospholipids	1253Å^3
Surface area (outer)	Phospholipids	62.7Å^2
	Amino acids	15.6Å^2
Surface area (inner)	Phospholipids	68.5Å^2
	Cholesterol, unesterified	39.1Å^2

of a complete space-filling model of one of the HDL subclasses. In particular I will focus the discussion on HDL_3, a lipoprotein subclass which can be purified to a reasonable degree of homogenity. Current ideas on the origin and functions of HDL will be examined elsewhere in this Symposium and therefore will not be dealt with in this article.

FACTS

The most pertinent physico-chemical properties of HDL_3 are given in Table I, which comprises old published data from our laboratory (4) and those on HDL fraction III which was recently isolated by Anderson et al. (5) by means of density gradient ultracentrifugation. The molecular weight value of 1.75 x 10^{-5} obtained earlier by sedimentation equilibrium analysis was confirmed by small-angle x-ray scattering and electron-microscopic studies (1-3). Recently, a detailed comparative analysis of human HDL_3 was carried out on both negatively stained and freeze-etched preparations, with attention paid to defocusing and phase contrast effets in conventional transmission electron microscopy, field emission electron microscopy, and scanning transmission electron microscopy (6). All of these methods have shown evidence that HDL_3 has a well defined surface substructure characterized by recurring domains about 28Å in diameter. Moreover, the freeze-etched specimens recealed an inner core 40±2Å in diameter, consistent with the core measurements of the HDL_3 model proposed by Shen et al. (7; also see below).

An important insight into the long range organization of the major classes of serum lipoproteins has been derived from small-angle x-ray scattering studies. For HDL_3, the diffraction patterns were found to be compatible with a sphere having an outer polar shell of 11.2Å and a core 74Å in diameter (1-3). More recent studies by Tardieu et al. (8) appear to support the existence of surface convolutions which penetrate to about 25Å from the center of the particle. This interesting proposal opens challenging perspectives for future experimental studies.

Additional information on HDL structure has been obtained by nuclear magnetic resonance (NMR) techniques. All of the NMR studies have shown that the phospholipid head group has unrestricted motion, indicating that it has minimal interaction, if any, with the neighboring molecules (2,3). The results of ^{31}P-NMR studies have shown, in addition, that the phospholipids are located on the surface; this finding was further documented by the kinetic analysis of the action of snake venom phospholipase A_2 on HDL_3 (9).

The surface location of the apolipoproteins is supported by small-angle x-ray scattering studies and by work on chemical modification with succinic anhydride (1-3). The results of cross-linking experiments also appear to support this view (10). It is clear, however, that none of these studies can distinguish between protein locations at the surface and near the surace. In fact, if the interpretation of the most recent small-angle x-ray scattering proves to be correct, the presence of convolutions calls for

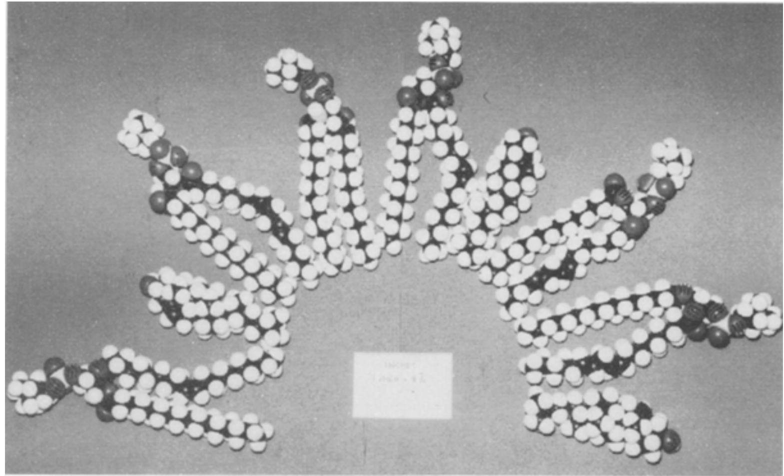

FIG. 1. Space-filling atomic models of phospholipids and unesterified cholesterol arranged in a radial distribution and surrounding a portion of a spherical core (not shown).

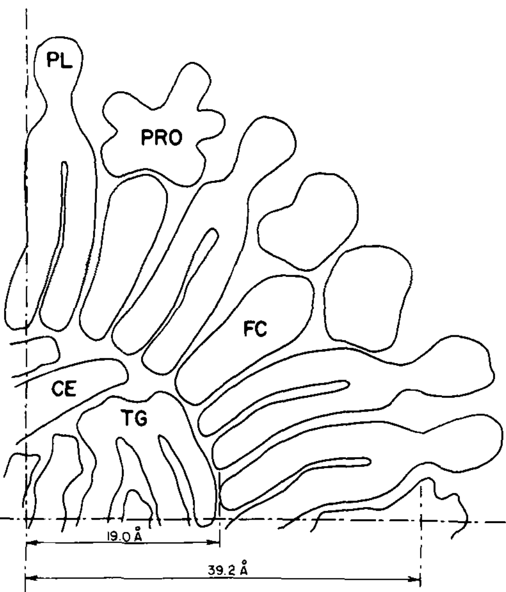

FIG. 2. Model of HDL_3 according to Shen et al. (7). The dimensions of all components are derived from space-filling atomic models. PL = phospholipids; FC = free cholesterol; PRO = protein; CE = cholesteryl esters; TG = triglycerides.

qualifying statements when the lipoprotein surface is referred to.

All of the above findings, together with recent calorimetric data (11), seem to support the conclusion that HDL_3 is a spherical particle having an apolar core and a polar shell, a concept which forms the basis for the models of HDL structure proposed thus far. In view of some uncertainties in the experimental premises, it is not surprising that the various proposed models, although having a gross geometrical organization in common, diverge from each other in important details. These will be examined in the following section.

MODELS

Assman and Brewer proposed a model for HDL based upon the results of ^{31}P- and ^{13}C-NMR studies (12). The model emphasizes the predominantly hydrophobic nature of the protein-lipid interactions occurring between the nonpolar portion of the polypeptides and the alkyl chains of the phospholipids; all are considered to be oriented in parallel and in a direction perpendicular to the curved surface of the lipoprotein molecule. In consequence, the HDL surface is viewed as consisting of protein "icebergs" immersed in a "sea" of lipids. Such a model does not consider major interactions between the ionic charges in the proteins and the polar head group of phospholipids at the lipoprotein surface. In addition, such a model favors the occurrence of electrostatic interactions between helical chains of apo A-I and apo A-II and the formation of secondary, tertiary, and quaternary structures which permit a deep penetration of these apoproteins toward the core of the HDL particle.

The model proposed by Stoffel et al. (13) also emphasizes the importance of hydrophobic protein-lipid interactions in the overall stabilization of HDL and, like the model of Assman and Brewer, rules out the occurrence of strong interactions between the polar head group of phospholipids and apoproteins. According to Stoffel et al., the cholesteryl esters would be

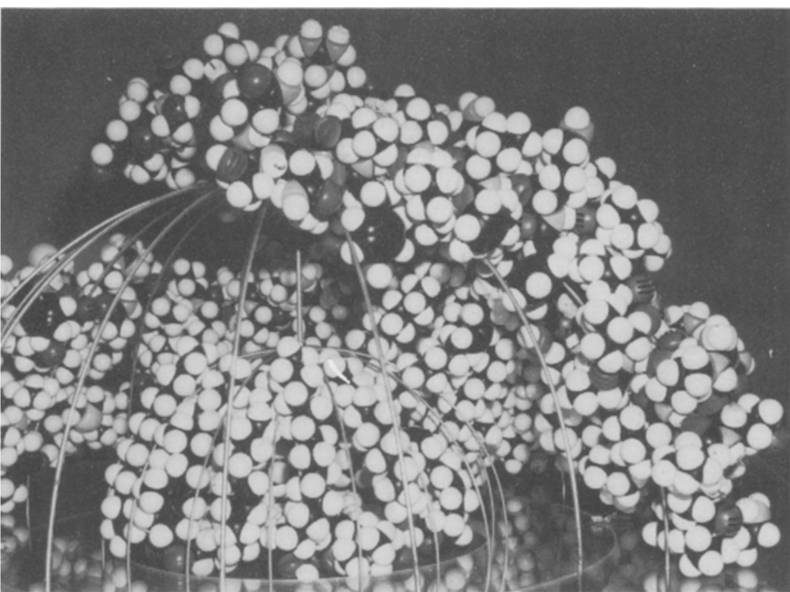

FIG. 3. Partial representation of HDL_3 based on space-filling atomic models. The inner spherical core is clearly visible, as are the phospholipids extending radially from the core surface. Protein and the polar head group of phospholipids are at the surface. (The data are from ref. 17.)

organized in the interior of the particle, whereas the phospholipids, together with unesterified cholesterol, would form a monolayer that covers half of the surface of the particle, the remainder being occupied by the apoproteins. Based on the results of reassembly studies, these authors also emphasize preferential interactions between apo A-II and sphingomyelin.

Jackson et al. (14) and Segrest (15) have proposed a model of HDL which is based on the amphipathic helix theory. The authors envisage the various helical segments of each apoprotein as being oriented parallel to the curved surface of the lipoprotein molecule, and thus perpendicular to the radially oriented phospholipids. In such an arrangment, the nonpolar surface of the amphipathic helical segments would interact with the fatty acid chains of the phospholipids, whereas the positive and negative charges on the polar surface of the helix would interact ionically with the zwitterionic polar head group of phospholipids. In their formulation the authors relied heavily on the examination of space-filling models of amphiphilic segments of the apoproteins.

Verdery and Nichols proposed a model of HDL (16) which, based on considerations of size and composition, has the basic surface-core features of the other models. However, it adds the detail that, in HDL_3, because of surface constraints, the alkyl chain of the cholesteryl ester is folded over the steroid nucleus. This would not be the case with HDL_2, where the alkyl chains are expected to have a greater freedom of motion.

Recently, Shen et al. (7) proposed a structural model that is applicable to all human circulating lipoproteins, at least in normal steady-state conditions. The model is based on compositional analyses and on a knowledge of the molecular volume of each HDL constituent and of the surface areas (see Tables II and III). In the case of HDL_3, the model predicts a spherical or quasi-spherical particle having a spherical fluid core of cholesteryl esters and triglycerides with a 19Å radius, surrounded by a monolayer 20-2Å thick composed of phospholipids, unesterified cholesterol, and protein. A partial representation of the arrangement of phospholipids and cholesterol based on the space-filling model is given in Figure 1. The hydrophobic ends of both cholesterol and phospholipids are closely packed at the surface of the core; in consequence, their hydrophilic groups have distinct positions on the surface monolayers which lead to wedge gaps occupied by proteins (see Fig. 2). The model also predicts that protein and phospholipid head groups do not interact with each other, but compete for space on the lipoprotein. A main stabilizing force in such a model of HDL is the hydrophobic boundary between the core and the inner surface of the phospholipid-cholesterol monolayer. In this model no attempts were made to describe the

surface organization of the apoproteins.

In recent years, semiempirical methods have been applied with reasonable success to the prediction of the probability of secondary structure from aminoacid sequence data. Therefore, we considered it appropriate to examine the primary structure of apo A-I and apo A-II and to complement the results of these analyses with those derived from experiments on both apoproteins at the air-water interface. Moreover, by using atomic space-filling models, we assembled each of the lipid and apoprotein constituents of HDL_3 into an overall model in which two concentric hemispheres defined the core and the surface, respectively (17). The construction of the model verified the predictions by Shen et al. (7) regarding the location of the core and the surface lipids. In addition, it was shown that both apo A-I and apo A-II, constructed to comply with the amphiphilicity of the whole molecule and the predicted distribution of the α-jelical segments, β- and random coil structures, and β-turns, could readily be placed at the surface of the HDL_3 particle between the areas occupied by the phospholipid head groups. The salient features of the model are the following: (a) The surface of the HDL particle is tightly packed with apoproteins and phospholipid head groups; (b) the phospholipids are distributed randomly at the lipoprotein surface and interact neither with themselves nor with the apoproteins in any specific way c(c) the best packing arrangement for the cholesterol head goup is a conformation in which the alcohol function is shielded from the solvent by the helical polypeptide segments; (d) the helical structure of the apoproteins is ideally suited to occupy the wedge-shaped gaps created by the large curvature of the HDL particle and by the disposition of phospholipids; (e) the random distribution of the phospholipid head groups does not favor a specific tertiary or quaternary structure of the apoproteins. A detailed description of the model is reported elsewhere (17); a partial representation is given in Figure 3.

GENERAL CONSIDERATIONS

It is evident from the studies outlined above the HDL_3 is amenable to model building, which provides principles that can also be used for other plasma lipoproteins. Obviously, however, such a model or models may not be a true representation of the actual lipoprotein structure. More realistically, models in general should be viewed as sources of novel ideas which, in turn, are amenable to new experimental designs and tests. One should also keep in mind that plasma lipoproteins are fluid structures which cannot be adequately represented within the rigid confines of space-filling models. Nonetheless, inspection of such models permits an estimate of the degree of freedom of each molecular constituent and the dismissal of improbable organizational schemes. It should now be easier to consider the problem of the structural interrelationship between HDL_3 and other HDL subclasses, and to obtain information which is likely to have functional significance. The construction of a complete space-filling model, now achieved for HDL_3 may be extended to other serum liporpoteins. In the case of LDL, the lack of knowledge of the primary structure of its apoprotein (apo B) unfortunately makes such an approach unrealistic at this time.

During the past few years, several models of HDL have been proposed. Only recently has sufficient experimental data accumulated to permit the formulation of models that provide a more realistic perception of the overall structural organization of HDL particles. As further work progresses, one feels optimistic that a complete understanding of the structure of HDL on a molecular level is within experimental reach.

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Quantitation of High Density Lipoproteins

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ABSTRACT

The demand for high density lipoprotein (HDL) quantitation has dramatically increased with the renewed awareness of the importance of HDL as a negative risk factor for coronary heart disease. HDL is usually estimated by specific precipitation of the non-HDL apoB-containing lipoproteins by polyanions and divalent cations followed by measurement of cholesterol in the supernatant. A common procedure involves precipitation with sodium heparin at 1.3 mg/ml and $MnCl_2$ at 0.046 M (final concentrations). This method is appropriate for serum but less than ideal for plasma because of incomplete precipitation and sedimentation of the apoB-containing lipoproteins. A two-fold increase in Mn^{2+} to 0.096 M improves precipitation of the apoB-associated lipoproteins from plasma without excessive precipitation of HDL. This modified heparin-Mn^{2+} procedure gives results nearly identical to the results with the ultracentrifugal reference method (cholesterol in the d>1.063 fraction corrected for losses and the presence of apoB-associated cholesterol). The dextran sulfate 500-Mg^{2+} and the sodium phosphotungstate-Mg^{2+} procedures give results consistently 2-4 mg/dl lower than does the reference method. In contrast, a heparin-Ca^{2+} method gives results 5-8 mg/dl higher than does the reference method. Immunochemical analysis of apoA-I in the precipitate and apoB in the supernatant indicates that lower values for the phosphotungstate-Mg^{2+} procedure is due to partial precipitation of the A-I-containing lipoproteins, while higher values by the heparin-Ca^{2+} method are due to incomplete precipitation of the apoB-containing lipoproteins. Quantitation of the principal apoproteins of HDL, A-I and A-II, represent an important additional index of HDL concentrations and composition. Quantitation of plasma A-I and A-II concentrations by radial immunodiffusion indicates that women generally have higher HDL concentrations than men (women, A-I, 135 ± 25, A-II, 36 ± 6; men, A-I, 120 ± 20, A-II, 33 ± 5; mean ± S.D., in mg/dl). A-I and A-II do not increase with age in men but show a slight increase with age in women. Estrogen increases HDL cholesterol and protein and may in part account for the higher HDL in women. The lighter density HDL subclass has a higher A-I/A-II ratio than the denser HDL subclass, with women generally having significantly more of the lighter HDL subclass. Density-gradient ultracentrifugation in $CsCl_2$ gradients indicates that HDL contains subpopulations of differing hydrated density which vary in the A-I/A=II ratio. Immunoassay of A-I and A-II when used in combination with HDL cholesterol analysis is a powerful tool for studies of HDL structure, epidemiology and metabolism.

INTRODUCTION

High density lipoproteins (HDL) are generally defined in terms of one or more of their physico-chemical properties; i.e., hydrated density, flotation characteristics in the analytical ultracentrifuge, electrophoretic mobility, and apolipoprotein composition. The fraction of plasma lipoproteins with d 1.063-1.21 has been operationally defined as the high density lipoproteins (1,2). Since HDL have alpha-electrophoretic mobility, they are aften alternatively called alpha lipoproteins. More precisely, HDL can be defined in terms of apoprotein composition as the apolipoprotein A (apoA)-containing lipoproteins which are free of apolipoprotein B (apoB) (3). However, the HDL ultracentrifugal fraction contains some apoB-containing lipoproteins, principally the Lp(a) lipoprotein found primarily in the d 1.050-1.090 plasma fraction (4,5) and may also contain some non-Lp(a) apoB-containing lipoproteins (6). Thus, if we define HDL (or more appropriately the alpha lipoproteins) in terms of their apoprotein composition, then the apoB-containing lipoproteins in the d 1.063-1.21 fraction must be excluded from our definition of HDL. For the purpose of this treatise, HDL will generally be considered synonymous with the alpha lipoproteins, but special emphasis will be placed upon techniques which optimize the quantitation of lipoproteins that contain apoA and are free of apoB.

HDL CHOLESTEROL QUANTITATION

With the new awareness of the importance of HDL as a negative risk factor for coronary heart disease (7-9), the demand for HDL quantitation has dramatically increased. HDL is generally estimated by measuring its cholesterol content. A practical approach which avoids ultracentrifugation is the selective precipitation of the non-HDL apoB-associated lipoproteins with sulfated polysaccharides and divalent cations and the measurement of cholesterol in the supernatant solution. A common procedure involves precipitation of the apoB-associated lipoproteins with sodium heparin at 1.3 mg/dl

TABLE I

Heparin-Mn^{2+} Precipitation Procedure

Standard Method	Modified Method
1. 3 ml plasma	1. 2 ml plasma
2. 0.12 ml 5000 units heparin/ml	2. 0.2 ml combined heparin-Mn^{2+} reagent (0.6 ml 40,000 units heparin/ml, 10 ml 1.06M $MnCl_2 \cdot 4\ H_2O$)
3. 0.15 ml 1 M $MnCl_2 \cdot 4\ H_2O$	3. Incubate 10 min at 23 C
4. Incubate 30 min at 4 C	4. Centrifuge 1500 X g for 30 min at 4 C
5. Centrifuge 1500 X g for 30 min at 4 C	

and $MnCl_2$ at 46 mM (10-12).

This method (Table I), originally applied to serum (10), appears reasonably specific and not often subject to large errors, precipitating generally less than 2% of the HDL (13,14), yet producing nearly complete removal of the apoB-associated lipoproteins. However, if this precipitation method is applied to plasma containing EDTA, it is less than ideal, since about 10% of the plasma samples so treated have supernatants with obvious turbidity, indicating incomplete sedimentation. Furthermore, about half of the samples with nonturbid supernatants still contain ca. 2 mg/dl apoB, leading to slight overestimation of HDL cholesterol. A two-fold increase in Mn^{2+} concentration to 92 mmol Mn^{2+}/liter improves precipitation of the apoB, without substantial precipitation of HDL, and sediments better the apoB associated lipoproteins from hypertriglyceridemic plasma (14). Therefore, a more accurate and more convenient modified version of the heparin Mn^{2+} precipitation technique is recommended for plasma, namely a smaller sample volume, 2 ml instead of 3 ml, a two-fold increase in the Mn^{2+} reagent concentration, addition of heparin Mn^{2+} as a combined reagent, followed by incubation at room temperature for 10 min instead of 30 min at 4 C (Table I). A collaborative study by eight laboratories to evaluate the modified heparin Mn^{2+} precipitation procedure also shows the modified procedure to be both more specific and more convenient. By the standard method, 32 of 375 (9%) of the samples had turbid supernatants, and 72% of the samples had detectable apoB in nonturbid samples. On the other hand, of the plasma samples precipitated by the modified procedure, only 2% had turbid supernatants and only 15% had detectable apoB in nonturbid supernatants (15).

With hypertriglyceridemic samples, the density of the heparin-Mn^{2+} lipoprotein aggregate frequently is too low for it to sediment. These lipoproteins thus remain suspended in the solution, producing obvious turbidity. A number of procedures have been described for circumventing the problem of turbid supernatants (Table II). Method 1 is expensive and time consuming. Method 2 magnifies the imprecision of the measurement of cholesterol in the heparin-Mn^{2+} supernatant. Method 3 is satisfactory for the majority of samples, but requires high speed centrifugation (14). Method 4 removes essentially all of the apoB-associated lipoproteins without removing appreciable amounts of HDL (16). It appears to be the simplest and most convenient procedure for the clinical laboratory.

TABLE II

Methods for HDL Estimation in Hypertriglyceridemic Samples

1. Centrifuge plasma 105,000 X g for 18 hr. Do precipitation on the d>1.006 fraction.
2. Dilute plasma with an equal volume of isotonic saline before precipitation.
3. Perform precipitation. Centrifuge turbid supernatants 12,000 X g for 10 min. Determine cholesterol on clear subnatant solution.
4. Perform precipitation. Filter turbid supernates with 0.22 μm filter protected by two depth prefilters. Determine cholesterol on clear filtrate.

Since Mn^{2+} interferes with the enzymatic cholesterol procedure, other polyanion-cation combinations including dextran sulfate 500 (Pharmacia, Piscataway, NJ 08854)-$MgCl_2$ (17), sodium phosphotungstate-$MgCl_2$ (18), and heparin-$CaCl_2$ (19), along with an ultracentrifugal method at d 1.063 were evaluated (Table III). The ultracentrifugal approach was improved as a reference method by correcting the cholesterol of the d>1.063 g/ml fraction for losses (5%) and the presence of apoB-associated cholesterol (average 5.7 mg/dl, as determined

TABLE III

Comparison of Mean HDL Cholesterol by Several Techniques[a]

Samples							
Source	n	Corrected d>1.063 fraction[b]	Heparin-Mn^{2+} .046 M[c]	Heparin-Mn^{2+} .092 M[d]	Dextran Sulphate[e] 500 Mg^{2+}	Phosphotungstate[f] Mg^{2+}	Heparin[g] Ca^{2+}
Plasma							
Women	30	61.7	63.9	60.7	58.1	57.4	66.7
Men	27	44.4	45.7	44.1	43.6	42.8	51.5
Children	8	52.2	53.0	50.8	48.0	49.0	57.2
All Subjects	65	53.4	55.0	52.6	50.9	50.3	59.2
Serum							
Women	10	70.0	70.5	66.9	62.4	64.7	75.0
Men	10	44.7	44.2	42.8	40.0	41.6	53.1
All Subjects	20	57.4	57.4	54.8	51.2	53.2	64.0

[a]Results expressed in mg/dl *
[b]Corrected for losses of cholesterol during ultracentrifugation and the presence of apoB-associated cholesterol.
[c]According to the Lipid Research Clinics Procedure (12).
[d]According to Warnick and Albers (14).
[e]According to Kostner (17).
[f]According to Lopes et al. (18).
[g]According to Srinivanson et al. (19).

TABLE IV

Rid Assay for Human Plasma A-I and A-II

1. 50 μl plasma or test sample.
2. 50 μl tetramethylurea, mix.
3. 400 μl 8 M urea, 10 mM Tris pH 8.0, mix.
4. Incubate 30 min.
5. Add 4 μl of the mixture to the antibody-containing Agarose plate.
6. Incubate 24-72 hr.
7. Measure diameter of precipitation rings.

TABLE V

Cholesterol and A-I and A-II in Ultracentrifuged Subfractions of HDL[a]

	d 1.063 - 1.10		d 1.10 - 1.21	
	Men	Women	Men	Women
Cholesterol	9.9	18.6	32.3	36.1
A-I	10.3	23.8	97.2	104.4
A-II	2.0	3.9	26.6	27.4
A-I/A-II	5.1	6.1	3.7	4.0

[a]Results expressed in mg/dl. Each number is a mean and represents the analysis of six ultracentrifuged fractions from six plasma pools. Each pool consisted of plasma samples from three or four healthy normolipidemic adults.

by radial immunodiffusion with anti-apoB). The dextran-sulfate 500-Mg^{2+} method consistently gave HDL cholesterol values on plasma samples 2-4 mg/dl lower and on serum samples 4-8 mg/dl lower than the HDL values estimated by centrifugation. Similarly, the sodium phosphotungstate-Mg^{2+} procedure consistently gave lower results than did the ultracentrifugal method. In contrast, the heparin-Ca^{2+} method gave results 5-8 mg/dl higher than the centrifugal reference method. Analyses of apoA-I in the precipitates and apoB in the supernatant fluid by immunoassay suggest that lower values by the phosphotungstate-Mg^{2+} procedure are due in part to increased precipitation of the apoA-I-containing lipoproteins, while higher values by the heparin-Ca^{2+} method are due to incomplete precipitation of the apoB-containing lipoproteins.

On plasma samples, the modified heparin Mn^{2+} method, which uses 0.092 M Mn^{2+}, was in excellent agreement with the estimation of HDL cholesterol by the ultracentrifugal method, while the Lipid Research Clinic heparin Mn^{2+} method using 0.046 M Mn^{2+} gave 1-to-3 mg/dl higher values. However, on serum samples, the heparin Mn^{2+} procedure using 0.046 M Mn^{2+} agreed the best with the ultracentrifugal estimation of HDL cholesterol. Thus, 0.046 M Mn^{2+} appears adequate for precipation of HDL from serum but a higher Mn^{2+} concentration is needed for complete precipitation of apoB-containing lipoproteins from plasma, probably because Mn^{2+} is bound by EDTA (14,15). Yet, EDTA-treated plasma is preferred to serum for lipid and lipoprotein determinations since, during clot formation,

TABLE VI

Mol Wt, Apolipoprotein and Cholesterol Analyses on HDL Subfractions Isolated by Density Gradient Centrifugation[a]

Density of Subfraction	10^{-5} x Mol Wt	A-I (mg/dl)	A-II (mg/dl)	CH (mg/dl)	A-I/A-II (Molar Ratio)	CH/(A-I+A-II) (Wt. Ratio)
(Male)						
1.151-1.200	1.2	16.2	3.9	4.1	2.6	0.21
1.119-1.150	1.4	29.3	8.4	11.9	2.2	0.32
1.106-1.118	2.0	23.4	6.3	11.4	2.3	0.38
1.093-1.105	2.4	14.6	3.4	7.4	2.7	0.41
1.083-1.092	3.0	9.9	1.4	5.9	4.4	0.53
1.077-1.082	3.3	7.3	0.8	4.9	5.7	0.63
1.063-1.076	3.7	3.9	0.4	3.5	6.0	0.82
(Female)						
1.151-1.200	1.2	13.2	3.1	3.0	2.6	0.19
1.119-1.150	1.4	27.9	7.9	10.7	2.2	0.30
1.106-1.118	2.0	22.6	6.7	11.3	2.1	0.38
1.093-1.105	2.4	14.4	3.0	6.5	2.9	0.38
1.083-1.092	3.0	11.1	1.5	5.9	4.6	0.47
1.077-1.082	3.3	8.4	0.9	5.6	5.5	0.61
1.063-1.076	3.7	4.2	0.5	3.5	5.3	0.75

[a]Mean values expressed in mg/dl from six healthy normolipidemic adult volunteers (three male and three female). Mol wt estimated by density gradient electrophoresis. A-I and A-II determined by radial immunodiffusion. HDL of d 1.063-1.25 was placed in a CsCl gradient and centrifuged in a SW 41 rotor at 40,000 rpm at 16 C for 72 hr.

chylomicrons can be removed by the clot, and changes may occur in the lipoprotein distribution. Furthermore, serum contains heavy metals that are known to promote auto-oxidation, whereas EDTA in plasma chelates metal ions, minimizing auto-oxidation. Therefore, the most appropriate procedure for quantitation of HDL cholesterol is precipitation of the apoB-containing lipoproteins from EDTA-plasma by the modified heparin-Mn^{2+} method and determination of the cholesterol in the supernatant fraction. Since Mn^{2+} interferes with the enzymatic cholesterol procedure, other polyanioncation combinations need to be considered for laboratories using the enzymatic cholesterol procedure.

APOLIPOPROTEIN A-I AND A-II QUANTITATION

HDL is usually estimated in terms of its lipoprotein cholesterol content. The cholesterol moiety is, however, only 15-20% of the total weight of HDL. Thus, a change in the HDL cholesterol could reflect a change in HDL composition or a change in whole HDL concentration, or both. Assessment of the principal apoproteins of HDL, apoproteins A-I and A-II, together comprising 50% of the weight of HDL, thus respresents an important additional index of alteration in HDL concentration or composition. We developed a simple yet precise and accurate radial immunodiffusion assay for human plasma A-I and A-II (Table IV) (20,21). The diameter of the precipitation rings are measured in 0.1-mm units, using a calibrated viewer. Since usually less than 1% of the total plasma A-I and A-II levels are found in the d<1.063 plasma fraction, quantification of total plasma A-I and A-II is representative of total HDL A-I and A-II levels (21). It has been estimated that ca. 10% of the plasma A-I and 3% of the plasma A-II is in the d>1.21 fraction (21). However, it is likely that most of the A apolipoproteins found in the d>1.21 fraction were those that had become dissociated from HDL during ultracentrifugation. Analysis of ultracentrifuged subfractions of the HDL preparations from normolipidemic adult subjects indicates that men and women have similar A-I, A-II and HDL levels in the d 1.10-1.21 subfraction (Table V). However, women have approximately twice as much A-I, A-II and HDL choelsterol in the d 1.063-1.10 fraction as men. Also for both men and women the A-I/A-II molar ratio of the lighter density HDL subfraction was significantly greater ($p<0.01$) than that in the heavier density HDL subfraction. It is clear, therefore, that apoprotein compositions differ significantly within the HDL hydrated density subclasses. Fractionation of HDL on the basis of hydrated density by CsCl density-gradient centrifugation and subsequent A-I and A-II analysis shows that the A-I/A-II ratio varies with the lipoprotein hydrated density (Table VI). The A-I/A-II molar ratio of HDL lipoproteins banding between d 1.106-1.150 was nearly constant at 2.2 ± 0.2. In the density range 1.51-1.25, the A-I/A-II

TABLE VII

Plasma A-II in Normal Subjects
(mean ± S.D., mg/dl)

Age	Number		Plasma A-II	
	Men	Women	Men	Women
20-29	27	82	34 ± 4	35 ± 6
30-39	53	33	33 ± 5	35 ± 7
40-49	55	44	34 ± 5	37 ± 5
50-59	35	27	33 ± 5	38 ± 6

TABLE VIII

Plasma A-I Levels in Normal Subjects
(mean ± S.D., mg/dl)

Age	Number		Plasma A-I	
	Men	Women	Men	Women
20-29	50	114	117 ± 18	132 ± 26
30-39	77	39	117 ± 19	135 ± 26
40-49	77	62	120 ± 10	137 ± 22
50-59	55	37	125 ± 22	140 ± 32
60-65	4	5	126 ± 20	168 ± 23

TABLE IX

Plasma HDL-Cholesterol, A-I and A-II Levels in Normal Subjects[a]

Subject group	n	HDL CH	A-I	A-II
All men	192	45	120	33
All women	188	55	135	36
Women taking no estrogen[b]	92	54	130	34
Women on estrogen	19	61	149	39
Women on estrogen and progesterone	56	54	140	39

[a]Normal subjects refers to a subset of an industrial population who were selected independently of their lipid levels. Results expressed as mean levels in mg/dl.

[b]Refers to a subset of women from the population who had taken no medication for 2 weeks before blood drawing.

ratio increased as the density increased. However, in the density range between d 1.077 and d 1.105, the A-I/A-II ratio increased as the density decreased. The cholesterol/(A-I + A-II) ratio decreased as the density increased in all six HDL samples examined. Gradient gel electrophoresis of the density-gradient fractions showed that as the density of the HDL particle increased, the apparent mol wt decreased. Thus, HDL contains subpopulations which differ not only in molecular weight but also in the A-I/A-II molar ratio. HDL subfractions with the same hydrated density had comparable A-I/A-II and cholesterol/protein ratios whether isolated from men or from women. These results suggest that the differences between HDL concentrations in men and those in women primarily reflect differences in the relative proportions of HDL subclasses rather than intrinsic male-female differences in HDL structure (22).

Table VII presents total plasma A-I levels by age decade in the population tested. Among the men, the older age groups (5th to 7th decades) had slightly higher A-I values. The women also had slightly higher A-I values in the older age groups, with an average annual increment of 0.33 mg/dl/yr. A-II did not increase with age in men, but showed a slight increase with age in women (Table VIII). HDL cholesterol was highly correlated with A-I (r=0.72) and A-II (R=0.60); also, A-I was highly correlated with A-II (r=0.72). Women had significantly higher HDL cholesterol and apolipoprotein A-I and A-II levels than did men (Table IX). Women taking estrogen-containing medication had significantly higher HDL cholesterol and A-I concentrations and somewhat higher A-II concentrations than those not taking estrogen. The observation that women taking estrogen had significantly higher HDL cholesterol levels and

somewhat higher A-I/A-II ratios suggests that estrogen preferentially increases the lighter density HDL-2 subclass. On the other hand, women on combination contraceptives had the same HDL cholesterol concentrations as those on no medication, but somewhat higher A-I and A-II levels, suggesting that progestins modify the estrogen response and promote an increase in the heavier density HDL-3 subclass (21). The work of Krauss et al. confirms this suggestion (23). Fourteen women studied before and after 14 days of estrogen therapy (ethinyl estradiol 1 μg/kg/day) had a mean A-I increase of 24%, whereas the HDL cholesterol increased by an average of 19%, supporting the concept that estrogen is in part responsible for the higher HDL_2 levels observed in women (20,21).

There are many conditions or diseases in which HDL concentrations or compositions are altered; e.g., persons with existing coronary heart disease and those with many of the conditions associated with increased risk of coronary heart disease have reduced concentrations of HDL cholesterol (24-26).

Quantitation of A-I and A-II in 90 male survivors of myocardial infarction (MI) sampled at least 3 months after an acute MI indicated that MI survivors had significantly lower ($p<0.01$) A-I (112 ± 2 mg/dl, mean ± S.E.M.), A-II (29 ± 1 mg/dl) and HDL CH (39 ± 1 mg/dl) than did a plasma-choelsterol and triglyceride-matched control group, and the HDL cholesterol of the MI survivors was significantly related to log triglyceride (4 = -0.442 lipid matched controls, r = -0.520 MI survivors). These results are consistent with a relative decrease of HDL in MI survivors over and above that attributable to their increased triglyceride levels. Furthermore, the low HDL cholesterol/protein ratio observed in the HDL of MI survivors suggests a relatively greater decrease of the cholesterol-rich HDL_2 subclass than that of HDL_3 (24).

CONCLUSIONS

Recent epidemiological studies have emphasized the importance of HDL as a negative risk factor (7-9). As a result, clinical laboratories have experienced an increased demand for quantitation of this lipoprotein. HDL is usually estimated by quantitation of its cholesterol. Precipitation techniques for estimating HDL cholesterol are used widely. However, they do not give equivalent results. Thus, careful validation of specific precipitation conditions is necessary in order to ensure an accurate estimate of HDL cholesterol.

Quantitation of the principal apolipoproteins of HDL, A-I and A-II, together comprising ca. 50% of the weight of HDL, represents an important additional index of alterations in HDL concentration or composition. The radial immunodiffusion assays for A-I and A-II do not require prior extraction of lipids, and use only 50 μl of plasma. These assays therefore are suitable for mass screening. Therefore, the quantitation of A-I and A-II shows particular promise as methods for complementing the HDL cholesterol procedure.

The concentration, composition and subclass distribution of HDL changes in response to a variety of physiological, pharmacological, pathological and dietary perturbations. Thus, the application of assay methods for HDL are numerous. The functional role of HDL in health and disease will become clearer as accurate and precise assays for HDL become widely available.

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The Role of High Density Lipoprotein Apolipoprotein CII in Triglyceride Metabolism

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ABSTRACT

The purpose of these studies was (a) to examine the relationship between total plasma triglycerides (TG) and the amount of apolipoprotein CII (apo CII) in triglyceride rich lipoproteins (TRL), and (b) to determine whether TRL could be enriched with apo CII in vitro. In 13 patients with primary endogenous hypertriglyceridemia, (log_{10}) total plasma TG correlated inversely with the amount of apo CII per unit very low density lipoprotein (VLDL) protein ($r = -0.76$; $p<0.005$) and VLDL TG ($r = -0.75$; $p<0.005$). The potency of VLDL to activate milk lipoprotein lipase (LPL) in hydrolyzing triolein was studied in vitro. LPL activator potency per unit VLDL protein or VLDL TG correlated inversely with (log_{10}) total plasma TG ($r = -0.86$ and $r = -0.76$, respectively; $p<0.005$). LPL activator potency per nM VLDL apo CII also correlated inversely with (log_{10}) total plasma TG ($r = -0.49$; $p<0.01$). In seven patients with familial type V hyperlipoproteinemia, the average amount of apo CII in TRL protein was subnormal (5.86 ± 0.62% vs 10.0 ± 0.51% in normal subjects). The higher the (log_{10}) total plasma TG, the lower was the apo CII content in TRL protein ($r = -0.93$; $p<0.01$). To determine the factors governing the distribution of apo CII between lipoproteins and whether TRL could be enriched with apo CII, five approaches were undertaken: (a) ^{125}I apo CII was added to mixtures of VLDL and HDL. The amount of labelled apo CII in VLDL was proportional to the ratio of VLDL to HDL. (b) TRL from four patients with familial type V hyperlipoproteinemia was incubated with high density lipoprotein (HDL) from a normal subject. An increase in the TRL/HDL ratio was associated with transfer of apo CII from HDL to TRL and a reciprocal transfer of non-apo CII protein from TRL to HDL. Net apo CII enrichment of TRL protein was possible below a HDL/TRL protein ratio of ca. 6 under the experimental conditions. (c) A fixed amount of normal plasma freed of TRL was incubated with different amounts of TRL from two patients with familial type V hyperlipoproteinemia. The amount of apo CII that transferred from normal TRL free plasma to the patient's TRL was proportional to the amount of TRL in the mixture. (d) A doubling and tripling in the amount of apo CII in TRL was found when apo CII was added directly to TRL from a normal subject and TRL from a patient with familial type V hyperlipoproteinemia, respectively. (e) When apo CII was added directly to normal plasma and plasma from a patient with primary type IV hyperlipoproteinemia, the peptide was taken up mainly by VLDL and HDL, indicating enrichment of these fractions. The distribution of the added apo CII in each lipoprotein fraction resembled the distribution in the native plasma. TRL was isolated after addition of apo CII to plasma from two patients with familial types IV and V, respectively. Enrichment of TRL with apo CII was associated with an approximate 1.5-fold increase in the LPL activator potency per unit TRL protein. These studies suggest that firstly, the amount of apo CII in TRL is inversely related to the severity of hypertriglyceridemia. Secondly, the distribution of apo CII between TRL and HDL is governed by the mass ratios of these two lipoprotein classes. Thirdly, plasma TRL and HDL have a reserve binding capacity of apo CII and fourthly, it is possible to enrich these lipoproteins with this functionally important peptide. Whether net enrichment of TRL with apo CII and also an increase in its biological activity to activate LPL in vitro is related to increased in vivo catabolic rate requires to be determined.

INTRODUCTION

The early observations of Hahn (1) and later of Korn et al. (2) and Anderson et al. (3) demonstrated that lipoprotein lipase (LPL) in extrahepatic tissues, e.g., adipose tissue and muscle, plays a major role in promoting the breakdown of transported triglyceride (TG) into free fatty acids and glycerol. In vitro, if serum is not added to LPL and TG, no appreciable hydrolysis occurs. The necessary serum cofactor for the LPL-TG interaction resides in the protein moiety of high density lipoprotein (HDL) (4,5) and in very low density lipoproteins (VLDL) (6). Additional work demonstrated that the cofactor activity resided in the C group of apolipoproteins which are constituents of both HDL and VLDL (7,8). Subsequently, Havel et al. (9) and Krauss et al. (10) demonstrated that the principal activator of the LPL-TG interaction of the C apolipoproteins is a peptide with a carboxy terminal amino glutamic acid (apo CII). They also showed that other apolipoprotiens inhibited LPL. Recently Jackson et al. (11) have described the primary structure of apo CII. The peptide consists of 78 amino acid residues. Further, Kinnunen et al. (12) have

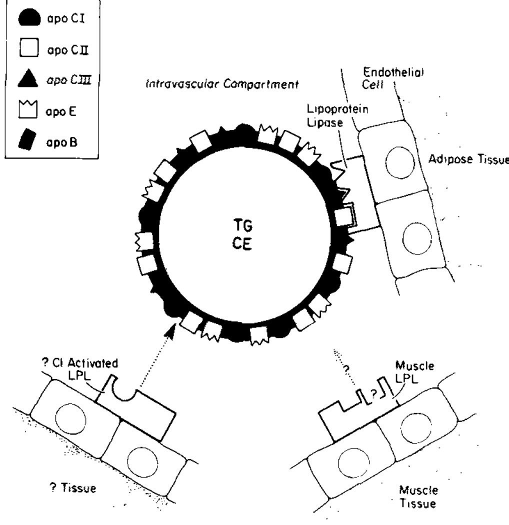

FIG. 1. VLDL-LPL interaction is shown schematically. Catabolism of VLDL is mediated by extrahepatic LPL. Adipose tissue, skeletal and cardiac muscle are the major sites of extrahepatic LPL. Apo CII activates these lipases. Some VLDL apolipoproteins (e.g., apo CIII) inhibit LPL activity. An apo CI activated LPL has been described (left bottom). Theoretically, tissue LPL levels or VLDL apolipoprotein composition may influence the site and rate of lipoprotein TG hydrolysis.

shown that the fragment (both natural and synthetic) corresponding to residues 50-78, activated LPL similar in extent to the complete molecule. This portion does not bind phospholipid. Kinnunen et al. (12) proposed that one portion of the molecule is used for lipid binding and the other for activation. A carboxyterminal 29 amino acid fragment obtained after tryptic cleavage of apo CII also has significant LPL activation property whereas the amino-terminal fragment did not (13).

Following fat ingestion in normal subjects, Havel et al. (14) demonstrated that LPL activator property is transferred from HDL_2 to chylomicrons. These observations led to the suggestion that HDL plays an important role in the transport of TG by supplying apo CII to chylomicrons and VLDL for their subsequent hydrolysis of lipoprotein lipase. Thus, in a sense, apo CII behaves as a hormone in that it is known to be synthesized by the liver (15-17), and its action is in other target LPL synthesizing organs where it enhances the breakdown and eventually utilization of transported TG.

According to current concepts, the catabolism of VLDL – which contains apolipoprotein B (apo B) and apo C – is mediated by LPL. During this VLDL-LPL interaction, VLDL TG hydrolysis is associated with loss of the C apolipoprotein to HDL, and the end product of this process is the formation of low density lipoprotein (LDL) which contains apo B. Recent evidence indicates that HDL is partly formed as an end product of chylomicron TG hydrolysis (18). Thus, recycling of C apolipoproteins occurs between HDL and TG rich lipoproteins (VLDL or chylomicrons) during synthesis and catabolism of transported TG (19,20).

Whereas apo CII activates LPL, apolipoprotein CIII (apo CIII) inhibits LPL activity (21) (Fig. 1). Apo CI and E have also been shown to inhibit human adipose tissue LPL (22). Thus, it appears that these apolipoproteins may modulate the metabolic clearance of TG. Pathologic disturbances of LPL activator system and its antagonists or abnormalities in the exchange of apolipoproteins between VLDL, chylomicrons and HDL could theoretically lead to hypertriglyceridemia. A deficiency of extra hepatic LPL (23,24) or absolute deficiency of apo CII (25) is associated with severe hypertriglyceridemia.

Recent studies using a specific radioimmunoassay (26) have shown that the concentration of total human plasma apo CII is ca. 5 mg/dl. It is mainly in triglyceride rich lipoproteins (TRL) and HDL. With progressive hypertriglyceridemia, the total plasma apo CII concentration rises, and apo CII is redistributed from HDL to TRL (26).

The overall objective of this investigation was to test the hypothesis that TRL (VLDL and/or chylomicrons) in some patients with primary and familial hypertriglyceridemia have subnormal amounts of apo CII and to determine whether TRL could be enriched with apo CII.

MATERIAL AND METHODS

Study Subjects

Studies were made in 15 healthy asymptomatic subjects and 21 patients with primary hypertriglyceridemia who were classified according to Lipid Research Clinics criteria (27) who were different from those previously described (26). There were no patients with types I or III in this study. Secondary causes for hyperlipoproteinemia were excluded by appropriate tests (28). The familial nature of the patients with type V hypertriglyceridemia was confirmed by the presence of at least one additional first degree relative who also had primary hypertriglyceridemia. All patients fasted except for water at least 12 hr. Venous blood was drawn in tubes containing EDTA to give a

blood concentration of 1 mg/ml and refrigerated at 4 C immediately.

Analytical Methods

Cholesterol, TG, lipoprotein electrophoresis and plasma HDL cholesterol were measured according to Lipid Research Clinics methodology (27). Preparative ultracentrifugation was performed by sequential gradient ultracentrifugation (29). TRL were isolated by ultracentrifugation of plasma and 'washed' by re-ultracentrifugation of the supernate with EDTA saline. IDL, LDL, HDL and 'residue' were isolated at d 1.019, 1.063, 1.210 with single ultracentrifugation after density adjustment and >1.210, respectively, unless otherwise stated. Protein was measured by Lowry's method (30).

Apo CII was measured by a specific double antibody radioimmunoassay (26). LPL activator potency of VLDL or TRL was measured using milk LPL and emulsified ^{14}C labelled triolein substrate as previously detailed (26). Fixed amounts of milk LPL and ^{14}C labelled sonicated triolein substrate were incubated with varying amounts of plasma (containing the cofactor). A standard curve was obtained. The activator property of an unknown sample was measured against the plasma which was given an arbitrary activator concentration of 100 units/ml. Aliquots of the same plasma (for the standard curve) were used in all determinations. ^{125}I apo CII was made using chloramine T as described previously (26) and radioactivity was measured using a Packard Autogamma scintillation spectrometer 5230 (Packard Instrument Co., Inc., Downers Grove, IL).

The molecular weight of apo CII was assumed to be 8837 based on the primary structure of the peptide by Jackson et al. (11). For TG, the molecular weight of triolein was used for calculating molar ratios of TG and apo CII.

Statistical Analyses

Studies of correlations, differences between means, and differences between slopes were carried out as per Snedecor and Cochran (31). Differences between means were tested using Student's t test. Correlation co-efficients were computed by Pearson's product moment method. In addition to correlations, regression lines were fitted by the method of least squares.

RESULTS

A. Total Plasma TG and APO CII in TRL

I. *Total plasma TG and VLDL apo CII in primary endogenous hypertriglyceridemia.*

As shown in the lower panel of Figure 2, in 13 hypertriglyceridemics the content of apo CII in VLDL protein decreased as total plasma TG increased ($r = -0.76$; $p<0.005$). Correlation between apo CII in VLDL was done with $\log_{10}$ transformed TG concentration. In 15 control subjects, no significant correlation between $\log_{10}$ total plasma TG and VLDL apo CII content was noted ($r = 0.10$). Mean apo CII content in VLDL protein in hypertriglyceridemics was 7.4 ± 0.6%, significantly lower than 10.0 ± 0.5% in normal subjects (M ± S.E.M.; $p<0.05$).

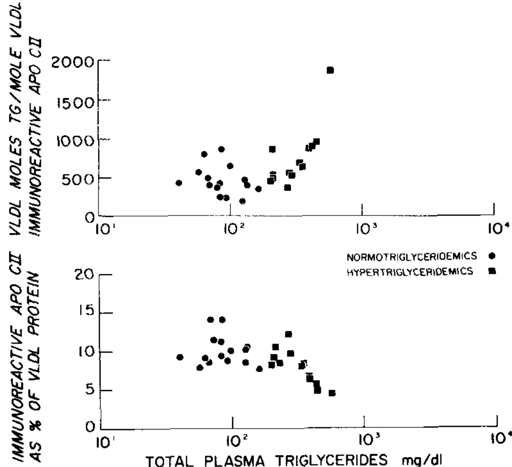

FIG. 2. Relationship between (a) total plasma TG and the molar ratio of VLDL TG/apo CII ratio (upper panel) and (b) apo CII as percent total VLDL protein in patients with primary endogenous hypertriglyceridemia. See text for data analysis.

In the upper panel (Fig. 2) is shown the relationship between the VLDL TG/apo CII molar ratio and total plasma TG on a semilogarithmic scale. In hypertriglyceridemics the VLDL TG/apo CII ratio increased with $\log_{10}$ total plasma TG ($r = 0.75$, $p<0.005$). For normal subjects the correlation coefficient was not significant ($r = -0.29$). Mean VLDL TG/apo CII molar ratio in hypertriglyceridemics 734 ± 106 was significantly higher than normals 435 ± 38; $p<0.001$. The relationships between total plasma TG and VLDL LPL activator per unit VLDL protein or per unit VLDL TG are shown in Figure 3. In 13 hypertriglyceridemics with increasing $\log_{10}$ total plasma TG concentration, LPL activator potency (per mg VLDL protein or μM VLDL TG $r = -0.86$ and $r = -0.76$, respectively) fell, $p<0.005$. Normal subjects also had more LPL activator potency (159 ± 11 units per mg VLDL protein; 37 ± 4 units per μM TG) than did hypertriglyceridemic subjects (95 ± 8 units and 18 ± 2 units, respectively, $p<0.001$, <0.0001). To further explicate the absolute fall in

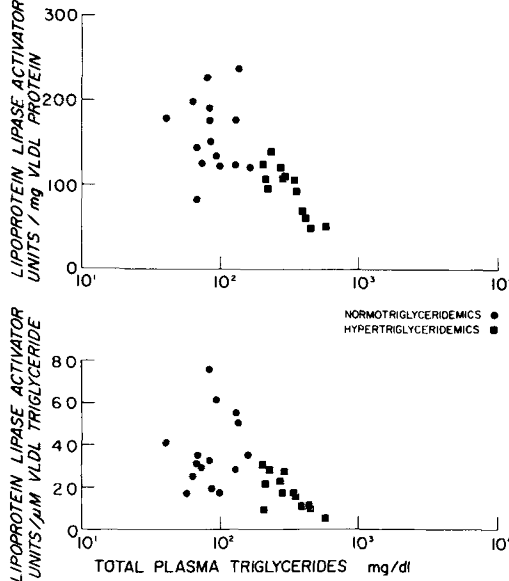

FIG. 3. Relationship between total plasma TG and LPL activator potency per unit VLDL protein (upper panel) and per unit VLDL TG (lower panel). See text for data analysis.

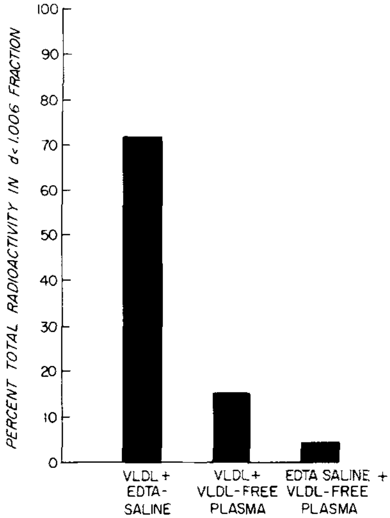

FIG. 4. *Left bar.* VLDL (1.5 mg VLDL protein) was incubated with ^{125}I apo CII for 2 hr at 37 C, in EDTA saline pH 7.4 in a cellulose nitrate tube. VLDL was reisolated by ultracentrifugation. 72% of total radioactivity was in the d<1.006 fraction. *Middle bar.* 1.5 mg VLDL protein was similarly incubated with VLDL free plasma obtained from 6 ml normal plasma. 15% of total radioactivity was found in the d<1.006 fraction. *Right bar.* VLDL free plasma from 6 ml normal plasma was incubated with ^{125}I apo CII without VLDL. 4% of total radioactivity was found in the d<1.006 fraction.

LPL activator potency per mg VLDL protein or per μM VLDL TG, the LPL activator potency was calculated per nM apo CII for both normal and hypertriglyceridemic subjects. As total plasma TG rose, the LPL activator potency per nM apo CII fell ($\log_{10}$ total plasma TG vs. LPL activator potency per nM apo CII; r = -0.49; $p<0.01$). Normals also had more LPL activator potency per nM apo CII than hypertriglyceridemics (14.2 ± 0.9 units vs.10.7 ± 0.6 units; $p<0.005$).

II. *Apo CII content in TRL protein in patients with familial chylomicronemia and hypertriglyceridemia (type V hyperlipoproteinemia).*

TRL apo CII, protein and total plasma TG were measured in 7 patients with familial type V hyperlipoproteinemia. The mean apo CII content in TRL protein was 5.86 ± 0.62%, significantly lower than in 15 normal subjects ($p<0.01$). An inverse correlation between $\log_{10}$ total plasma TG and apo CII content in TRL protein was found (r = -0.93; $p<0.01$).

B. Apo CII Distribution Following Incubation of ^{125}I apo CII with TRL, TRL Free Plasma and HDL

I. *Effect of VLDL-free plasma on binding of apo CII to VLDL*

^{125}I apo CII was incubated in 3 sets of cellulose nitrate tubes containing (1) VLDL (2) VLDL and VLDL free plasma and (3) VLDL free plasma alone. After the incubation the tubes were ultracentrifuged and the d<1.006 fraction reisolated. As shown in Figure 4, 72% of total radioactivity was found in the d<1.006 supernate of the tube containing VLDL alone. Fifteen percent of total radioactivity was noted in the d<1.006 supernate of the tube containing VLDL and VLDL free plasma. In the tube containing VLDL free plasma, only 4% of total radioactivity was found in the d<1.006 supernate.

II. *Distribution of ^{125}I apo CII incubated with VLDL free plasma*

^{125}I apo CII was incubated with VLDL free plasma. Lipoprotein fractions were isolated by sequential gradient ultracentrifugation and the amount of radioactivity in each fraction measured. The results showed that 4.6%, 13.8%, 55.3%, 7.9%, and 18.9% of the total radioactivity was recovered in the IDL, LDL, HDL, VHDL and 'residue' fractions, respectively.

III. *Relationship of VLDL/HDL protein ratio to distribution of ^{125}I apo CII*

HDL was incubated with increasing concentrations of VLDL from a normal subject. The percent total radioactivity in the VLDL fraction increased in proportion to the VLDL/HDL

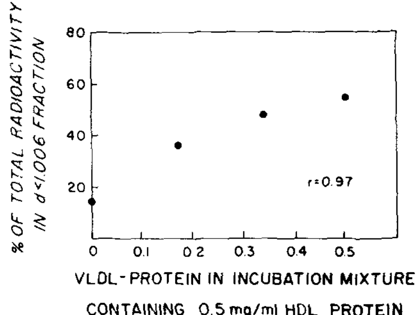

FIG. 5. ^{125}I apo CII was incubated with HDL in EDTA saline alone or with increasing amounts of VLDL from a normal subject. VLDL and HDL were separated by ultracentrifugation, and amount of radioactivity in each fraction was measured.

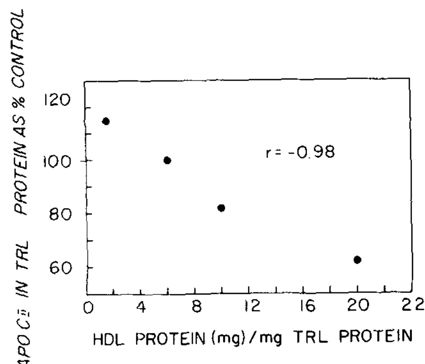

FIG. 7. Relationship between the HDL/TRL protein ratio in incubation mixture and the content of apo CII in TRL protein.

ration (r = 0.97; p<0.001)(Fig. 5).

C. In Vitro Studies on Enrichment of TRL with apo CII

I. *Apo CII distribution following incubation of hypertriglyceridemic TRL with HDL from normal subjects*

HDL isolated from a healthy normolipidemic subject was incubated with TRL from 4 patients with familial type V hyperlipoproteinemia. An increasing ratio of HDL to TRL protein was used (Fig. 6). The results (Fig. 6) in the left panel indicate that, with increasing ratio of HDL to TRL protein, the amount of apo CII in the d<1.0u6 supernate after ultracentrifugation decreased. However, as shown in the right panel, the amount of total protein in the d<1.006 supernate increased. With a progressive increase in HDL to TRL ratio, apo CII transferred from TRL to HDL whilst HDL protein transferred to TRL.

The net effect of increasing HDL concentration of the apo CII content in TRL protein is shown in Figure 7. The content of apo CII in TRL protein was inversely correlated with HDL/TRL protein ratio in the incubation mixture (r = -0.98; p<0.001).

II. *Apo CII distribution following incubation of hypertriglyceridemic TRL with TRL free plasma from normal subjects*

In this set of experiments (Fig. 8), TRL free plasma (d> 1.006) from 2 healthy normolipidemic subjects was incubated with increasing concentrations of TRL isolated from 2 patients with familial type V hyperlipoproteinemia. As shown in Figure 8, increasing concentrations of TRL to a fixed volume of normal TRL free plasma resulted in a progressive de-

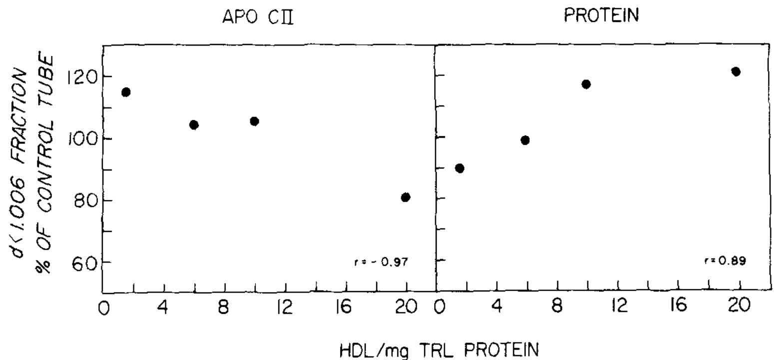

FIG. 6. TRL from four hypertriglyceridemic patients was incubated with HDL isolated from a healthy normolipemic subject. Incubation was in 0.15 M sodium chloride in 1 mM disodium EDTA, pH 7.4 at 37 C for 2 hr in cellulose nitrate tubes. TRL and HDL were separated by ultracentrifugation. Total protein and apo CII were measured in the supernate. Control tubes containing TRL were incubated in EDTA saline alone.

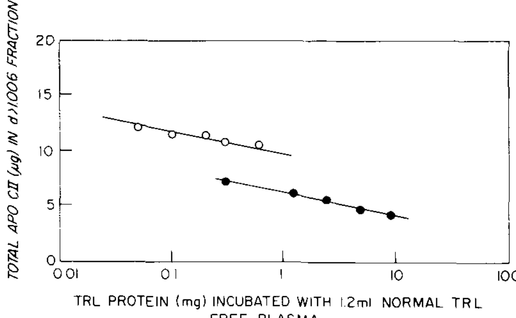

FIG. 8. 1.2 ml plasma freed of TRL was taken from two healthy normolipidemic subjects and incubated with different amounts of TRL from two patients with familial type V hyperlipoproteinemia. Incubation was done at 37 C for 2 hr in EDTA normal saline, pH 7.4. TRL were removed by ultracentrifugation. Apo CII was measured in the TRL free (d>1.006) infranate. Control tubes contained only TRL. Apo CII was measured in control infranates also. Apo CII in d>1.006 fraction shown in this figure was derived by subtracting control infranate apo CII from apo CII measured in the infranates of experimental tubes. The amount of apo CII in the d>1.006 fraction decreased linearly with the $\log_{10}$ of TRL protein in the incubation mixture.

crease in the absolute total amount of apo CII in normal TRL free plasma. The correlation is negative with ($\log_{10}$) TRL protein concentration and significant statistically ($p<0.01$ for both experiments).

III. *Addition of apo CII to TRL (Table I)*

TRL was isolated from one normal subject and a patient with familial type V hyperlipoproteinemia. The TRL fractions were added to apo CII and the mixture incubated. TRL was reisolated and dialyzed after ultracentrifugation. TRL, apo CII, and protein concentrations were measured in the d<1.006 supernate. Control TRL was incubated in vials without apo CII. As shown in Table I, a two- to threefold increase in the amount of apo CII in TRL protein was observed in the experimental tubes.

IV. *Distribution of apo CII added to plasma (Figure 9)*

Figure 9 shows the distribution of apo CII as percent of total apo CII in each lipoprotein density fraction after addition of apo CII to plasma. Most of native and added apo CII was distributed in VLDL and HDL fractions. The pattern of distribution of apo CII added to plasma was similar to the distribution of apo CII in native plasma. Thus, VLDL and HDL were enriched with apo CII.

V. *LPL activator potency of TRL from apo CII enriched plasma*

In two patients with familial types IV and V hyperlipoproteinemia, respectively, TRL was isolated from 1.0 ml plasma which had been preincubated with apo CII. TRL was isolated and its ability to activate hydrolysis of emulsified triolein by milk LPL was measured in vitro. TRL from apo CII enriched plasma showed a 1.5-fold increase in LPL potency per mg TRL protein compared to TRL from native plasma (Table II).

DISCUSSION

Total Plasma Triglycerides and Apo CII in TRL

The relative reduction of apo CII content in TRL in hypertriglyceridemics has also been noted using a different method. Using polyacrylamide gel electrophoresis to separate and quantitate TRL apolipoproteins, Kane et al. (32) found that hypertriglyceridemic patients with type IV lipoprotein patterns had increased Arginine-Rich peptide (apo E) and apo CI in VLDL and relative reductions of apo CII. Carlson and Ballantyne (33) reported that the TRL apo CII/apo CIII ratio was decreased in proportion to levels of plasma TRL TG concentration. Both groups of investigators found that larger TRL particles from normal and hypertriglyceridemic subjects had a higher content of apo CII per unit protein. However, hypertriglyceridemics had lower amounts of apo CII per unit TRL protein than normolipidemics in each

TABLE I

APO CII Content in TRL Following Incubation of APO CII with TRL[a]

Subject	Total cholesterol mg/dl	Total TG mg/dl	LPE	APO CII as % Total TRL protein	
				Control sample	Experimental sample
R.C.	130	88	Normal	10.8	21.6
G.G.	356	2850	Type V	5.9	17.7

[a]TRL (150 μg protein) obtained from one normal subject and a patient with familial type V hyperlipoproteinemia were incubated with 30 μg of apo CII. Control tubes contained only TRL. TRL was reisolated by ultracentrifugation, dialyzed, and apo CII and protein was measured in control and experimental samples.

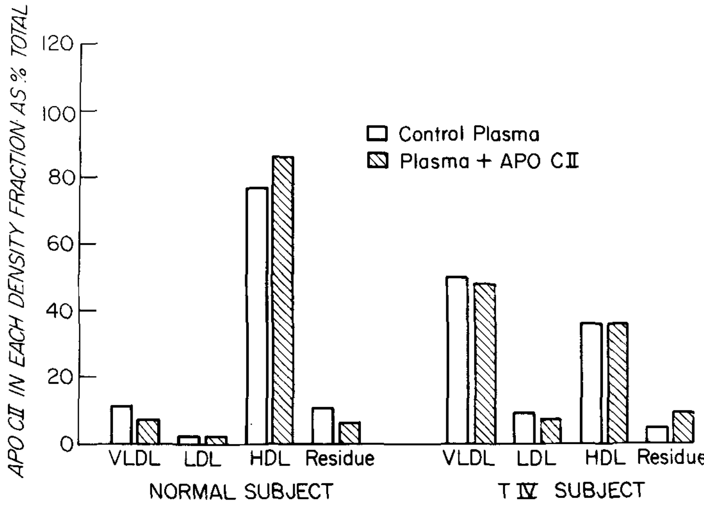

FIG. 9. One ml plasma from a normal subject and 1.0 ml from a patient with primary type IV hyperlipoproteinemia were added to 30 μg lyophilized apo CII in a vial, mixed and incubated for 2 hr. Control plasma was added to an empty vial. Sequential density ultracentrifugation was done and TRL (d<1.006), LDL (d 1.006-1.063), HDL (1.063-1.210) and 'Residue' (d>1.210) fractions isolated. Apo CII was measured in each fraction and results expressed as percent of total apo CII recovered.

TABLE II

APO CII in TRL Following Incubation of APO CII with Plasma[a]

Subject	Total plasma cholesterol mg/dl	Total plasma triglyceride mg/dl	L.P.E.	APO CII as % total TRL protein		LPL activator potency units/mg TRL protein	
				Control sample	Experimental sample	Control sample	Experimental sample
T.M.	187	308	Type IV	5.42	11.8	107	157
F.W.	259	776	Type V	6.16	9.2	98	144

[a]1.0 ml plasma from patients with primary types IV and V were incubated with 30 μg apo CII. TRL was isolated, dialyzed and apo CII, protein and activator potency on milk lipoprotein lipase (LPL) was measured.

lipoprotein size class. The average amount of apo CII in TRL protein from hypertriglyceridemics was less than that in normal subjects. A reduced apo CII/CIII ratio was also noted in the hypertriglyceridemia associated with normal human gestation (34). These observations are in contrast to what is observed when hypertriglyceridemia is produced acutely after a high carbohydrate diet in normal healthy subjects. Schonfeld et al. (35) observed that the VLDL apo CII/apo CIII ratio increased under these experimental conditions.

Several lines of evidence suggest that TRL are spherical particles with a hydrophobic TG and esterified cholesterol 'core' and an outer amphipathic 'shell' of protein, phospholipids and cholesterol (36-40). The thickness of the outer monolayer is invariant (37-39), and the amount of apo B per TRL particle is constant. The data in this study indicate that in hypertriglyceridemic subjects, the higher the total plasma TG, the lower the content of apo CII in TRL protein. Since protein is part of the outer layer of the TRL particle, the data are consistent with the concept that the amount of apo CII per mean unit surface area of TRL particles is reduced in hypertriglyceridemia. This hypothesis is based on the above assumptions regarding the lipid core structural model of TRL and will require more direct proof.

A decreased content of apo CII in VLDL protein presumably indicates a relative increase in the other VLDL apolipoproteins, including apo CIII, apo E, and apo CI. These three apolipoproteins inhibit human adipose tissue LPL (22), and apo CIII inhibits bovine milk LPL as well (21).

In this study, progressive hypertriglyceridemia was associated with concomitant reduction in LPL activating potency per unit VLDL protein or VLDL TG. This reduction could be secondary to lower apo CII content in

hypertriglyceridemic VLDL. However, there was also lower net LPL activator property per unit hypertriglyceridemic VLDL apo CII. This reduction might be accounted for by relatively increased amounts of apo CIII, apo E, and apo CI, each of which inhibits LPL. Alternately, the possibility that milk LPL used in our assay might use hypertriglyceridemic VLDL TG as a preferred substrate resulting in an apparent decrease in lipolytic potency cannot be excluded.

Breckenridge et al. (25) reported a severely hypertriglyceridemic patient with undetectable apo CII in TRL and HDL by polyacrylamide gel electrophoresis, by double immunodiffusion using apo CII antibody, and inability of plasma and TRL to activate LPL. Infusion of one unit of normal plasma dramatically lowered the total plasma TG, and apo CII was detected in the patient's TRL after the infusion. Infused normal plasma raised the patient's apo CII concentration to approximately one tenth of the normal concentration. Addition of natural apo CII or synthetic fragments containing the carboxyterminal third portion of apo CII converted this patient's TRL from an inactive substrate for LPL to an active substrate (41). The studies in this patient indicate that an apo CII deficiency was the fundamental pathophysiologic mechanism leading to hypertriglyceridemia. Can milder degrees of apo CII deficiency lead to less severe hypertriglyceridemia? Our studies indicate that progressive hypertriglyceridemia is not only associated with a progressive decrease in the amount of apo CII in TRL, but apo CII is also inhibited in its ability to activate LPL. As indicated earlier, apo CIII (and other apolipoproteins), which is a potent inhibitor of LPL, is increased relative to apo CII in TRL of hypertriglyceridemic patients. Further, Huttunen et al. (42) found that as total plasma TG rise, there is a progressive fall in extra hepatic LPL. Thus, it is conceivable that the combination of a subnormal apo CII content in TRL, hypernormal amounts of LPL inhibitory peptide(s) levels, and low levels of extra hepatic LPL in aggregate may be important pathophysiologic mechanisms leading to hypertriglyceridemia in these patients.

Fielding and Fielding (43) showed that LPL activator property of rat lymph chylomicrons (which did not contain apo CII) could be restored to maximal levels by the addition of a fraction of apo CII present in plasma chylomicrons. Their work and that of Breckenridge et al. (25) would thus appear to suggest that a deficiency of apo CII *per se* in TRL may have to be severe to result in hypertriglyceridemia. Thus, other mechanisms as postulated above may be present in the more common forms of hypertriglyceridemia.

Studies on the Distribution of Apo CII between Lipoproteins and Enrichment of TRL with Apo CII

In one set of experiments, the distribution of apo CII in plasma lipoprotein fractions was studied using ^{125}I labelled apo CII. In an experiment, ^{125}I labelled apo CII was incubated with lipoproteins. VLDL from a normal subject bound ^{125}I labelled apo CII (Fig. 4). HDL competed with VLDL for ^{125}I apo CII binding. These observations support the view that the ratio of HDL/TRL governs the distribution of apo CII between these lipoprotein classes.

In two studies, transfer of HDL apo CII or apo CII in TRL free plasma to TRL was studied (Figs. 6-8). When TRL from hypertriglyceridemic patients was incubated with normal HDL, the amount of apo CII found in TRL after incubation was related to the TRL/HDL ratio. The higher the TRL/HDL ratio, the greater was the TRL apo CII content (Fig. 7). That an increase in the TRL/HDL ratio was associated with a transfer of apo CII from HDL to TRL was further confirmed in experiments in which increasing amounts of TRL were incubated with normal TRL-free plasma (Fig. 8). Progressive transfer of apo CII from TRL-free plasma to TRL was observed. The amount of apo CII in TRL free plasma was inversely related to the $\log_{10}$ of TRL protein used in the incubation mixture. The greater the TRL concentration, the lower the amount of apo CII in the d>1.006 plasma fraction. Thus, although TRL can be enriched with apo CII from TRL free plasma, high ($\log_{10}$) concentrations of TRL relative to HDL are required to achieve this enrichment. In other words, the availability to TRL of apo CII in TRL free plasma was limited by the nature of the mass relationship of TRL and HDL. This concept is supported by our earlier observations that, in human plasma samples with varying total plasma TG, the amount of apo CII in TRL free plasma is inversely related to the $\log_{10}$ of total plasma TG (26).

In addition to transfer of apo CII between HDL and TRL, we also observed the transfer of non-apo CII protein between HDL and TRL when different ratios of these lipoproteins were incubated. The nature of the apolipoproteins was not determined and would be the subject of future studies. Transfers of apo E from HDL to TRL in the presence of lecithin cholesteryl acyl transferase (LCAT) have been observed by Glomset (44). Our studies (Fig. 6) on transfer

of apolipoproteins were conducted with isolated lipoproteins in an EDTA saline medium, and it is unlikely that LCAT was present. The results suggest that besides LCAT, the mass ratio of HDL to TRL also influences apolipoprotein transfer.

Enrichment of TRL by addition of apo CII to plasma was also possible (Fig. 9 and Table II). The data indicate that plasma TRL and HDL possess a reserve binding capacity for apo CII which was not saturated under the experimental conditions described. The possibility that part of the increased amount of apo CII found mainly in the TRL and HDL ultracentrifugal density fractions was not in these lipoproteins cannot be entirely excluded. Glangeaud et al. (45) observed that, after in vitro rat VLDL lipolysis with LPL in a system without HDL, a small fraction of C apolipoproteins was found in the HDL density range.

Taken together, our experimental observations are consistant with the dynamic role of apo CII in vivo. Nascent VLDL secreted by the liver or chylomicrons by the intestine do not contain apo CII. The exact step of incorporation of apo CII into HDL during its synthetic assembly is not known with certainty, and it is possible that HDL may acquire the peptide after its secretion. Thus, our experiments would be consistent with the concept that apo CII distributes in circulating plasma according to the amounts of TRL and HDL in plasma. The greater the load of TG (e.g., after a fatty meal or during increased VLDL secretion), the greater is the relative amount of total plasma apo CII in TRL. Upon lipolysis of TRL particles, the decreased ratio of TRL to HDL would result in more apo CII in HDL. Newly secreted apo CII would bind to HDL and TRL, since these lipoproteins appear to possess a reserve apo CII binding capacity as indicated by the data.

In this study, in vitro enrichment of TRL with apo CII was associated with an increased potency to activate milk LPL (Table II). The possiblity that such enrichment may be associated with accelerated TRL catabolic rate in vivo is an attractive one. However, caution is necessary in the extrapolation of this data to the in vivo situation. There are obvious differences between the in vitro TRL-LPL interaction and the interaction of TRL with membrane bound LPL in vivo. For example, the exact number of apo CII molecules on a TRL particle interacting with LPL may be limited by the exact site and molecular distribution of tissue LPL (46) and also by the stoichiometric relationship between apo CII and LPL (47-48). In the very rare patient with an absolute deficiency of apo CII, Breckenridge et al. (25) were successful in lowering total plasma TG. In the hypertriglyceridemic subjects described in this study, other experimental approaches maybe necessary, e.g., infusion of apo CII into the circulation. This is because, as pointed out earlier, a combination of apo CII deficiency, excessive LPL inhibitors in TRL and possibly a fall in the amount of extrahepatic LPL may be pathogenetic mechanisms in these patients.

It will be important to determine experimentally whether in vivo enrichment of TRL with apo CII leads to a lowering of total plasma TG in the far more common patient with hypertriglyceridemia.

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Interaction of Plasma High Density Lipoprotein HDL_{2b} (d 1.063-1.100 g/ml) with Single-Bilayer Liposomes of Dimyristoylphosphatidylcholine

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ABSTRACT

Incubation of a major subfraction, HDL_{2b} (d 1.063-1.100 g/ml), of human plasma high density lipoproteins, HDL (d 1.063-1.21 g/ml), with single-bilayer liposomes of dimyristoylphosphatidylcholine (DMPC) resulted in uptake of DMPC by the HDL_{2b} and dissociation of lipid-free apolipoprotein A-I (apoA-I). In the presence of excess DMPC, the dissociated apoA-I was also incorporated with DMPC into discoidal complexes. Preliminary studies with model apoA-I-DMPC complexes indicated that they also can interact with native HDL_{2b} with the resultant transfer of their DMPC to HDL_{2b} and the concomitant release of their apoA-I. After interaction of HDL_{2b} with DMPC liposomes, the DMPC-enriched HDL_{2b} product showed a lower hydrated density and a larger particle size than the control HDL_{2b}. The molecular properties of the lipoprotein product suggest that stabilization of the apoA-I-depleted HDL_{2b} probably occurred via substitution of DMPC for the apoA-I at the HDL_{2b} surface rather than by fusion of the apoA-I-depleted HDL_{2b}. The above interactions of HDL_{2b} with single-bilayer liposomes and discoidal complexes indicate pathways of phospholipid transfer relevant to the possible role of HDL in the metabolism of lipoprotein surface components in vivo.

INTRODUCTION

The interaction between apolipoproteins and phospholipids contributes significantly to the structure of human plasma lipoproteins (1). Recent models of lipoprotein structure consist essentially of a monolayer of phospholipids and apolipoproteins at the lipoprotein surface, surrounding a core of apolar lipids, cholesteryl esters and triglycerides (2,3). In the absence of apolar lipids, interaction between phospholipids and apolipoproteins can result in structures which include discoidal (4) as well as vesicular (5) complexes.

Many studies on the interaction between plasma apolipoproteins and phospholipids have used dimyristoylphosphatidylcholine (DMPC), because its physical-chemical properties are well defined, and it readily interacts with the different apolipoproteins (6-8). Multilamellar liposomes of DMPC have also been reported to interact with intact high density lipoproteins (HDL) to produce discoidal complexes enriched in apolipoprotein A-I (apoA-I) and residual lipoproteins of increased size and decreased density (9). Tall et al. (10) have proposed that apoA-I partitions from HDL to DMPC liposomes to form thermodynamically more stable discoidal complexes of DMPC and apoA-I, and that the apoA-I-depleted HDL then fuse to form stable larger lipoprotein species. Our preliminary analytic ultracentrifugal studies indicated that the products formed following interaction of DMPC liposomes with HDL were dependent on the stoichiometry of the reactants and the conditions of incubation (11). In this report, we describe our studies on the changes in physical-chemical properties of a major subfraction (d 1.063-1.100 g/ml) of human plasma HDL, HDL_{2b} [approximate molecular weight, 420,000; hydrated density, 1.09 g/ml; average percent protein, 37% (12)], when it interacts with single-bilayer liposomes of DMPC.

EXPERIMENTAL PROCEDURES

Preparation of HDL_{2b}

Blood was drawn from healthy female donors (age 21-56) into heparinized evacuated tubes, and the plasma was separated by centrifugation. Sodium azide (30 mM), ethylmercurisalicylic acid (0.124mM) and EDTA (0.27mM) were added to the plasma. All subsequent solutions and incubation mixtures contained the above concentrations of these reagents. HDL were isolated by sequential ultracentrifugation using modified procedures of Lindgren et al. (13). To isolate HDL_{2b} (12), the HDL preparation was adjusted by dialysis to a background salt (NaBr-NaCl) of d 1.110 g/ml, and 2 ml of this solution were layered between 2 ml of NaBr-NaCl solutions of d 1.105 and 1.115 g/ml. Following preparative ultracentrifugation (50.3 Ti rotor, Beckman Instruments, Inc., Palo Alto, CA; 50,000 rpm, 48 hr, 17 C), the top 1 ml containing the HDL_{2b} was removed and dialyzed to 5 mM NH_4HCO_3, pH 8.6.

Preparation of DMPC Liposomes

Dimyristoylphosphatidylcholine (98% purity) was obtained from Sigma Chemical, St. Louis, MO; L-a-dimyristoyl-1-^{14}C phosphatidylcholine was obtained from Applied Science Laboratories, Inc., State College, PA. DMPC liposomes were prepared by sonication (maximum energy level of 50 W for 20-30 min) in 5 mM NH_4HCO_3 using a Branson Sonifer Cell Disruptor (Model W 185) equipped with a Ti microprobe. The temperature of sonication was maintained at 24 C, and the sonication chamber was continuously flushed with nitrogen. All liposome preparations were ultracentrifuged (16,000 x g, 15 min) to remove undispersed DMPC and Ti metal. Chromatography of the sonicate on Sepharose 6B and subsequent electron microscopy of the eluted fractions showed that 80-85% of the DMPC was in the form of single-bilayer liposomes. Thin layer chromatography of sonicated DMPC gave a single spot at the position of unsonicated DMPC.

Incubation Procedures

Most samples were incubated at 37 C for 1 and/or 4.5 hr, under nitrogen, in 5 mM NH_4HCO_3 in a shaking water bath. After incubation these samples were maintained at 24-26 C during all analytic and preparative ultracentrifugal procedures. This was done to avoid possible structural changes which might occur when DMPC-containing products are subjected to one or more cooling-heating cycles through the transition temperature of DMPC (23 C). As a result, all such samples were exposed to additional incubation at 24-26 C during the various ultracentrifugal procedures. While analytic ultracentrifugal procedures required an overall processing time of ca. 2 hr, preparative ultracentrifugal procedures usually required at least 48 hr. In most experiments, samples were incubated for no longer than 4.5 hr at 37 C, because maximal changes in analytic ultracentrifugal parameters (flotation rate of the major component and total pattern area) were observed under these conditions.

Concentrations of HDL_{2b} used in the incubation mixtures ranged from 1.5-2.3 mg apo HDL_{2b}/ml; DMPC liposome concentrations used were 2.4-7.0 mg/ml. Weight ratios used in the text represent weight of DMPC: weight of apoHDL_{2b}.

Analytic Ultracentrifugation

Samples for analytic ultracentrifugation were adjusted to appropriate background solution density by addition of solid NaBr and NaCl. Analytic ultracentrifugal analysis was performed according to Lindgren et al. (13). Computer representations of the schlieren patterns were prepared as described elsewhere (14). The overall methodologic reproducibility of duplicate samples was within 5-10% for the area measurements and 0-5% for the flotation rate measurements.

Gradient Gel Electrophoresis

Gradient gel electrophoresis was performed according to Anderson et al. (12) on a Pharmacia Electrophoresis Apparatus GE-4 using gradient gels PAA 4/30, and calibrated with the high molecular weight Electrophoresis Calibration Kit, Pharmacia Fine Chemicals, Uppsala, Sweden. Sizes of electrophoresed particles were estimated from a calibration curve of migration distance of the reference proteins vs. their Stokes' diameter.

Electron Microscopy

Incubated mixtures and ultracentrifugal fractions, in 5 mM NH_4HCO_3, were negatively stained (15) and examined with a JEM 100C electron microscope, JEOL, Tokyo, Japan. Particle size determinations were usually performed on at least 100 particles within representative fields.

Chemical and Other Analyses

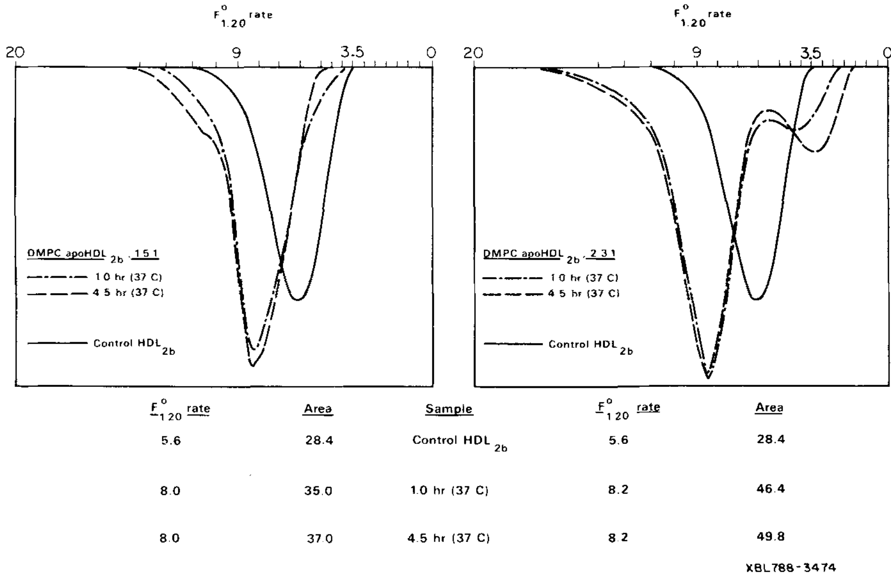
Protein and phosphorus content of incubation mixtures and ultracentrifugal fractions were analyzed according to the methods of Lowry et al. (16) and Bartlett (17), respectively.

Polyacrylamide gel electrophoresis was performed according to the method of Kane (18).

RESULTS

Analytic Ultracentrifugation

Ultracentrifugal patterns of control HDL_{2b} showed a single major peak with flotation rate of ca. $F^{\circ}_{1.20}$ 5.6. Incubation of control HDL_{2b} for periods up to 18 hr at 37 C did not significantly alter this pattern either in area or flotation rate. In these ultracentrifugal studies, the interaction between HDL_{2b} and DMPC liposomes was evaluated at two different weight ratios of DMPC:apoHDL_{2b}, 1.5:1 and 2.3:1. The patterns (Fig. 1) of an incubation mixture at the lower weight ratio showed increases in both area (1 hr, 23%; 4.5 hr, 30%) and flotation rate (1 and 4.5 hr, 43%) of the major peak. The above data indicated that the major portion of the observed changes occurred within 1 hr of incubation at 37 C (plus, of course, the analysis time). In these patterns little or no material was detected in the flotation rate range, $F^{\circ}_{1.20}$ 30-136, where the schlieren peak of control



$F^o_{1.20}$ rate	Area	Sample	$F^o_{1.20}$ rate	Area
5.6	28.4	Control HDL_{2b}	5.6	28.4
8.0	35.0	1.0 hr (37 C)	8.2	46.4
8.0	37.0	4.5 hr (37 C)	8.2	49.8

FIG. 1. Analytic ultracentrifugal patterns of incubated mixtures of HDL_{2b} and DMPC liposomes. $F^o_{1.20}$ is the HDL flotation rate, expressed in svedbergs (10^{-13} cm/sec/dyne/g), fully corrected for concentration and to standard conditions of 0.195m NaCl and 2.762m NaBr at 26 C and d 1.200 g/ml. Schlieren pattern areas are given in corrected relative refractive index increment units.

preparations of DMPC liposomes was clearly observed at the concentrations used. Hence, the above increases in area were consistent with a shift of DMPC into the flotation rate interval of the peak ($F^o_{1.20}$ 4.8-20), which now includes the major part of the HDL_{2b}.

For the mixture at weight ratio 2.3:1, the increases in total pattern area (Fig. 1) were considerably greater (1 hr, 63%; 4.5 hr, 75%) than for similarly incubated mixtures at the lower weight ratio. In addition to the major peak, the total pattern area now included a new slower floating component which was detected within 1 hr of incubation. The flotation rate of the major peak increased by 46% in both the 1 hr and 4.5 hr incubation samples. Interestingly, the flotation rate of the new peak decreased from $F^o_{1.20}$ 4.0 (1 hr) to $F^o_{1.20}$ 3.1 (4.5 hr). The area of the major peak (within the $F^o_{1.20}$ 4.8-20 interval) was almost identical in the 1 hr and 4.5 hr samples, and the larger total pattern area observed in the 4.5 hr sample resulted from a further increase in area of the new slow component. Two separate analytic ultracentrifugal studies on incubated mixtures of DMPC liposomes plus HDL_{2b}, isolated from plasma of two additional subjects, demonstrated similar increases in area and flotation rate when incubated under the conditions described above.

Based on the data for mixtures incubated 4.5 hr at 37 C, the values of total pattern area did not increase linearly with DMPC concentration in the incubation medium. Per unit DMPC concentration, the increase in area at the higher weight ratio was considerably larger (65%) than at the lower ratio.

Ultracentrifugal analysis of the lower weight ratio mixture incubated at 24 C showed increases in area comparable to those at 37 C (1 hr, 24%; 4.5 hr, 32%), but smaller increases in flotation rate of the major peak (1 hr, 20%, 4.5 hr, 28%). At 24 C, the new slower floating component appeared in the higher weight ratio mixture only after 4.5 hr incubation. At this temperature, the total pattern area increased by 40% (1 hr) and 53% (4.5 hr); and the flotation rate of the major peak increased by 26% (1 hr) and 45% (4.5 hr).

Preparative Ultracentrifugation

The above changes suggested the possibility both of uptake of DMPC by HDL_{2b} and redistribution of HDL_{2b} components with formation of new particulate species. Since redistribution of apoA-I, from HDL to discoidal complexes had already been noted in such incubation systems, we were particularly interested in correlating our analytic ultracentrifugal observations with information on the distribution of apoA-I during incubation of the two different weight ratio mixtures.

To evaluate if unbound apoA-I was present

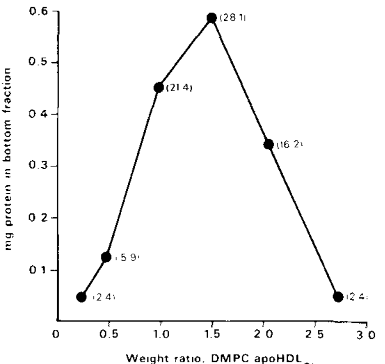


FIG. 2. Protein recovered in bottom 2 ml fraction following ultracentrifugation (50.3 Ti rotor, 50,000 rpm, 48 hr, 26 C) of mixtures of HDL_{2b} and DMPC liposomes incubated for 4.5 hr at 37 C. Density of the incubation mixtures was adjusted to d 1.21 g/ml by addition of solid NaBr-NaCl prior to preparative ultracentrifugation. Values in parentheses represent percent of initial $apoHDL_{2b}$ in incubation mixture.

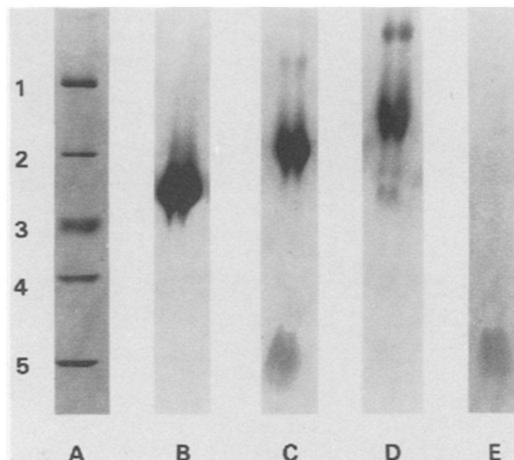


FIG. 3. Gradient gel electrophoretograms of: (a) reference protein mixture, (1) thyroglobulin, (2) apoferritin, (3) catalase, (4) lactate dehydrogenase, (5) human serum albumin; (b) control HDL_{2b}; (c) DMPC and HDL_{2b} (1.4:1) incubated for 4.5 hr at 37 C; (d) DMPC and HDL_{2b} (3.1:1) incubated for 4.5 hr at 37 C; (e) apoA-I.

in any of the incubation mixtures during its redistribution from HDL_{2b} to discoidal or other complexes, mixtures of HDL_{2b} with DMPC liposomes were incubated and ultracentrifuged at d 1.21 g/ml. Under these conditions, lipid-free apolipoprotein would sediment away from both floating lipoprotein and DMPC-apolipoprotein interaction products. As shown in Figure 2, there was a progressive increase in apolipoprotein in the ultracentrifugal bottom 2 ml fraction as the weight ratio of the incubation mixture was increased up to 1.5:1. At this weight ratio, a maximum of 28% of the total apo HDL_{2b} was dissociated. No apolipoprotein was detected in fractions intermediate to the top and bottom fractions. At weight ratios above 1.5:1, the amount of apolipoprotein in the bottom fraction decreased and was negligible at a weight ratio of 2.8:1. Again, no apolipoprotein was detected in the intermediate fractions. Negligible phospholipid phosphorus was detected in the ultracentrifugal bottom fractions. Polyacrylamide gel electrophoresis of the protein in the bottom fractions showed only one band whose position corresponded to that of apoA-I. Hence, at the maximal dissociation (28%) of apolipoprotein noted above, we estimate a release on the average of ca. 1.5 apoA-I molecules per HDL_{2b} molecule.

The increases in area and in flotation rate of the major HDL_{2b} peak noted in the analytic ultracentrifugal patterns of mixtures in the weight ratio range of 0.3:1 – 1.5:1 are compatible with an uptake of DMPC and a loss of some apoA-I by the HDL_{2b}. In mixtures of higher weight ratio, the released apoA-I is apparently incorporated into complexes of $d<1.20$ g/ml which probably appear as the slower floating component in the analytic ultracentrifugal patterns seen in Figure 1. Analytic ultracentrifugal patterns of model discoidal complexes of DMPC and apoA-I showed their flotation properties to be comparable to those of the slower floating component (Unpublished observations).

Gradient Gel Electrophoresis

Incubation mixtures were also analyzed by gradient gel electrophoresis to further evaluate apoA-I dissociation as well as the number and approximate size of the major lipoprotein interaction products. There was no change in migration properties of control HDL_{2b} after incubation (4.5 hr, 37 C). The estimated average diameter of the HDL_{2b} before and after incubation was ca. 106 Å. After incubation a mixture at the lower weight ratio (1.4:1) showed (Fig. 3) one major band migrating to a position corresponding to particles with average diameter of ca. 115 Å. This mixture also showed the presence of some minor bands at positions of particles with diameters of 66 Å and 135 Å. The former band was located at the migration distance of lipid-free apoA-I (probably in multimeric form). When a mixture of higher weight ratio (3.1:1) was electrophoresed, the migration of the major band corresponded to still larger particles with an average diameter of ca. 123 Å. At the higher

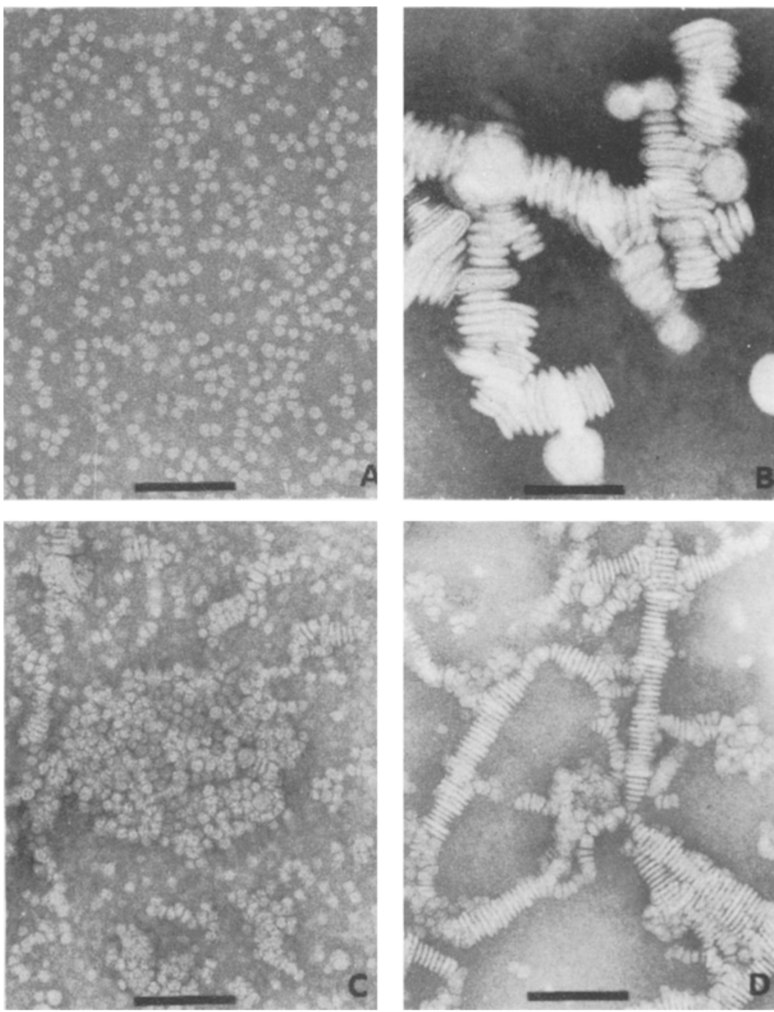


FIG. 4. Electron micrographs of: (a) control HDL_{2b}; (b) control DMPC liposomes; (c) DMPC and HDL_{2b} (1.4:1) incubated for 4.5 hr at 37 C; (d) DMPC and HDL_{2b} (3.1:1) incubated for 4.5 hr at 37 C. Bar markers represent 1000 Å.

weight ratio, no band was observed at the position previously noted for lipid-free ApoA-I. Additional minor bands, however, were observed at positions of particles with diameters of 105 Å and 144 Å. These results confirmed the preparative ultracentrifugal observation of apolipoprotein dissociation in mixtures at the lower weight ratio and indicated significant changes in particle size of the HDL_{2b}.

In separate electrophoretic experiments, the distribution of radioactive DMPC among the major bands was also evaluated in a mixture at the lower weight ratio (1.3:1, 4.5 hr, 37 C). Although quantitative recovery of the total radioactivity applied to the gel was not obtained (average recovery, 70%), there was significant radioactivity (71% of recovered activity) associated with the shifted HDL_{2b} band, suggesting uptake of DMPC by HDL_{2b} during apoA-I dissociation.

Electon Microscopy

The changes in HDL_{2b} particle size, as indicated by gradient gel electrophoresis, and the properties of the apoA-I-DMPC complexes at the different weight ratios were also investigated by electron microscopy (Fig. 4). Control HDL_{2b} were spherical particles of 105 ± 8 Å average diameter and showed no change in size or shape following incubation. DMPC liposomes were vesicular structures (400 ± 60 Å diameter) and frequently formed stacks when examined

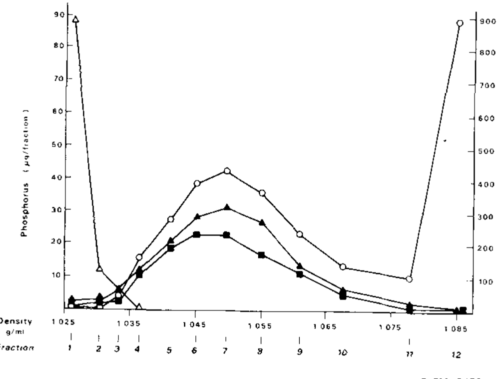


FIG. 5. Protein and phosphorus distributions following density gradient ultracentrifugation of the incubated mixture (1.4:1, DMPC:HDL_{2b}) described in Figures 3 and 4. Sucrose was added to the sample to give a background density of 1.050 g/ml, and 2 ml were layered between 2 ml of d 1.045 and 2 ml of d 1.055 g/ml sucrose solutions. Following ultracentrifugation (50.3 Ti rotor, 50,000 rpm, 48 hr, 26 C), 0.5 ml aliquots (fractions 1-12) were pipetted down the tube. Concentrations of DMPC phosphorus (▲ - ▲), HDL_{2b} phosphorus (■ - ■), and HDL_{2b} protein (○ - ○) were determined for each fraction. Distribution of phosphorus in control DMPC liposomes following ultracentrifugation is also shown (△ - △).

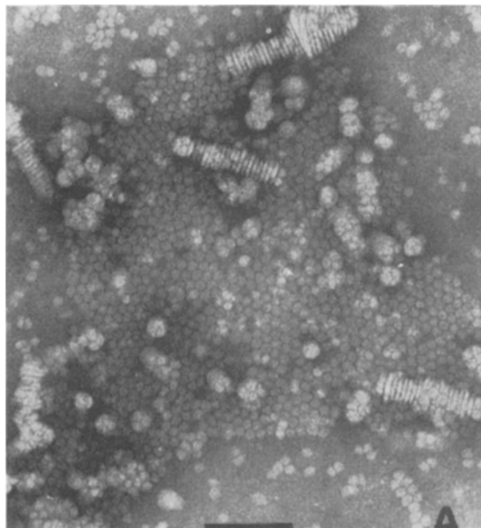
under negative staining. Micrographs of an incubated lower weight ratio mixture (1.4:1, 4.5 hr, 37 C) showed quite uniform spherical particles with an average diameter of 113 ± 10 Å. These particles tended to pack in hexagonal arrays. Some discoidal (186 ± 23 Å x 40 ± 3Å) but few liposomal particles were observed. Micrographs of an incubated mixture of higher weight ratio (3.1:1, 4.5 hr, 37 C) revealed two major populations of particles: spherical particles with an average diameter of 124 ± 12 Å and discoidal particles (277 ± 31 Å x 40 ± 3 Å) which frequently formed rouleaux. As in the mixture with the lower weight ratio, few DMPC liposomes were detected. The average dimensions of the predominant spherical particles in the various incubation mixtures obtained by electron microscopy were in close agreement with those estimated by gradient gel electrophoresis. The results from electron microscopy were also consistent with the ultracentrifugal and electrophoretic data in showing a marked increase in number of apoA-I-DMPC discoidal complexes in mixtures at the higher weight ratio.

Density Gradient Ultracentrifugation

Our analytic ultracentrifugal and gradient gel electrophoretic data suggested that the interaction process may involve uptake of DMPC by HDL_{2b} prior to or during release of some of its apoA-I. Such a substitution reaction may be a crucial initial step in the redistribution of apoA-I and, hence, we attempted to establish more definitively its occurrence in the incubation studies described above. Density gradient ultracentrifugation was used to separate the major lipoprotein reaction product from any of the other possible DMPC-containing particles, such as liposomes and/or discoidal complexes in the incubation mixture.

From a separate analytic ultracentrifugal study, the apparent hydrated density of the major lipoprotein product in a mixture at the lower ratio was estimated to be ca. 1.05 g/ml. Based on this information, control samples and an incubation mixture (1.4:1, 4.5 hr, 37 C) were adjusted with sucrose to d 1.050 g/ml and separately layered between two sucrose solutions of d 1.045 and 1.055 g/ml prior to ultracentrifugation. In the absence of HDL_{2b}, DMPC liposomes floated into the top 1 ml (d 1.026 g/ml) of the gradient (Fig. 5). Under the same conditions, HDL_{2b} sedimented into the bottom 2 ml (fractions 9-12 in Fig. 5). After ultracentrifugation, the incubated mixture of HDL_{2b} plus DMPC liposomes showed two protein peaks, one centered at d 1.050 g/ml and the other at d $\geqslant$ 1.085 g/ml (bottom 0.5 ml). Single peaks were obtained for DMPC and HDL_{2b} phospholipid phosphorus, and both showed the same configuration as the protein peak centered at d 1.050 g/ml. The protein in the bottom fraction was essentially lipid-free and, on polyacrylamide gel electrophoresis, gave a single band which corresponded to apoA-I. This protein represented ca. 30% of the total HDL_{2b} protein in the incubation mixture, indicating a dissociation, on the average, of ca. 1.6 apoA-I molecules per HDL_{2b} molecule.

To evaluate the presence and approximate extent of contamination of this product peak by other DMPC-containing particles, we examined the peak fractions by electron microscopy. Electron micrographs of the peak fractions from two separate density gradient studies (mixture A:1.4:1; mixture B:1.2:1) are presented in Figure 6. Figure 6A shows a representative field in which ca. 92% of the particles were highly uniform spheres (diameter 115 ± 6 Å) frequently packed in hexagonal array. Approximately 8% of the total particles were discoidal complexes with dimensions of ca. 253 Å x 40 Å. Figure 6B is representative of fields taken of a peak fraction from incubation mixture B and shows almost exclusively the presence of uniform spherical particles (diameter 117 ± 6 Å) also frequently packed in hexagonal array. Gradient gel electrophoresis of the peak fraction in Figure 6A showed a major



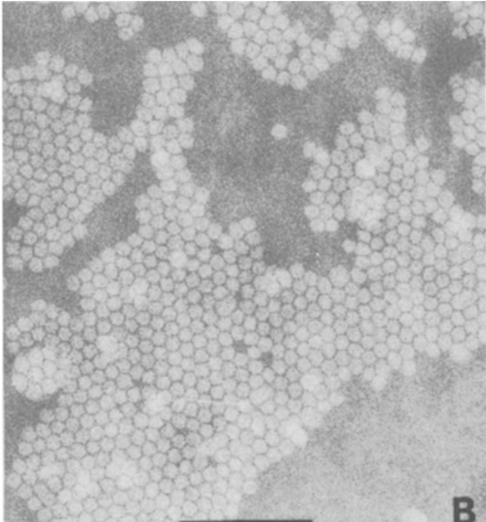


FIG. 6. Electron micrographs of the peak fraction (fraction 7, see Figure 5) isolated by density gradient ultracentrifugation from two separate incubation mixtures: (a) DMPC and HDL_{2b} (1.4:1) incubated for 4.5 hr at 37 C and (b) DMPC and HDL_{2b} (1.2:1) incubated for 4.5 hr at 37 C. Bar markers represent 1000 Å.

band (average particle size 113 Å) in the same size range as observed by electron microscopy, and minor bands at positions of particles of ca. 68 and 136 Å in diameter.

DISCUSSION

Discoidal complex formation during incubation of DMPC suspensions with intact total HDL was first described by Tall and Small (9). They also noted an increase in particle size and decrease in hydrated density of the HDL. More recently, Tall et al. (10) have reported studies on the interaction of DMPC multilamellar liposomes with HDL_3 and HDL_2 and have proposed that the discoidal complexes are formed upon transfer of apoA-I from HDL to DMPC liposomes, and that the larger lipoprotein products result from fusion of the apoA-I-depleted HDL.

Under the conditions of our experiments, we found that dissociation of apoA-I was closely linked with uptake of DMPC by the HDL_{2b}. At low levels of DMPC, the predominant interaction appeared to be uptake of DMPC by HDL_{2b} with destabilization and/or release of apoA-I. At higher levels of DMPC, DMPC, in excess of that taken up by the HDL_{2b}, appeared to interact readily with the released apoA-I to form discoidal complexes. Since some discoidal complexes were observed by electron microscopy even in mixtures at the lower weight ratio, some competition for available DMPC between HDL_{2b} and released apoA-I probably occurred. Our recent studies (19) indicate that intact HDL_{2b} can also take up DMPC from model discoidal complexes of DMPC and apoA-I. Such uptake results in an increase in HDL_{2b} particle size; reduction in number or disappearance of the discoidal complexes, depending on the relative proportions of HDL_{2b} and complexes in the incubation mixture; and the appearance of lipid-free apoA-I. The amount of lipid-free apoA-I is markedly greater than that released during interaction of HDL_{2b} with liposome preparations which contain the same amount of DMPC as the discoidal complex preparation. These observations raise the possibility that some of the lipid-free apoA-I detected in our lower weight ratio mixtures may be derived from discoidal complexes which have been depleted of their DMPC.

Tall et al. (10) noted large increases (65-76%) in apparent diameter for HDL_3 (control diameter 91 Å) incubated for 12-18 hr, 26 C, at a weight ratio of 1.5:1 (DMPC:apoHDL_3). No data on changes in particle size for HDL_2 were reported by these workers. In our studies the increase in apparent particle diameter of the HDL_{2b} (control diameter ca. 106 Å) following incubation for 4.5 hr at 37 C was 7% (at 1.4:1) and 15% (at 3.1:1). These changes were substantially less than those described for HDL_3 by Tall et al. (10) even though apoA-I dissociation from HDL_{2b} was clearly demonstrable. Hence, it is possible that, during short term incubation of HDL_{2b} with DMPC liposomes, the surface of this lipoprotein may be stabilized

without fusion by substitutaion of DMPC for apoA-I. How such substitution may lead to an increase in HDL_{2b} particle size is not clear, but it is possible that a relaxation or expansion of the lipoprotein structure may occur in response to loss of surface apolipoprotein.

Estimates of the molecular weights of the control HDL_{2b} and the major lipoprotein product observed in a lower weight ratio incubation mixture (1.5:1, 4.5 hr, 37 C) were calculated from the Svedberg equation using constants previously reported (12). Values of hydrated density and particle diameter (assuming spherical shape) used for the above estimates were: control HDL_{2b}, 1.09 g/ml, 106 Å; product, 1.05 g/ml, 115 Å. The molecular weights obtained were ca. 433,000 and 475,000 daltons, respectively. The estimated molecular weight for the product was substantially lower than would be expected for a dimer fusion product (ca. 764,000, assuming loss of 1 apoA-I per HDL_{2b}; 708,000 with loss of 2 apoA-I per HDL_{2b}). The above estimates must be considered highly approximate, but do suggest that, under the conditions of our experiments, fusion was not a major interaction process.

Since discoidal complexes have been proposed as possible precursor forms of HDL (20), the observation of transfer of DMPC from DMPC-apoA-I complexes to HDL_{2b} may have relevance to our understanding of the origin of the HDL distribution. In addition, if comparable phospholipid transfer occurs between HDL species and discoidal complexes containing physiologic phospholipids, then our observations would suggest a mechanism for incorporation by HDL of surface components generated during degradation of chylomicrons or very low density lipoproteins.

ACKNOWLEDGMENTS

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Plasma, Apolipoprotein A-I and A-II Levels in Hyperlipidemia

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ABSTRACT

Some of the component moieties of high denisty lipoproteins (HDL) were analyzed in normal subjects and in patients with hyperlipidemia. Apoproteins A-I and A-II were quantified by radioimmunoassay, HDL cholesterol and triglycerides were assessed on heparin-$MnCl_2$ supernates of fasting plasmas. We found that HDL is enriched in triglycerides in all forms of hyperlipidemia, while the proportion of ApoA-II is unaltered and the proportion of ApoA-I is decreased. Thus, the composition of HDL is altered in hypertriglyceridemia. The molecular associations of ApoA-I and ApoA-II in plasma were also examined by assaying the apoprotein contents of plasma fractions prepared by ultracentrifugation and by gel filtration column chromatography. The ApoA-I contents of d<1.063 fraction increased in hyperlipidemia from <0.5% to ~2%, but the ApoA-I contents of the d>1.21 fraction remained at <12% of total in plasmas with triglyceride levels <1500 mg/dl. d>1.21 ApoA-I rose to 23% in one plasma with a triglyceride level of >1700 mg/dl. On column chromatography, ApoA-I eluted with the lipoproteins and also in a fraction whose molecular weight (MW) appeared to be ~50,000 daltons. The proportion of plasma ApoA-I which eluted in the 50,000 MW peak was positively correlated with plasma triglyceride levels, but at triglyceride levels of <1500 mg/dl, <20% of ApoA-I was in the 50,000 MW peak. Between levels of ~2000 and 12,000 mg/dl, the percentage "50,000 M.W. ApoA-I" was 20-25%. The ApoA-II contents of d<1.063 fractions were also increased in hyperlipidemia, but >95% of ApoA-II was found in the HDL fractions in both normal and hyperlipidemic plasma both by column chromatography and ultracentrifugation. Thus, the molecular association of ApoA-I appears to be altered in hyperlipidemia.

INTRODUCTION

The high density lipoproteins (HDL) have received attention in recent years because of the strong inverse relationship between levels of HDL cholesterol and clinically manifest coronary atheroscerosis (angina pectoris, myocardial infarction, and sudden death) in free-living populations (1-3). Selected populations whose coronary risk may be lower than average also have high levels of HDL cholesterol; among these are subjects with familial hyperalphalipoproteinemia (defined as HDL cholesterol in the top 5th percentile) (4,5), women (6), and joggers (7). While measurements of HDL cholesterol may be useful as indices of HDL levels, no information is obtained about other HDL lipids or about the levels of HDL apoproteins, which together constitute ca. 70% by weight of HDL (8). Yet it is clear that such information would be useful. In recent years, immunoassays have been developed for the major apoproteins of HDL, ApoA-I (9-12) and ApoA-II (13-15), which make up 65% and 25% of the HDL apoprotein, respectively. These assays have yielded interesting data on sex differences in the ApoA-I and ApoA-II contents of HDL isolated from plasma (14). Measurements of these apoproteins have also allowed the detection of alterations of HDL composition in patients with renal disease (16), in survivors of myocardial infarction (17), and in vegetarian populations (18). Thus, alterations of the levels of HDL cholesterol are frequently accompanied by alteractions in the relative proportions of the other component moieties which comprise HDL. Since interactions between cells and HDL may play an important role in atherogenesis (19-21), and since the compositions of HDL may have an important bearing on its interactions with cells, it is necessary to assess changes not only in HDL-cholesterol levels, but also in the levels of the other components of HDL. In this report, we present data on the ApoA-I and ApoA-II levels in plasmas of normolipidemic subjects and of patients with the various forms of primary hyperlipidemia. In addition, we have examined the molecular associations between plasma lipoproteins and ApoA-I and ApoA-II by ultracentrifugation and column chromatography. Our findings indicate that HDL structure is altered in hyperlipidemia.

METHODS

Normolipidemic subjects were volunteers recruited from various prevalence or clinical studies. They were drawn from the community at large. All had normal values by local Lipid Research Center laboratory standards (cholesterol and triglycerides <90th percentile), and none were taking drugs known to have altered lipid levels. Hyperlipidemic subjects were referred either by physicians of by other patients of the clinic. Some subjects were discovered to

TABLE I

Lipoprotein Lipid Levels in Hyperlipidemia (Men)[a]

Phenotype	TC	LDL-C	HDL-C	HDL-TC
N (41)	90 ± 34	121 ± 33	47 ± 12	9 ± 5
IIa (10)	141 ± 30	240 ± 49[b]	36 ± 7[c]	10 ± 5
IIb (10)	258 ± 111[b]	242 ± 69[b]	37 ± 8[c]	13 ± 4[c]
III (6)	223 ± 59[b]	126 ± 27	42 ± 6	18 ± 4[b]
IV (14)	393 ± 130[b]	123 ± 29	34 ± 15[b]	17 ± 6[b]
V	2310 ± 884[b]	57 ± 19[b]	21 ± 5	26 ± 6[b]

[a]Results are in mg/dl; mean ± 1 S.D.; TG = triglyceride; C = cholesterol; LDL = low density lipoproteins; and HDL = high density lipoproteins. Type II patients had LDL-C > 190 mg/dl. Types III were diagnosed by VLDL-C/VLDL-TG > 0.42 and isoelectric focusing of VLDL apoprotein. Types IV and V had TG > 250 mg/dl. Means of patient groups are compared to the mean of normolipidemic controls (N). Numbers of individuals per group are in parenthises. (The same designations pertain in Tables II, IV-VIII).
[b]$p<0.0001$.
[c]$p<0.005$.

TABLE II

Lipoprotein Lipid Levels in Hyperlipidemia (Women)[a]

Phenotype	TG	LDL-C	HDL-C	HDL-TG
N (33)	74 ± 32	117 ± 29	54 ± 15	10 ± 5
IIa (12)	136[b] ± 31	230[b] ± 35	50 ± 10	13 ± 5
IIb (9)	243[b] ± 40	225[b] ± 58	44[c] ± 15	17[b] ± 4
III (5)	505[d] ± 456	123 ± 26	45[d] ± 11	17[d] ± 6
IV (5)	430[d] ± 204	105 ± 45	30[b] ±5	23[b] ± 8
V	1419	81	24	22

[a]Results are in mg/dl; mean ± 1 S.D.; TG = triglyceride; C = cholesterol; LDL = low density lipoproteins; and HDL = high density lipoproteins. Type II patients had LDL-C > 190 mg/dl. Types III were diagnosed by VLDL-C/VLDL-TG > 0.42 and isoelectric focusing of VLDL apoprotein. Types IV and V had TG > 250 mg/dl. Means of patient groups are compared to the mean of normolipidemic controls (N). Numbers of individuals per group are in parenthises. (The same designations pertain in Tables II, IV-VIII).
[b]$p<0.0001$.
[c]$p<0.05$.
[d]$p<0.01$.

have abnormal lipid values during various population surveys. Families were not studied, but 47% of the cases with hyperlipidemia reported that one or more first degree relatives had hyperlipidemia and/or a coronary episode (angina pectoris, myocardial infarct or sudden death) before the age of 60 years. However, since we could not be certian of the genetic categories of the study subjects, data are presented only for the NIH "phenotypes" (22). At the time of sampling, none of the patients were under any dietary or drug therapy. In most cases of hyperlipidemia, the samples represent the second blood sample drawn in the clinic. (Three baseline measurements are routinely made before any treatment is given.) Cases with seconday hyperlipidemia were ruled out by appropriate clinical tests. Venous bloods were drawn after 12-14 hr of fasting into EDTA-containing tubes (1 mg/ml). Total plasma and lipoprotein cholesterol and triglyceride determinations were carried out in the Core Laboratory of the Lipid Research Center using the combined ultracentrifugal and precipitation procedures of the Lipid Research Center (23). HDL lipids were measured on heparin-$MnCl_2$ supernates of d>1.006 fractions using a technician II Autoanalyzer. Apoprotein A-I (9)

TABLE III

Correlations between ApoA-I, ApoA-II, and Lipoprotein Lipids[a]

All males (n=80)	ln ApoA-II	ln VLDL-TG	ln HDL-TG	ln HDL-C
ln ApoA-I	+0.51[b] (+0.58[b])	-0.37[c]	-0.21	+0.59[b]
ln ApoA-II	—	-0.32[c]	-0.06	+0.51[b]
ln VLDL-TG	—	—	+0.70	-0.73[b]
All females (n=59)				
ln ApoA-I	+0.12 (+0.40[c])	-0.30[c]	-0.13	+0.58[b]
ln ApoA-II		-0.05	-0.27	+0.12 (+0.41[c])
ln VLDL-TG			+0.70[b]	-0.57[b]

[a]Pearson correlations coefficients were calculated on ln transformed data because of the log normal distribution of lipid values. ApoA-II vs HDL-C and ApoA-I vs ApoA-II correlation coefficients in parentheses are values for the hyperlipidemic subjects.

[b]$p<0.001$.

[c]$p<0.01$.

and A-II (12) levels were measured by radioimmunoassays developed in this laboratory.

Survey data were entered into the SAS statistical analysis and data management system for subsequent display and statistical analysis (24). Groups' means and standard deviations were calculated and, nonpaired t-tests were performed on values that had been subjected to natural logarithmic transformation, because of the well known log normal form of the distribution of lipid values (25). Correlation coefficients (Pearson) were also calculated (see table legends). Indices of HDL composition were calculated using the values for HDL lipids, ApoA-I and ApoA-II. This is valid because, in plasmas taken from normolipidemic and hyperlipidemic people with triglycerides <1500 mg/dl, ~90% of the ApoA-I and ApoA-II appear to be associated with HDL (see below and Refs. 9 and 13).

The association of ApoA-I and ApoA-II with HDL or other lipoproteins was determined by two methods. First, selected plasma samples were subjected to single ultracentrifugations at d 1.063 and d 1.21 in a 40.3 rotor for 16 hr at 10 C in a Beckman L265B ultracentrifuge. The apoprotein contents of both the supernate and infranate fractions (separated by tube slicing) were measured. Second, to assess the association between lipoprotein and ApoA-I and ApoA-II by another means, 1 ml of plasmas was filtered on a 2.5 x 90 cm column of Sephadex G-100 equilibrated with 0.05 M phosphate buffer pH 7.4, 0.16 M NaCl. The columns were "presaturated" with 5 ml of plasma to decrease any adsorptive losses of apoproteins. The columns were also calibrated for the determinations of apparent molecular weights. For this purpose, proteins of known molecular weight (MW) were filtered and a MW vs K_d plot was constructed (26). Following the filtration of plasmas, individual elution fractions were assayed for their contents of ApoA-I (9) and ApoA-II (13) and for total protein (27). Ninety-seven to 100% of the total protein and 79-92% and 82-113% of ApoA-I and ApoA-II, respectively, were recovered from these columns. Assays to determine the ApoA-I contents of individual fractions were initially carried out on unextracted samples; these results were used solely for identifying the elution positions of ApoA-I because, in our ApoA-I assay, HDL-containg fractions have to be delipidated in order to detect all their contents of ApoA-I. Therefore, to obtain the quantities of ApoA-I in each elution peak, the peaks containing ApoA-I were pooled, delipidated, and assayed for ApoA-I by radioimmunoassay. In order to assess further the column recoveries of ApoA-I and ApoA-II, ^{125}I-ApoA-I and ^{125}I-ApoA-II were also filtered on the column. Recoveries of label ranged from 87-92% (2 experiments per label).

RESULTS

In population studies, HDL cholesterol levels have been shown to be higher in women than in men (28); therefore, the data were calculated separately for the two sexes. The mean lipid values of patients and the cutoff points used to diagnose then are given in Tables I and II. Other diagnostic criteria were also used. In addition to the lipid levels shown, Type III patients had VLDL cholesterol/total triglyceride ratios of >0.3 (29), and ratios of the VLDL-ApoE-

TABLE IV

Apolipoprotein A-I and A-II Levels in Hyperlipidemia (Men)[a]

Phenotype	ApoA-I	ApoA-II
N (19)	109 ± 24	40 ± 8
IIa (8)	108 ± 37	38 ± 10
IIb (13)	124 ± 44	44 ± 7
III (6)	121 ± 26	41 ± 9
IV (17)	104 ± 30	36 ± 8
V	90[b] ± 14	30[b] ± 12

[a]Results are in mg/dl given as means ± 1 S.D.; nonpaired t-tests are calculated using log transformed data.
[b]$p<0.05$.

TABLE V

Apolipoprotein A-I and A-II Levels in Hyperlipidemia (Women)[a]

Phenotype	ApoA-I	Apo-A-II
	mg/dl	
N (21)	122 ± 27	37 ± 6
IIa (11)	111 ± 19	45[b] ± 10
IIb (8)	109 ± 24	45 ± 13
III (5)	102 ± 14	43 ± 12
IV (5)	96[c] ± 18	38 ± 14
V (1)	81	44

[a]Results are in mg/dl given as means ± 1 S.D.; nonpaired t-tests are calculated using log transformed data.
[b]$p<0.03$.
[c]$p<0.01$.

III/ApoE-II bands were <0.2 on isoelectric focusing (normals are >1.2) (30-32). Type V patients had fasting chylomicronemia (22). The mean ages of the groups were between 47 and 56 years, and there were no significant differences between them.

HDL cholesterol levels in normolipemic women tended to be higher than in normolipemic men (Tables I and II). HDL cholesterol levels in hypertriglyceridemic subjects of either sex were lower than normal, whereas HDL triglycerides were elevated. Indeed, the Pearson correlation coefficients (Table III) between total triglycerides and HDL cholesterol were -0.73 in men and -0.57 in women, whereas correlations between total triglycerides and HDL triglycerides were +0.70 for both sexes. Thus, HDL cholesterol and HDL triglyceride levels diverged with increasing plasma triglyceride concentrations.

Mean ApoA-I and ApoA-II levels in normal and hyperlipidemic subjects were similar to each other, with the exception of mean levels of Type V, which were low (Tables IV and V). Mean ApoA-I/ApoA-II ratios ranged from 2.5-3.2 for the different groups, and there were no significant differences.

ApoA-I and ApoA-II levels were significantly positively correlated (Table III) with each other in both normal and abnormal men, and with levels of HDL cholesterol; the apoproteins of men were also negatively correlated with VLDL-TG (HDL-C were also negatively correlated with VLDL-TG). But, in women, for reasons which are not clear, the correlation of ApoA-I vs ApoA-II, and ApoA-II vs HDL-C were significant only in the hyperlipidemic population, but not in normals. The correlations of ApoA-I with HDL-C and with VLDL-TG were similar in men and women.

The relative proportions of the components of. HDL were altered by increasing hyperlipidemia, in both sexes (Tables VI and VII). The relative contribution of HDL-C decreased and that of HDL-TG rose significantly. The relative proportions of the apoproteins changed less dramatically. There were strong *inverse* correlations between VLDL-TG and HDL-C (expressed as a proportion of the calculated HDL components), and there were equally strong *positive* correlations between VLDL-TG and the proportion of HDL-TG.

ApoA-I and ApoA-II in HDL

Upon ultracentrifugation, the vast majority of the ApoA-I and ApoA-II in plasma were found to be in the d>1.063 fractions (Table VIII) and in the fractions which floated at d 1.21 (Table IX). However, the amounts of the ApoA-I in d<1.063 and the proportions of total ApoA-I in the d>1.21 fractions were greater in hyperlipidemic plasmas than in normals. One plasma from a Type V patient contained ~15% of the two apoproteins in the d<1.063 fraction, and 23% of the ApoA-I in the plasma of another patient with Type V was in the d>1.21 fraction. But, in general, ~85% of ApoA-I was found in the d 1.063-1.21 density range.

The results of plasma filtrations on Sephadex G-100 columns are illustrated (Figs. 1 and 2). Two normal HDL_3 preparations (d 1.12-1.21) were similarly examined (elution profile not shown). In addition to ApoA-I and ApoA-II, the elution profile of ApoB was also obtianed by radioimmunoassay (33). The

TABLE VI

Relative Proportions of Lipids and Apoproteins A-I and A-II in HDL (Men)[a]

Phenotype	ΣHDL[b]	A-I/Σ	A-II/Σ	C/Σ	TG/Σ	HDL-C/HDL-TG[c]
		proportion of mass				mass ratio
N (19)	205 ± 47	0.55 ± 0.03	0.18 ± 0.02	0.24 ± 0.04	0.03 ± 0.01	6.7 ± 3.5
IIa (8)	195 ± 8	0.54 ± 0.08	0.22 ± 0.09	0.20 ± 0.03	0.04 ± 0.03	7.0 ± 6.7
IIb (10)	180 ± 12	0.52 ± 0.07	0.22 ± 0.05[d]	0.19 ± 0.04[d]	0.07 ± 0.03[d]	2.9 ± 0.8[d]
III (6)	228 ± 35	0.54 ± 0.05	0.19 ± 0.03	0.20 ± 0.04	0.07 ± 0.02[d]	2.6 ± 0.8[d]
IV (14)	191 ± 53	0.53 ± 0.03	0.21 ± 0.04[d]	0.18 ± 0.03[d]	0.09 ± 0.03[d]	2.3 ± 1.3[d]
V (6)	167 ± 31	0.54 ± 0.02	0.18 ± 0.05	0.12 ± 0.02[d]	0.16 ± 0.04[d]	0.8 ± 0.3[d]

[a]Results are means of mass ratios ± 1 SD.
[b]Σ - ΣHDL = A-I + A-II + HDL - C + HDL - TG.
[c]C = cholesterol, TG = triglyceride
[d]Means significantly different from normals $p < 0.02$.

TABLE VII

Relative Proportions of Lipids and Apoproteins A-I and A-II in HDL (Women)[a]

Phenotype	ΣHDL[b]	A-I/Σ	A-II/Σ	C/Σ	TG/Σ	HDL-C/HDL-TG[c]
		proportion of mass				mass ratio
N (20)	232 ± 45	0.55 ± 0.04	0.15 ± 0.02	0.25 ± 0.04	0.04 ± 0.02	6.5 ± 3.4
IIa (11)	217 ± 26	0.48 ± 0.06[d]	0.22 ± 0.05[d]	0.23 ± 0.04	0.07 ± 0.02[d]	8.0 ± 6.9
IIb (8)	212 ± 26[d]	0.53 ± 0.05	0.20 ± 0.05	0.20 ± 0.02[d]	0.07 ± 0.02[d]	2.6 ± 1.0
III (5)	205 ± 31	0.50 ± 0.05	0.21 ± 0.03[d]	0.21 ± 0.05[d]	0.09 ± 0.03[d]	2.8 ± 1.3
IV (5)	188 ± 31[d]	0.51 ± 0.02[d]	0.20 ± 0.04[d]	0.16 ± 0.01[d]	0.13 ± 0.05[d]	1.5 ± 1.0
V	90	0.50	0.21	0.13	0.16	1.2

[a]Results are means of mass ratios ± 1 SD.
[b]Σ = ΣHDL = A-I + A-II + HDL · C + HDL · TG.
[c]C = cholesterol, TG = triglycerides.
[d]Means significantly different from normals $p < 0.02$.

TABLE VIII

Distribution of ApoA=I and ApoA-II among $<$ d 1.063 and $>$ d 1.063[a]

Subjects		Total TG	ApoA-I Plasma Total	ApoA-I d $<$ 1.063	ApoA-I d $<$ 1.063	ApoA-II Plasma Total	ApoA-II d $<$ 1.063	ApoA-II d $>$ 1.063
Normal	DG	61	142	0.2	140	39	0.02	38
	RS	55	128	0.3	126	41	0.03	43
IIa	RR	79	86	0.3	84	32	0.03	34
	VW	139	139	0.4	120	52	0.04	50
IIb	BC	227	91	1.0	90	37	0.13	32
III	JF	960	96	0.7	96	43	0.20	45
	RS	1310	93	2.4	85	52	0.80	49
IV	CH	379	101	0.5	100	49	0.08	35
	ES	403	108	0.5	101	54	0.10	50
V	CB	1370	116	1.6	125	48	0.62	43
	EG	3420	83	12.8	64	26	6.80	20

[a]All values are given in mg/dl.

elution peak of ApoB (detected only in plasma and not in the HDL_3 preparations) coincided with the elution peak of Dextran Blue. The former peak was, therefore, used as an index of the void volume. In each of the eleven plasmas and the HDL_3 preparations examined, ApoA-II eluted in a single peak near ApoB. No immunologically reactive ApoA-II was detected in any smaller fraction which would have corresponded to monomers (MW 17,000), polymers (up to MW 85,000 could have been distinguished from HDL), or fragments (MW $\leqslant$ 4,500 could have been detected).

ApoA-I in normal plasma (Fig. 1) and in HDL_3 (not shown) eluted in two distinct peaks. The first peak co-eluted with ApoA-II; the second corresponded to a MW of ~50,000. Following pooling and delipidation, the second peak was found to contain 5-10% of the total ApoA-I of normal plasma and of HDL_3. The elution profile of ApoA-I in hyperlipidemic plasma was both quantitatively and qualitatively different from normal plasma. Frequently a third peak was found, which eluted between the void volume and the 50,000 MW peak. This peak was not present in normal plasma. The 50,000 MW peak appeared to be usually large. Upon pooling and delipidation, the 50,000 MW peak was found to contain 10-50% of total ApoA-I. In fact, the proportion of ApoA-I in the 50,000 MW peak was strongly correlated with plasma TG concentrations (Fig. 3). However, in general, >80% of the ApoA-I peak eluted with HDL in plasma with triglyceride contents of <1000 mg/dl. No immunologic activity due to ApoA-I was found to elution volumes which would have corresponded to ApoA-I monomers (MW ~28,000) or fragments in either normal or hyperlipidemic plasmas.

DISCUSSION

One of the aims of these studies has been to ascertain whether the relative proportions of HDL apoproteins and lipids are altered in hyperlipidemia. It has been reported that levels of HDL are inversely related to levels of total plasma TG or VLDL in unselected populations (35), a finding we have confirmed in our group of subjects (Tables I-III). It has also been reported that HDL-TG rise as total TG increase in response to metabolic factors (16,17). Thus, our finding of a strong correlation between total or VLDL-TG and HDL-TG (Table III) agrees with previous findings. ApoA-I and ApoA-II levels were not significantly different from normal in all but the most severe forms of hypertriglyceridemia (Type V) (Tables VI and

TABLE IX

Distribution of ApoA-I among d < 1.21 and d > 1.21 Fractions[a]

Subjects	Total plasma TG	Total plasma	ApoA-I		
			d < 1.21	d > 1.21	% d > 1.21
Normal					
RF	132	88	75	7	8
MT	93	110	97	11	10
SH	87	127	123	10	8
IIa					
AK	164	113	8	13	12
IIb					
JM	217	114	107	12	11
VC	190	135	131	8	
III					
DD	542	118	95	20	17
PM	296	121	105	12	10
JF	2105	102	---	16	16
IV					
VN	400	89	80	8	10
V					
HJ	1021	73	65	6	8
WM	1759	120	92	27	23
DK	1500	114	101	10	9

[a]All values are given in mg/dl.

VII), and ApoA-I and ApoA-II both varied independently of HDL-TG (Table III). Thus, the proportions of HDL components were altered in all forms of hypertriglyceridemia (Types IIB-V), whether results were computed as relative proportions of the masses of ApoA-I, ApoA-II, HDL-C, and HDL-TG, or as HDL-C/HDL-TG mass ratios (Tables VI and VII). The change appears to consist of a relative decrease in cholesterol and an increase in TG without consistently significant changes in the proportions of either ApoA-I or ApoA-II. We interpret these results as showing that the composition of HDL (rather than the relative proportions of HDL_2 and HDL_3) are altered in hypertriglyceridemia (see below).

Another aim of these studies was to ascertain whether the association between the ApoA proteins and HDL is altered in hyperlipidemia. We (9,13) and others (14) have previously shown that, in normolipidemic subjects, >90% of ApoA-I and >98% of ApoA-II are isolated with the d 1.063-1.21 density fractions. However, it could no be assumed that a similar situation existed for the hyperlipidemias because apoproteins are known readily to exchange between HDL subfractions (36), and between the TG-rich lipoproteins and HDL (37). The distribution of apoproteins among the lipoprotein classes is probably determined at least in part by the levels of the various lipoproteins relative to each other and by the affinities between ApoA-I or ApoA-II and the individual lipoproteins. Hyperlipidemia by definition involves alterations in lipoprotein levels and, as we have shown, changes in the compositions of HDL. Both circumstances could alter the distribution of ApoA-I between lipoproteins.

There is no way to quantify the distribution of apoproteins between the lipoprotein classes, as this exists in plasma, without the introduction of artefacts. Ultracentrifugation "strips" apoproteins from lipoproteins (38), and column chromatography may affect apoprotein-lipoprotein interactions by dilution or by selective adsorption and retardation of eluted components. Electrophoresis, too, may dissociate various moieties from each other. Nevertheless, one can compare the behaviors of different plasmas subjected to identical manipulations. Ultracentrifugation at d 1.063 revealed that increased amounts of ApoA-I and ApoA-II were present in the d<1.063 fractions in hypertriglyceridemia (Table VIII), and similarly, that there were increased proportions of ApoA-I in many of the d>1.21 fractions (Table IX).

When plasmas were filtered on columns of Sephadex G-100, all of the ApoA-II was found in the lipoprotein fractions (Figs. 1 and 2) (for ApoA-I, see below). One can conclude from the ultracentrifugal and filtration experiments that >90% of ApoA-II in fasting plasma is probably associated with HDL in all but the very hypertriglyceridemic plasmas. For this reason, total plasma ApoA-II can be included with confidence in calculations of HDL "composition."

The column filtration results are more difficult to interpret for ApoA-I, because, on column chromatography, 5-50% of ApoA-I eluted with the 50,000 MW "small" fraction

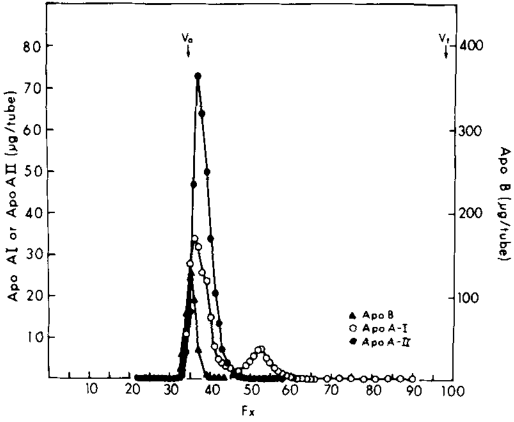

FIG. 1. Elution profile of normal plasma (2.5 x 90 cm column, Sephadex G100, 0.16 M NaCl, 0.05 M phosphate, pH 7.4). Apoprotein contents were determined by radioimmunoassay without prior delipidation of the fractions. Thus, ApoA-I levels as shown are grossly under estimated, but ApoA-II and ApoB levels are accurate. Although not shown, fractions 1-100 were assayed. ApoB and ApoA-II were found only in the void volume (V_0). To assay ApoA-I accurately, the ApoA-I elution peaks were pooled and their ApoA-I contents determined after delipidation; 95% of ApoA-I was in peak 1, but about 5% of ApoA-I was also present in a second peak (tubes 46-60, MW ~50,000).

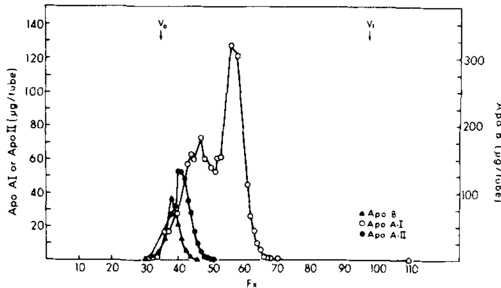

FIG. 2. Elution profile of a type V plasma (fasting triglycerides 3124 mg/dl with chylomicronemia, see Figure 1 for elution and assay conditions). ApoA-II and ApoB elute with or near the void volume. ApoA-I elutes in three peaks (tubes 36, 46, and 56). Each peak was pooled, delipidated, and assayed for ApoA-I. Peak 3 (tubes 51-64) contained 24% of the total. Recovery of ApoA-I from the column was 92%.

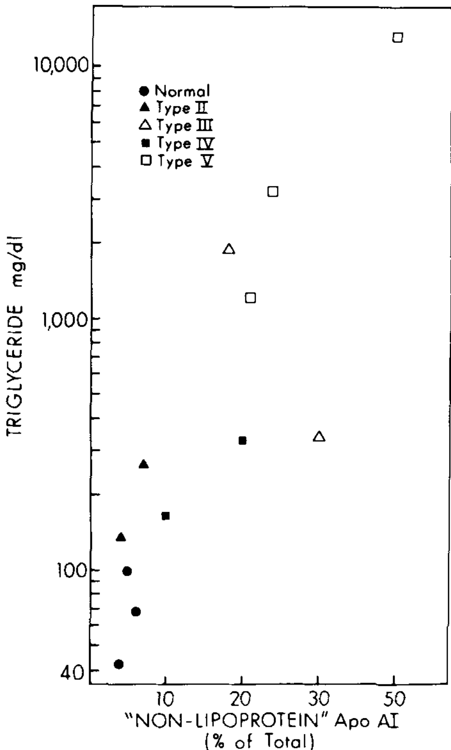

FIG. 3. Relationship between "50,000 MW" ApoA-I (see Figures 1 and 2) and total plasma triglyceride concentrations.

(Figs. 1 and 2). In fact, the proportion of "small" ApoA-I in plasma was directly correlated with total TG or TG-rich lipoprotein levels (Fig. 3). For the normal plasmas, "small" ApoA-I was probably produced during the column filtration process because ~5% of the ApoA-I of HDL_3 was also found in the 50,000 MW fraction when normal HDL_3 was filtered on the same columns. The source of the "small" ApoA-I in hyperlipidemic plasmas is not clear. It could have come from any of the lipoproteins or from the d>1.21 fractions of plasma. Filtrations of HDL and other lipoproteins isolated from a variety of plasmas could help to settle the issue. On the other hand, all of the "easily" removable ApoA-I in HDL may be "stripped" off during isolation. Therefore, the results of filtration of isolated lipoproteins may also be equivocal. Clearly, a method is needed for assessing the apoprotein distributions in plasma without perturbing the system. Thus, at this point, one cannot assign an exact quantitation to the density distribution of ApoA-I in hyperlipidemic plasma. But it is probably fair to say that more of the total ApoA-I is found in the non-HDL fractions of hypertriglyceridemic than of normolipidemic plasmas.

Cheung has shown that in normolipidemic subjects, ApoA-I/ApoA-II ratios are inversely related to *the density of the* HDL fractions (14). Changes in the density distributions of HDL in plasmas of hyperlipidemic subjects would be expected to be accompanied by alterations in the ApoA-I/ApoA-II ratios. The ApoA-I/ApoA-II ratios of HDL in the hyper-

lipidemic subjects could not be determined with certaintly, because of the uncertainty about the density distributions of ApoA-I. But, if anything, the ratios were lower than normal because total ApoA-II values could be used in the calculations, whereas the total ApoA-I values would be diminished by however much ApoA-I is present in non-HDL fractions. A decrease in ApoA-/ApoA-II ratio would imply that the mean density of HDL had increased or that HDL_2/HDL_3 ratios fell. But the demonstrated rises in HDL-TG should have produced the opposite effect, namely a lowering of mean density. Thus, the changes in the relative proportions of HDL components were probably due to alterations in the compositions of the HDL particles rather than to changes in the density distributions of HDL particles of normal compositions. These changes in compositions probably arose in connection with the metabolic defects in hyperlipidemia and are likely to affect the metabolism of HDL by further affecting the interactions of HDL with other lipoproteins, with lecithin cholesterol acyl transferase, and with tissues.

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Biosynthesis of Chenodeoxycholic Acid: Side-Chain Hydroxylation of 5β-Cholestane-3α,7α-Diol by Subcellular Fractions of Guinea Pig Liver

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ABSTRACT

Side-chain hydroxylation of 5β-cholestane-3α,7α diol was studied in subcellular fractions of guinea pig liver. The purity of the microsomal and the mitochondrial fractions was determined with marker enzymes, and relatively little cross contamination between the particulate fractions was detected. Methods for the analysis of the incubation mixture by thin-layer chromatography and gas-chromatography-mass spectrometry were developed. Optimal assay conditions were established for the major hydroxylation reactions, namely the mitochondrial 26-hydroxylation and the microsomal 25-hydroxylation. It was found that the most active side-chain hydroxylation in the guinea pig was the microsomal 25-hydroxylation. The mitochondrial ω-hydroxylation was stereospecific, in that the rate of formation of (25R)-5β-cholestane-3α,7α,26-triol was 8 times greater than that of the 25S isomer. The microsomal "26"-hydroxylation was not stereospecific under the conditions employed. It is concluded that the mitochondrial "26" hydroxylation (leading to the formation of (25R)-5β-cholestane-3α,7α,26-triol) plays an important role in the biosynthesis of chenodeoxycholic acid. The participation of microsomal 25-hydroxylation in the formation of chenodeoxycholic acids requires further investigation.

INTRODUCTION

Previous studies suggest that the last intermediate common to both cholic acid and chenodeoxycholic acid is 7α-hydroxy-4-cholesten-3-one (1). If this compound fails to undergo 12α-hydroxylation, it can be converted to 7α-hydroxy-5β-cholestan-3-one and 5β-cholestane-3α,7α-diol. The diol is probably the preferred substrate for 26-hydroxylation (2), and 5β-cholestane-3α,7α,26-triol then undergoes further transformation into chenodeoxycholic acid. The side-chain degradation of 5β-cholestane-3α,7α-diol has not been studied in detail, but the steps are assumed to be analogous to those described for cholic acid biosynthesis (2).

The guinea pig appears to be a suitable animal model for studies of chenodeoxycholic acid biosynthesis, since guinea pig bile contains merely traces of cholic acid (3). The major bile acid is chenodeoxycholic acid and the 12α-hydroxylase activity is quite low (4).

This paper describes in vitro studies of the initial reaction leading to side-chain degradation during chenodeoxycholic acid biosynthesis, namely the 25- and 26-hydroxylation of 5β-cholestane-3α,7α-diol. It was found that the major product of the microsomal hydroxylation of 5β-cholestane-3α,7α-diol was 5β-cholestane-3α,7α, 25-triol, while the chief product with the mitochondrial fraction was (25R)-5β-cholestane-3α,7α,26-triol. The latter fraction also produced relatively large proportions of 5β-cholestane-3α, 7α, 24S-triol.

EXPERIMENTAL PROCEDURES

Material

Unlabeled Compounds. 5β-Cholestane-3α,7α,25-triol was prepared from chenodeoxycholic acid as described previously (5). This compound was employed for the preparation of 24R- and 24S-5β-cholestane-3α,7α,24-triols and 25R- and 25S-5β-cholestane-3α,7α,26-triols (6).

5β-Cholestane-3α,7α-diol, m.p. 86-88 C [reported m.p. 84-86 C (7)], was prepared by dehydration of 5β-cholestane-3α,7α,25-triol (6) followed by catalytic hydrogenation of the resulting isomeric mixture of 5β-cholest-24-ene-3α,7α-diol and 5β-cholest-25-ene-3α,7α-diol.

Labeled Compounds. 5β-[G-^{3}H] cholestane-3α,7α-diol was prepared according to the method of Wilzbach (8) and purified by successive preparative thin layer chromatography (TLC) with the solvent systems: benzene-ethyl acetate (4:6, v/v) and chloroform-ethanol (10:1, v/v) to constant specific activity (2.16 μCi per mg).

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TABLE I

Composition of Guinea Pig Gallbladder Bile[a]

Bile acid	5 of total bile acids
Chenodeoxycholic acid	94.0
Ursodeoxycholic acid	2.2
Cholic acid	0.1
Lithocholic acid	0.1
7-Ketolithocholic acid	2.0
Unidentified	1.8

[a]Average values from five male guinea pigs (average weight 350 g). For analytical procedures see (9).

TABLE II

Subcellular Distribution of Side-Chain Hydroxylation Systems Acting upon 5β-Cholestane-3α,7α- Diol[a]

Fraction	Formation of 5β-cholestane-			
	3α,7α,26-triol	3α,7α,25-triol	3α,7α,24-triol	3α,7α,12α-triol
	pmole/mg protein/min			
600 x g Supernatant[b]	5.8	3.6	2.4	1.5
Mitochondria[c]	51.2	7.2	28.5	0.81
Microsomes[b]	24.8	146.6	13.3	9.3
100,000 x g Supernatant[b]	0.15	0.90	0.23	1.9

[a]Subcellular fractionation was performed as described in "Methods."
[b]Standard assay system as described in "Methods." 3.0 mM NADPH was employed as the electron donor.
[c]Twice washed mitochondria were used. DL-Isocitrate was employed to generate intramitochondrial NADPH.

Reagents and Cofactors. NADPH, NADH, DL-isocitrate were purchased from the Sigma Chemical Co. (St. Louis, MO). All other reagents were obtained from Fisher Scientific Co. (Pittsburgh, PA).

Methods

The bile acid composition of gall bladder bile of the guinea pig was analyzed as described by Hofmann et al. (9).

Male guinea pigs (250 g) were obtained from the Otisville Laboratories of the New York City Health Department. Liver homogenates (20%, w/v) were prepared in 0.25 M sucrose solution with a Potter-Elvehjem homogenizer having a loosely fitting Teflon pestle. Fractionation of each homogenate was performed according to the procedure described by Wilgram and Kennedy (10). The average protein concentration of the mitochondria (twice washed) and the microsomes was about 10 mg per ml, determined according to the method of Lowry et al. (11).

In the standard incubation procedure for the mitochondrial fraction, [^{3}H]5β-cholestane-3α,7α-diol (100 nmol, 1.61 x 10^7 dpm/μmole), dissolved in 15 μl of acetone, was incubated in a total volume of 1.0 ml with 85 mM phosphate buffer (pH 7.4), 1.7 mM $MgCl_2$, 4.0 mMDL-isocitrate , and 0.1 ml of the mitochondrial fraction. Incubations was carried out in air at 37 C for 15 min with shaking, except in the case of carbon monoxide inhibition. For the microsomal fraction, 1.0 mM NADPH replaced the DL-isocitrate.

The reaction mixture was preincubated without substrate for 5 min at 37 C and the reaction was terminated by the addition of 0.1 ml of 1 N HCl. The unreacted cholestanediol and the reaction products were extracted with 2 x 5 ml of ethyl acetate, the extracts were washed with water, and the solvent was evaporaated under nitrogen (50-60 C).

The incubation products were separated by TLC on alumina G plates (0.25 mm thick, Analtech, Inc., Newark, DE) which were developed twice, first with chloroform-acetone-methanol [35:25:35(v/v/v)] and then with benzene-ethyl acetate ethanol [90:20:7 (v/v/v)]. The R_f values of the steroids were as follows: 5β-cholestane-3α,7α-diol (0.86), 5β-cholestane-3α,7α,24S-triol (0.70), 5β-cholestane-3α,7α,25-triol + 5β-cholestane-3α,7α,24R-triol (0.61), 5β-cholestane-3α,7α,26-triol (25R), 0.44, 5β-cholestane-3α,7α,26-triol (25S), 0.51, and 5β-cholestane-3α,7α,12α-triol (0.37). Activation of the TLC plate at 105 C for 15 min was required, and samples

TABLE III

Effect of Repeated Washings on the Removal of Microsomal Enzymes from Guinea Pig Liver Mitochondria[a]

Particulate fraction	No. of washes	26-Hydroxylation	25-Hydroxylation	Monoamine oxidase sp. act.[d]	Glucose-6-phosphatase sp. act.[e]
		pmole/mg protein/min			
Mitochondria[b]	0	47.9	14.1	1.40	0.42
Mitochondria[b]	1	50.1	7.6	1.46	0.23
Mitochondria[b]	2	49.7	7.1	1.47	0.21
Mitochondria[b]	3	49.0	6.9	1.44	0.20
Microsomes[c]	0	25.1	139.6	0.03	5.60

[a]The mitochondrial and microsomal fractions were prepared as described in "Methods."
[b]DL-Isocitrate was employed to generate intramitochondrial NADPH.
[c]NADPH was the electron donor.
[d]Marker enzyme for mitochondrial contamination: specific activity of monoamine oxidase is expressed as nmoles of benzaldehyde produced per minute per mg protein.
[e]Marker enzyme for microsomal contamination: specific activity of glucose-6-phosphate is expressed as nmoles of inorganic phosphate released per 15 min per mg protein.

were applied when the plate had cooled to 40 C on a constant temperature hot plate. Unlabeled 5β-cholestane-3α,7α-diol and 5β-cholestane triols (10 μg each) were applied with the extracts and to each side of the plate as markers. The markers along the sides of the plate were made visible with spray reagent consisting of 3.5% phosphomolybdic acid in isopropanol. To separate 5β-cholestane-3α,7α,24R-triol from 5β-cholestane-3α,7α,25-triol, the spot containing these two compounds was removed from the alumina plate and eluted with methanol. After concentrating the methanol solution, the compounds were reapplied to a 0.25 mm thick Silica Gel G plate (Brinkmann, Westbury, NY) and developed twice with benzene-acetone-methanol, 70:50:1.5 (v/v/v). R_f values of 5β-cholestane-3α,7α,24R-triol and 5β-cholestane-3α,7α 25-triol were: 0.58 and 0.54, respectively.

Enzyme activities were calculated by removing individual spots from the TLC plates and measuring their radioactivity in a liquid scintillation counter (Beckman LS-200B, Beckman Instruments Fullerton, CA). Since the specific radioactivity of the substrate was known, the radioactivity data could be expressed in terms of pmoles of products formed.

Marker enzymes. Monoamine oxidase was assayed according to the method described by Schnaitman et al. (12). Glucose-6-phosphatase activity was measured at 37 C (13). Inorganic phosphate liberated during incubation was determined by the method of Fiske and SubbaRow (14).

RESULTS

Table I illustrates the biliary bile acid composition of the strain of guinea pig used in this study. As expected, the major bile acid was chenodeoxycholic acid which accounted for 94% of the total bile acids. Cholic acid was present only in trace amounts.

The subcellular distribution of the hepatic side chain hydroxylation system is shown in Table II. In the mitochondria, 26-hydroxylation predominated, but the 24-hydroxylation was also significant. In contrast, the microsomal fraction catalyzed mainly the 25-hydroxylation of 5β-cholestane-3α,7α-diol. As expected, 12α-hydroxylation was quite low.

In order to demonstrate that the mitochondrial hydroxylations were not ascribable to large scale contamination by microsomes, the mitochondrial fraction was washed 4 times and 25- and 26-hydroxylase activities were determined after each wash. As summarized in Table III, repeated washings did not decrease mitochondrial 26-hydroxylase activity, but the first wash removed 50% of the 25-hydroxylase activity. Additional washings did not result in further loss of 25-hydroxylase activity. The microsomal marker enzyme glucose-6-phosphatase was removed from the mitochondria in approximately the same proportions as the 25-hydroxylase. On the basis of these findings, we estimated that the mitochondrial fraction was contaminated by about 3% with the microsomal fraction.

Table IV illustrates the effect of increasing concentrations of carbon monoxide in the gas phase on the major mitochondrial and microsomal side-chain hydroxylations. All hydroxylations studied were progressively inhibited as the percentage of carbon monoxide in the gas phase was increased from 3% to 16%.

The conversion of labeled

TABLE IV

Inhibition of Mitochondrial 26-Hydroxylation and Microsomal 25- and 26-Hydroxylations by Carbon Monoxide

Gas mixture			Mitochondria[a]		Microsomes[a]			
CO	O_2	CO/O_2	26-Hydroxylation	Inhibition	25-Hydroxylation	Inhibition	26-Hydroxylation	Inhibition
%[b]	%[b]		pmole/mg/min	%	pmole/mg/min	%	pmole/mg/min	%
...	5.58		52.4	...	148.1	...	25.3	...
3.03	5.28	0.57	38.8	25.9	91.5	38.2	17.7	30.0
5.54	5.31	1.04	22.5	57.1	83.5	43.6	11.3	55.3
15.57	5.42	2.88	9.4	82.1	67.4	54.5	5.1	79.8

[a]For details of enzyme assays, see "Methods" section. All assays were carried out in gastight containers.
[b]Percent of total. N_2 gas was used in all cases as the diluent.

5β-cholestane-3α,7α-diol to the various 5β-cholestanetriols by hepatic mitochondria and microsomes is summarized in Table V. In the guinea pig, the major reaction product with the mitochondrial fraction was clearly the 25R-isomer of 5β-cholestane-3α,7α,26-triol. Appreciable amounts of 5β-cholestane-3α,7α,24-triol(24S) were also produced. On the other hand, incubations with the microsomal fraction yielded predominantly 5β-cholestane-3α,7α,25-triol. As predictable, the 12α-hydroxylation was quite low and amounted to less than 5% of the total hydroxylations. The two 26-hydroxylated diastereomers accounted for only 13% of the total and the ratio of the 25S to 25R isomer was ca. 2:1.

DISCUSSION

The guinea pig is very suitable for studies of chenodeoxycholic acid biosynthesis because, in most strains as in the one used in the present study, the major bile acid is chenodeoxycholic acid (Table I). In most mammalian species, cholic acid and chenodeoxycholic acid are the primary bile acids and occur in varying proportions which are relatively species-specific. The virtual absence of cholic acid from guinea pig bile can most probably be ascribed to the very low levels of 12α-hydroxylase in the liver of this species (Tables II and V).

In the present paper, the activities of the hepatic mitochondrial and microsomal enzymes converting 5β-cholestane-3α,7α-diol to a mixture of isomeric 5β-cholestanetriols were studied by an isotope incorporation procedure. Side-chain hydroxylation activity was present in the mitochondrial and microsomal fractions, while the 100,000 x g supernatant solution was inactive (Table II). Care was taken to prepare particulate subcellular fractions of high purity using the procedure of Wilgram and Kennedy (10). It seems likely, therefore, that the twice washed mitochondrial fraction used in these experiments was only minimally contaminated with microsomes. By the use of a marker enzyme, glucose-6-phosphatase, this contamination was estimated to be about 3% (Table III).

The exact nature of the enzyme systems catalyzing the side-chain hydroxylations is not known. It seems probable, however, that the 26-hydroxylases (mitochondrial and microsomal) and the 25-hydroxylase (microsomal) function in conjunction with a cytochrome as a terminal oxidase, since they are sensitive to carbon monoxide (Table IV).

The results of the present paper demonstrate that in the guinea pig hepatic mitochondria catalyze predominantly the 26-hydroxylation

TABLE V

Conversion of 5β-[G-^{3}H]Cholestane-3α,7α-Diol to 5β-Cholestanetriols by Hepatic Mitochondria and Microsomes[a]

5β-Cholestanetriols formed	Rate of 5β-cholestanetriol formation in:	
	Mitochondria	Microsomes
	pmole/mg protein/15 min	
3α,7α,12α-triol	0.73[b]	8.00[b]
3α,7α,24-triol (24R)	5.70	5.87
3α,7α,24-triol (24S)	21.3	6.71
3α,7α,25-triol	5.64	123.3
3α,7α,26-triol (25R)	44.8	6.65
3α,7α,26-triol (25S	5.80	14.6

[a]Mitochondrial and microsomal fractions were prepared and products were analyzed as described in the "Methods" section. Standard assay conditions were employed.

[b]Each value represents the average of five experiments with guinea pig hepatic subcellular fractions.

of 5β-cholestane-3α,7α-diol. In contrast, the major side-chain hydroxylation product formed during the incubation of the diol with liver microsomes was 5β-cholestane-3α,7α,25-triol. Hydroxylation at C-24 or C-26 leads to the formation of two distinct pairs of diastereomers, 24R and 24S of 5β-cholestane-3α, 7α,24-triol, and 25R and 25S of 5β-cholestane-3α,7α,26-triol. The mitochondrial hydroxylations were stereospecific: the ratio of the 25R to 25S diastereomers of 5β-cholestane-3α,7α,26-triol was ca. 8:1, while the ratio of 24S to 24R of 5β-cholestane-3α,7α,24-triol was 4:1. The significance of the 24-hydroxylation of 5β-cholestane-3α,7α-diol in the biosynthesis of chenodeoxycholic acid is not known. On the other hand, if the side-chain degradation is initiated via the mitochondrial 26-hydroxylation of 5β-cholestane-3α,7α-diol, the 25R-isomer of 5β-cholestane-3α,7α,26-triol is obviously a key intermediate.

Under the conditions of the present experiments, the microsomal hydroxylations at C-24 and C-26 exhibited relatively low stereospecificity and relatively low reaction rates (Table V). The major product was 5β-cholestane-3α,7α,25-triol.

Thus, the high rate and stereospecificity of the 26-hydroxylation of 5β-cholestane-3α,7α-diol by guinea pig liver mitochondria suggests that the initial step in the degradation of the side-chain during chenodeoxycholic acid biosynthesis is the formation of the 25R-diastereomer of 5β-cholestane-3α,7α,26-triol, and that this step is catalyzed by the mitochondria. However, if the first step in the side-chain hydroxylation is mediated by the microsomes, then the major pathway of chenodeoxycholic acid formation would involve 25-hydroxylated intermediates as observed recently (15). This requires further study.

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Bile Acids in Tissues: Binding of Lithocholic Acid to Protein [1,2]

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ABSTRACT

Human liver contains two forms of lithocholic acid. One form is readily extractable by 95% ethanol/0.1% ammonia (soluble lithocholate, SL), while the other remains firmly bound to the residue (tissue-bound lithocholate, TBL). TBL could be hydrolytically released using clostridial cholanoyl-amino acid hydrolase, suggesting a peptide link between lithocholate and protein. With bovine serum albumin (BSA), lithocholic acid showed spontaneous amino group-modifying activity. When small molecular weight lysine (α-t-BOC-1-lysyl-β-naphthylamide) and arginine peptides (α-CBZ-di-arginyl-β-naphthylamide) were used in place of BSA, lithocholate bound specifically to the lysine peptide. The unusual affinity for lysine suggested that this amino acid might be involved as a residue in TBL. Synthesis of lithocholyl lysines and comparison with products of acid hydrolysis of TBL established ε-lithocholyl lysine as the predominant form in which lithocholic acid is found in tissue bound form.

The primary end products of cholesterol metabolism in the liver are cholic and chenodeoxycholic acids. The final step in this metabolic pathway involves the formation of water soluble conjugates with glycine and taurine. This selective conjugation with glycine and tauring is considered to be a normal function of hepatic cells. However, there are instances where hepatic and extrahepatic factors either alter the relative proportions of the glycine and taurine conjugates or induce the formation of atypical conjugates with other amino acids (basic) such as ornithine and arginine (1-5).

Until recently, it was generally thought that tissue bile acids were readily extractable with solvents such as 95% ethanol containing ammonium hydroxide (6). However, during the course of our studies on the nature of tissue bile acids, we observed that certain tissues, especially cirrhotic livers, yielded insignificant amounts of lithocholic acid, although we expected to see much higher concentrations.

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[2]The systematic nomenclature of bile acids referred to in this report by trivial names are as follows: Cholanic acid, 5β-cholan-24-oic acid; lithocholic acid, 3α-hydroxy-5β-cholan-24-oic acid; 3-epilithocholic acid, 3β-hydroxy-5β-cholan-24-oic acid; glycolithocholic acid, 3α-hydroxy-5β-cholan-24-oyl glycine; 3-ketocholanic acid, 3-keto-5β-cholan-24-oic acid; 12α-hydroxycholanic acid, 12α-hydroxy-5β-cholan-24-oic acid; chenodeoxycholic acid, 3α, 7α-dihydroxy-5β-cholan-24-oic acid; glycochenodeoxycholic acid, 3α, 7α-dihydroxy-5β-cholan-24-oyl glycine; deoxycholic acid, 3α, 12α-dihydroxy-5β-cholan-24-oic acid; glycodeoxycholic acid, 3α, 12α-dihydroxy-5β-cholan-24-oyl glycine; cholic acid, 3α, 7α, 12α-trihydroxy-5β-cholan-24-oic acid, glycocholic acid, 3α, 7α, 12α-trihydroxy-5β-cholan-24-oyl glycine; dehydrocholic acid, 3, 7, 12-triketo-5β-cholan-24-oic acid.

Our subsequent studies led us to suspect that lithocholic acid is firmly bound to the tissue in a form unextractable by the usual solvent systems. Tissues homogenized in 95% ethanol containing 0.1% ammonium hydroxide, when repeatedly extracted with this solvent system, leave a residue (Figure 1). Upon subjecting the residue to enzymatic hydrolysis with cholylglycine hydrolase (from C. perfringens ATCC 19574) (7,8,10), additional amounts of lithocholic acid were released. Since no bile acid was released from the tissue residue in the absence of enzymatic hydrolysis (peptide bond hydrolase), we postulated that this bile acid is probably bound to the tissue covalently through peptide bonds.

On the basis of these observations, an extraction procedure was developed (similar to that outlined in Figure 1) for the isolation of soluble lithocholate (SL) and tissue-bound lithocholate (TBL) from tissues (11). Analyses of human livers obtained at hospital autopsies gave tissue-bound lithocholate in all samples, the values ranging from 4-116 μg/g of liver weight. In six of the twelve tissues analyzed, this represented more than 50% of the total tissue bile acids. Since lithocholate is known to occur in the form of sulfates, we analyzed several samples of liver for bile salt sulfates. No sulfates were detected in the tissue-bound lithocholate fractions, whereas the soluble portion (soluble-lithocholate) yielded varying amounts of lithocholate sulfate. The absence of lithocholate sulfate in tissue-bound form might have some significance, because sulfation is considered to be a protective mechanism against liver injury by toxic monohydroxy bile salts.

In order to determine the nature of tissue-bound bile acids, it was initially necessary to

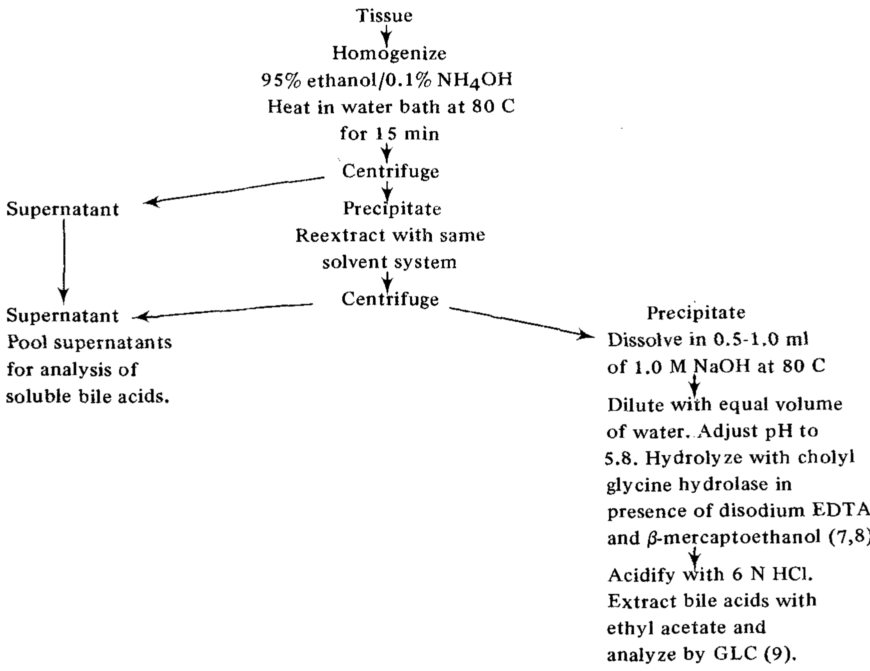

FIG. 1. Isolation of tissue-bound bile acids by cholylglycine hydrolase.

TABLE I

Modification of Bovine Serum Albumin by Bile Acids[a]

Bile acid	Number of $-NH_2$ groups substituted[b]	Relative Activity
Cholanic acid	26	100
Lithocholic acid[c]	19	73
3-Epilithocholic acid	15	57
Glycolithocholic acid	21	81
3-Ketocholanic acid	7	27
12α-Hydroxycholanic acid	12	46
Chenodeoxycholic acid	10	38
Glycochenodeoxycholic acid	5	19
Deoxycholic acid	20	77
Glycodeoxycholic acid	27	104
Cholic acid	19	73
Glycocholic acid	21	81
Dehydrocholic acid	Not Detectable	

[a]The conjugations were performed under identical conditions over a period of 24 hr.
[b]The values are expressed as the number of $-NH_2$ groups substituted per molecule of albumin, assuming a molecular weight of 69,000 and ϵ NH_2 = 9.95 x 10^3 at 335 nm, using TNBS.
[c]This reaction was conducted at pH 9.8.

establish that the enzyme, cholanoylamino acid hydrolase (also termed cholylglycine hydrolase), although active against several naturally occurring bile salt conjugates, is actually capable of releasing lithocholate from peptide linkage with model proteins. 24-[^{14}C]labeled lithocholate coupled to lipid-free bovine serum albumin (BSA), when subjected to the standard enzymatic hydrolysis in the presence of liver homogenate, released free labeled-lithocholate quantitatively (11). Similarly, in another model system, the enzyme cleaved labeled lithocholic acid from [24-^{14}C]lithocholyl polylysine which was synthesized in our laboratory. By analogy, in tissues the enzyme releases lithocholate from its conjugated form with basic side groups of amino acids such as lysine.

By this time, we were intrigued by the

TABLE II

Binding of Glycolithocholate to BLBNA and CBZ-DA-BNA[a]

Test Compound	OD_{540} at various time intervals		
	0	3 hr	24 hr
BLBNA	0.73	0.41	0.44
CBZ-DA-BNA	0.76	0.77	0.71

[a]Resistance to tryptic hydrolysis was measured by coupling Fast Blue B to the β-naphthylamine released by the action of trypsin on the test compound.

possibility that bile acids, under certain conditions, could remain immobilized on protein via peptide bonds. The question now was, what is the nature of the cholanoyl amino acid residue in protein? As far as amino acid residues were concerned, three possibilities involving lysine, arginine or histidine were considered. Since histidine-linked residues are generally unstable, we were left with lysine and arginine. There was also the theoretical possibility of ester bonds with acidic residues in proteins.

Our insight into these problems emerged from parallel studies on bile acid-protein interactions. When purified BSA is modified by coupling with bile acids in the presence of a water-soluble carbodiimide (1-cyclohexyl-3-(2-morpholino ethyl) carbodiimide, metho-p-toluenesulfonate) at pH 8.5-9.0, a significant number of the available free $-NH_2$ groups appear to be blocked (Table I). Amino group modification was followed spectrophotometrically by allowing aliquots of the incubation mixture to react with trinitrobenzene sulfonic acid (TNBS), as described by Habeeb (12). Control experiments were carried out in parallel, in the absence of the carbodiimide coupling agent. Cholanoic acid and a number of its hydroxylated derivatives readily coupled with BSA, while their oxidized forms carrying ketonic functions on the steroid nucleus were either nonreactive or reacted poorly. Furthermore, monohydroxy bile acids such as lithocholic acid exhibited the unusual behavior of being able to carry out amino group modification in the absence of the coupling agent.

In order to determine the extent of nonamide forming side reactions in this system, the uptake of [24-^{14}C]lithocholic acid by BSA was compared with the number of amino groups modified, as determined by the TNBS reaction. For 2.3 μmoles of labeled lithocholic acid bound to BSA, about 2.0 μmoles of $-NH_2$ groups were modified, showing close stoichiometric correspondence between bile acid uptake and amino group modification. From these results, it is apparent that nonamide forming side reactions are minimal.

Since lithocholic acid showed spontaneous amino group modifying activity, a series of experiments were conducted to determine the specificity of the amino acid attached. When lysine and arginine occur in a peptide or in a protein, only the side chain $-NH_2$ groups are available for coupling to carboxylic compounds through a peptide bond or for binding by ionic interaction. In order to simulate this, blocked chromogenic peptides of lysine and arginine (β-naphthylamides) were allowed to react with lithocholic acid. Amino group modification could be followed, either directly by measuring free $-NH_2$ groups by the TNBS reaction, or indirectly, by determining the resistance to tryptic cleavage of the substrate.

The two model peptides used in this test system were α-t-BOC-1-lysyl-β-naphthylamide (BLBNA) and α-CBZ-di-arginyl-β-naphthylamide (CBZ-DA-BNA). BLBNA was synthesized by first coupling α-t-BOC-ε-CBZ-1-lysine (I) to β-naphthylamine (13,14) via the mixed carboxylic-carbonic anhydride procedure (15). The product, α-t-BOC-ε-CBZ-1-lysyl-β-naphthylamide (II), upon catalytic hydrogenation, yielded α-t-BOC-1-lysyl-β-naphthylamide (III) (BLBNA). The synthetic steps are illustrated in the following scheme:

(I) BOC-NH-CH((CH_2)$_4$-NH-CBZ)-COOH + NH_2-(β-naphthyl)

↓ Mixed anhydride procedure

(II) BOC-NH-CH((CH_2)$_4$-NH-CBZ)-CONH-(β-naphthyl)

↓ [H]

(III) BOC-NH-CH((CH_2)$_4$-NH_2)-CONH-(β-naphthyl)

CBZ-DA-BNA was synthesized by the procedure described by Plapinger (13).

The procedure for determining the amino group modifying activity of bile acids using these two peptides is illustrated in the following example with BLBNA.

Trypsin cleaves this bond
when ϵ-NH_2 is free.

(III) BOC-NH-CH-CO | NH-[naphthyl]
α $(CH_2)_4$
NH_2
ϵ

Blocking of ϵ-NH_2 by bile acids confers resistance to tryptic cleavage of -CONH- linkage.

BLBNA (III) is an excellent substrate for trypsin, releasing β-naphthylamine as a product of tryptic activity, provided the ϵ-NH_2 group remains free. The β-naphthylamine released in the reaction is coupled to tetrazotized diorthodianisine (Fast Blue B), giving a diazo dye with an absorption maximum at 540 nm (16). Upon incubating BLBNA with bile acids such as lithocholic acid or glycolithocholic acid, either in the presence or absence of a carbodiimide (coupling agent), the bile acid could presumably couple into or bind to the ϵ-NH_2 group of BLBNA. As the ϵ-NH_2 group is progressively modified during the course of the incubation, the product becomes refractory to tryptic hydrolysis. We used this system to determine the -NH_2 group modifying property of glycolithocholate towards the lysine and arginine peptides. The data presented in Table II show that glycolithocholate preferentially attacks the ϵ-NH_2 of lysine. These results were verified by direct measurement of free amino groups (Table III).

These studies on lithocholate-peptide interactions showed that this bile acid has a strong affinity towards lysine residues. Now, our approach to the tissue-bound lithocholate phenomenon underwent some reexamination. It appeared to us that lithocholyl lysine might be involved as a residue in TBL. N-α-lithochoyl-l-lysine, N-ϵ-lithocholyl-l-lysine and N-α-ϵ-bislithocholyl-l-lysine were synthesized and characterized by mass spectrometry, infrared spectroscopy, and thin-layer chromatography (TLC) (11). The isomeric N-α- and N-ϵ-lithocholyl lysines could be readily distinguished by their mass spectra, since only the N-α-substituted compound gave evidence for the formation of a steroid lactam (17). In addition, on TLC as expected, only N-ϵ-lithocholyl lysine gave a ninhydrin positive spot. Human livers known to contain significant amounts of TBL were hydrolyzed with 6 N HCl and subjected to TLC. These samples gave a ninhydrin positive spot corresponding to ϵ-lithocholyl lysine.

TABLE III

Binding of Lithocholic and Glycolithocholic Acids to BLBNA[a]

Bile Acid	OD_{335} nm 0	24 hr	Δ OD
Lithocholic acid	0.55	0.31	0.24
Glycolithocholic acid	0.54	0.15	0.39

[a]Amino group modifying activity was measured by the TNBS reaction.

Isolation of this material from TLC, followed by enzymatic hydrolysis with cholanoylamino acid hydrolase, gave lithocholic acid. Upon co-chromatography of this substance with ϵ-^{14}C-lithocholyl lysine (prepared from ^{14}C-lithocholyl polylysine), all of the radioactivity was recovered from the ninhydrin positive zone. From the evidence, it was concluded that ϵ-lithocholyl lysine is the predominant residue in TBL.

Among the naturally occurring bile acids, lithocholic acid is unique. It exhibits unusual cellular toxicity, perhaps a manifestation of its strong affinity towards cellular proteins. The absence of lithocholic acid sulfate in tissue-bound form is also interesting because of the widely held view that sulfation is an intrinsic mechanism to protect the liver from toxic monohydroxylated bile salts.

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Bile Acids LVII. Analysis of Bile Acids by High Pressure Liquid Chromatography and Mass Spectrometry

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ABSTRACT

Several high pressure liquid chromatographic methods for the separation of conjugated and free bile acids are presented. A mixture of synthetic conjugated bile acids has been separated by reverse-phase systems consisting of either a Waters Associates' "fatty-acid analysis" or a μBondapak/C_{18} column eluted with a mixture of 2-propanol/potassium phosphate buffer (pH 2.5 or 7.0). The major conjugated bile acids present in the gallbladder bile of obese subjects have been analyzed each in less than 30 min and quantitated with a U.V. detector set at 193 nm. Some of the 5α- and 5β-isomers of conjugated bile salts could be resolved in straight-phase systems on Corasil II or μPorasil columns. Mass spectra of the conjugated bile acids obtained by electron impact were characteristic of the type of amino acid attached to the side chain, and the number of hydroxyl substituents on the nucleus. Most of the isomers could readily be differentiated by the relative intensities of the fragment ions.

Bile acids usually occur naturally in conjugation with glycine or taurine. The classical technique for the analysis of these conjugates involves vigorous basic hydrolysis and derivatization for analysis by gas liquid chromatography (GLC) (1). Disadvantages of this method include prolonged reaction time, loss of material, and production of artifacts (1-3). Attempts to resolve the conjugated compounds by other means, e.g., paper, thin layer, ion-exchange, column and GLC and electrophoresis (3,4) were less successful because of inadequate separation.

Recently, enzymatic (hydroxysteroid dehydrogenase) (5-7), and immunochemical methods (8-11), and an improved thin layer chromatographic solvent mixture (12) have greatly simplified in each case the procedure of measurement, improved the sensitivity of detection, or permitted the separation of the isomeric dihydroxy derivatives. However, none of these methods provides a rapid means for identification and quantitation of *each* conjugated bile acid. High pressure liquid chromatography (HPLC) offers the versatility needed for such a system of analysis. In the last two years, a number of publications (4,13-22) have appeared concerning this particular application of HPLC, with special emphasis on the use of octadecyl silyl bonded columns as a stationary phase in reverse-phase chromatography.

Okuyama et al. (16) were the first to report the separation of taurolithocholate from taurocholate on a μBondapak/C_{18} column with a mixture of methanol and 0.01 M potassium acid phosphate, and used as a detector a U.V. monitor set at 210 nm; however, taurodeoxycholate and taurochenodeoxycholate were eluted as a single peak. With a MicroPak NH_2 column, p-nitrobenzyl esters of the glyco analogs were resolved (16) with a mixture of methylene chloride and isooctane. Parris (17) utilized a DuPont ODS column to resolve taurocholate, taurodeoxycholate and taurolithocholate with a mixture of methanol, water and phosphoric acid at pH 2. Laatikainen et al. (18) and Bloch and Watkins (19) independently utilized a μBondapak/C_{18} column with a mixture of methanol, acetic acid and sodium hydroxide at pH 4.7 to separate taurocholate, glycocholate, taurochenodeoxycholate, taurodeoxycholate, glycochenodeoxycholate, and glycodeoxycholate in that order from human gallbladder bile. Shimada et al. (20) and Goto et al. (21) separated mixtures of free bile acids, glyco- and tauro-derivatives by thin layer chromatography (TLC), and then utilized a μBondapak/C_{18} column, and a mixture of 0.3% ammonium carbonate and acetonitrile (9:4) to separate taurochenodeoxycholate, taurodeoxycholate and taurolithocholate. Tauroursodeoxycholate and taurocholate were eluted first as a single peak, but this fraction was separated by rechromatography with the same solvents in the ratio 11:4. Their glyco-conjugates were separated similarly. Baker et al. (22) have reported the use of a straight-phase system in the gradient mode (chloroform with a mixture of ethyl acetate and ethanol) to effect the separation of conjugates by the number of hydroxyl substituents, but were unsuccessful in resolving conjugates of chenodeoxy- and deoxycholates.

We found that the separation of a mixture of samples of synthetic conjugated bile acids (CBA) could be achieved in less than 30 min (13) by the use of either a "fatty acid analysis" column or μBondapak/C_{18} column (each column 4 mm x 30 cm, Waters Associates) with a

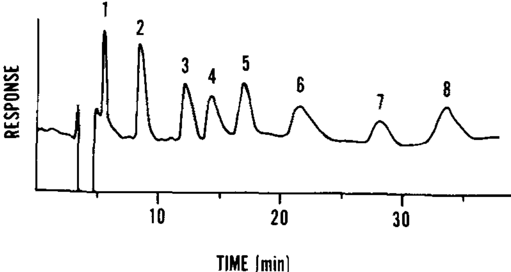

FIG. 1. Separation of a mixture of synthetic conjugated bile acids on a Waters Associates' "fatty acid analysis column" with solvent R_1. Peak 1 tauro-α-muricholate; 2 taurocholate; 3 taurochenodeoxycholate; 4 taurodeoxycholate; 5 glycocholate; 6 taurolithocholate; 7 glycochenodeoxycholate; 8 glycodeoxycholate.

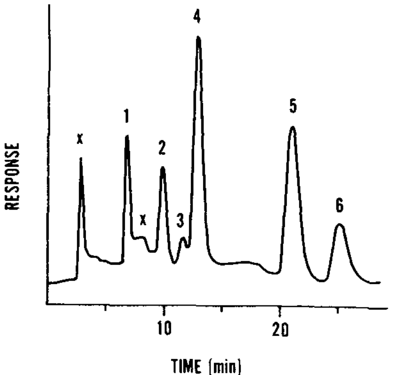

FIG. 2. HPLC of a deproteinated human gallbladder bile on a "fatty acid analysis column" with solvent R_1. X unknowns; 1 taurocholate; 2 taurochenodeoxycholate; 3 taurodeoxycholate; 4 glycocholate; 5 glycochenodeoxycholate; 6 glycodeoxycholate.

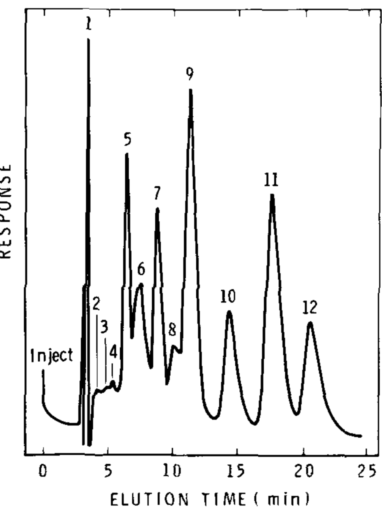

FIG. 3. HPLC of a deproteinated human gallbladder bile on a "fatty acid analysis column" with solvent R_1, demonstrating the material (peak 10) with mobility like taurolithocholate. Peaks 1-4 and 6 unknown; 5 taurocholate, 7 taurochenodeoxycholate; 8, taurodeoxycholate; 9 glycocholate; 11 glycochenodeoxycholate; 12 glycodeoxycholate.

Waters Model ALC 201 instrument equipped with a U6K loop injector and a model R401 differential refractometer or a Schoeffel Model 770 Spectroflow UV monitor with a water-jacketed lamp (13-15). The refractometer was set at attenuation 4X or 8X. The solvent mixture (R_1) was composed of 2-propanol/8.8 mM potassium phosphate buffer, pH 2.5 (160:340), flowing isobarically at 1 ml/min. The solvent system was degassed, saturated with nitrogen and again degassed prior to use. The organic solvent must be of high purity, and the water employed was deionized and doubly distilled in an all glass apparatus.

Figure 1 shows a separation of several tauro- and glyco-conjugates which may be found in human or rat bile. Note the elution of the tauro-derivatives generally before the glyco compounds; the more polar glycocholate, however, was eluted just before the less polar taurolithocholate. Tauroursodeoxycholate (relative retention volume, RR_V, 0.53, expressed in regard to standard taurodeoxycholate whose retention volume was 10.5 ml) was eluted before taurocholate (RR_V 0.62) (14) in agrement with the observations of Shimada et al. (20) and Bloch and Watkins (19). Material from each of the identifiable peaks was identical in mobility to a standard on TLC (13). Samples of synthetic CBA exhibited a linear response with the differential refractometer and with the UV monitor set at 192-3 nm and 0.02 AUF. The UV monitor was 30 to 40-fold more sensitive than the refractometer; the lower limit of detection of the UV monitor was at least 0.1 nmole for the tauro-conjugates and 0.2 nmole for the glyco-derivatives.

Analyses of human bile samples were carried out with samples deproteinized with ethanol (Fig. 2); the UV monitor was used in this case. The material eluted in each of the labeled peaks was identified by its mobility with a standard on TLC. Additional evidence was obtained for two of the substances by co-elution in HPLC with added radioactive taurochenodeoxycholate or glycocholate. Final confirmation of the substances eluted in HPLC awaits completion of mass spectral studies. Several peaks of unidentified materials may contain substances related to medication of the patients, and these were not further investigated.

A few observations of interest were noted

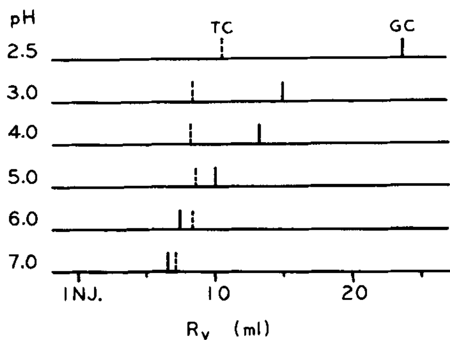

FIG. 4. Effect of pH on the retention volumes (RV) of taurocholate (TC; dashed line) and glycocholate (GC; solid line). Conditions; a μBondapak/C_{18} column; solvent R_1 with pH of the phosphate buffer preadjusted as indicated in the diagram.

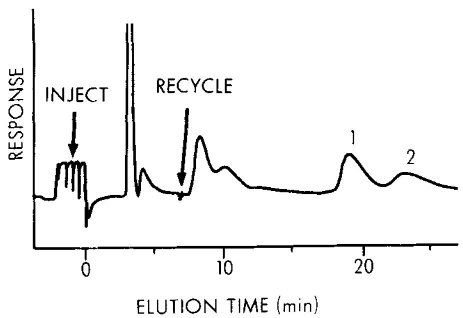

FIG. 5. Separation of glycoallodeoxycholate (peak 1) and glycodeoxycholate (peak 2) by recycling on five sequential columns of Corasil II of 1/8" x 2' each, with acetonitrile/acetic acid 400:10 at 2 ml/min isobaric flow. Multiple injection through septum by the stop-flow technique.

TABLE I

Retention Volumes (ml) of Common Conjugated Bile Salts in Neutral Medium

μBondapak/C_{18}, 4 mm x 30 cm; 2-Propanol/10 mM potassium phosphate buffer, pH 7.0

	System R_2 160:340	System R_3 300:200
	Tauro-	Methyl Glyco-
Cholate	6.4	7.0
Chenodeoxycholate	10.0	9.6
Deoxycholate	12.8	11.6

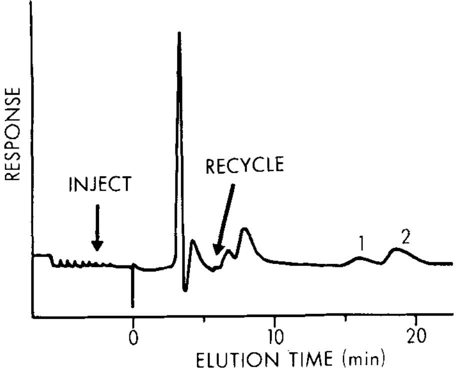

FIG. 6. Separation of glycoallolithocholate (peak 1) and glycolithocholate (peak 2) by recycling on HPLC. Conditions as for Fig. 5.

during the analyses of samples of gallbladder bile from 22 obese patients at surgery for small bowel by-pass. The ratio of glycine to taurine was constant in the cholate and chenodeoxycholate fractions (r = 0.5792; $p < 0.01$). One patient undergoing revision of small bowel by-pass had a significantly lower total bile acid concentration (at least 10-fold). Eight patients exhibited significant quantities (7-15% of total CBA) of a material with HPLC mobility similar to taurolithocholate (Fig. 3), but the material did not exhibit the characteristic stain with phosphomolybdic acid on a TLC plate. Two of the eight subjects suffered from cholelithiasis and choledocystitis; three had hypertension, two were diabetics, and one had psychiatric problems. The identity of this material is currently under investigation.

Changes in the ionic strength of the phosphate buffer from 7.5 to 8.5 mM at pH 3.1 appeared to have no effect on the retention volume (13) in agreement with findings of others (18-20). On the other hand, alteration of pH caused pronounced differences in the elution time of the glyco-derivatives (Fig. 4). Thus, glycocholate is eluted after taurodeoxycholate at pH 2.5 (13), but before taurochenodeoxycholate at pH 4.7 (18,19). An increase in pH resulted in unsatisfactory separations in our systems, but the deleterious effect of an acidic medium on the usable life of a bonded reverse-phase column necessitated employment of a more neutral pH range. Since the tauro-conjugates are not esterified (23,24) under conditions which provide methyl esters of the glycoconjugates (23), an investigation was carried out to separate these components at neutral pH. Table I shows the retention volumes on μBondapak/C_{18} of several tauro-conjugates and the methyl esters of their glyco-conjugates in solvent systems containing 2-propanol/10 mM phosphate buffer, pH 7.0, with different ratios of the components (R_2=160:340 and R_3=300:200). Replacement of the phosphate buffer with water in R_2 gave essentially similar results. Thus, in system R_2 the methyl esters are not eluted because the solvent is too polar,

TABLE II

Differentiation of Conjugates of Bile Acids

A. Chenodeoxycholic and Deoxycholic Acids									B. Conjugates of 5β- and 5α- Bile Acids										
	Glyco-			Methyl Glyco-			Tauro-			Glyco-			Methyl Glyco-			Tauro-			
m/e	CDC[a]	DC[a]	m/e	CDC	DC	m/e	CDC	DC	m/e	C[a]	AC[a]	m/e	DC	ADC[a]	m/e	CDC	ACDC[a]	DC	ADC
398	*45%*	*4%*	412	*22%*	*5%*	355	*100%*	*18%*	271	*91%*	*100%*	273	*29%*	*99%*	355	*100%*	*66%*		
255	45	*100*	255	22	98	340	56	4	253	*100*	50	255	98	53	273			*17%*	*100%*
117	*100*	46				301	42	6							255			*100*	4
						255	81	*100*							91	40	*100*		

[a]CDC=chenodeoxycholate; DC=deoxycholate; C=cholate; A=allo- (5α-).

whereas with system R_3 the tauro-derivatives are eluted in the solvent front; clearly the system R_2 can be followed by R_3 in a gradient mode to provide each of the components. Recent studies indicate that use of a gradient mode achieves the separation of all the major CBA of human bile; the UV monitor was used at 193 nm (13 and Shaw and Elliott, unpublished observations).

Application of HPLC to the isolation, quantitation, and determination of purity of free bile acids may be a useful complement to the existing chromatographic techniques. Numerous reports on the HPLC of free bile acids and their derivatives have appeared. Jefferson and Chang (25) achieved the resolution of methyl esters of several dihydroxy and trihydroxy bile acids on a μPorasil column eluted with a mixture of ethyl acetate and hexane. Shaikh et al. (26) investigated the mobilities of lithocholic acid and its 3β-isomer derivatized with UV-active tags (p-nitrobenzyl and p-chlorobenzoyl esters) on columns containing silica. Stellaard et al. (27) recently reported the separation of several bile acids as their phenacyl esters on a similar type of column. Parris (17), Okuyama et al. (16) and Shimada and coworkers (20) used reverse-phase systems similar to those described earlier for the conjugates, but were unable to separate the dihydroxy acids as their p-nitrophenyl esters (17), or cholate and chenodeoxycholate (16), or cholate and ursodeoxycholate (20), respectively. In our laboratory, about sixty of these compounds have been analyzed on μBondapak/C_{18} columns in solvents R_2, R_4 (130:370), and R_5 (200:300). Besides good resolution of cholic, chenodeoxycholic, deoxycholic and lithocholic acids with RR_V of 0.51, 0.81, 1.00 and 1.88, respectively, several general conclusions can be made: (a) orders of elution are trihydroxy > dihydroxy > monohydroxy; 3-OH > 6-OH ~7-OH > 12-OH; 3β > 3α; 7β > 7α; 6α > 6β; and (b) C-5 epimers were usually not separable (28).

Tauro- and glyco-conjugates of the allo- (or 5α-) bile acids have been prepared and initial studies on their mobility in HPLC have been carried out. By reverse phase chromatography with system R_2, conjugates of the 5β-acids were generally not separable from the 5α-isomers. In straight phase systems, the glyco-conjugates were resolvable upon recycling in solvent mixtures containing acetonitrile, acetic acid, and sometimes water (28). Figures 5 and 6 illustrate the separation of glyco-5β- and 5α-deoxycholate and glyco-5β- and 5α-lithocholate, respectively, after two cycles, and suggest the utility of HPLC to separate such materials more rapidly than existing techniques. Tauro-conjugates of

the C-5 epimers are not readily resolved in these systems, but partial resolution in certain cases could be achieved in a slightly alkaline medium (28). In these cases, straight-phase chromatography was carried out on five sequential columns of Corasil II (each 1/8"x2'), at an isobaric flow rate of 2 ml/min; the solvent mixture contained 2-propanol/ethyl acetate/water/7N ammonium hydroxide (260:600:50:3).

Because of the known advantages of coupling HPLC with mass spectrometry to provide characterization and identification of separated components of mixtures, the electron impact spectra of synthetic CBA of the 5β- and 5α-series were examined (23). Spectra were obtained via the direct probe in an LKB Model 9000 mass spectrometer coupled to a data acquisition system (23). Spectra of glyco-conjugates of deoxycholic (29), cholic, chenodeoxycholic, (30), lithocholic and their allo (5α) analogs exhibit small molecular ions with subsequent losses of molecules of water, the fragments [$NH_2CH_2CO_2H$] (75 amu) and [CH_2=C(OH) $NHCH_2CO_2H$] (117 amu) (McLafferty rearrangements), the side chain and elements of ring D, and finally the cleavage of the nucleus, as observed with the free bile acids (29). The methyl esters of the glycoconjugates fragmented similarly but with larger molecular ions. The tauro-conjugates were vaporized at higher temperatures with subsequent absence of molecular ions. Significant losses in conjuction with water molecules were the fragments [CH_2=$CHSO_3H$] (108 amu), [CH_2=C(OH) $NHCH_2CH_2SO_3H$] (167 amu), the side chain and elements of ring D. Table IIA shows the difference in abundance of fragment ions derived from the conjugates of the two common dihydroxy 5β- bile acids, which may be useful in identification of these acids. Table IIB provides similar data for conjugates of epimeric pairs of several 5β- and 5α-bile acids (23).

These data indicate the feasibility of the use of HPLC and mass spectrometry for the direct analysis of conjugated bile acids, especially in small samples of biological fluids, such as bile. Further refinement of the methodology and extension to the analysis of serum samples is an objective of this laboratory.

ACKNOWLEDGMENTS

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Bile Acids LVIII. Bile Acids and Colorectal Cancer

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ABSTRACT

Significant correlations have been reported by epidemiologists between the mortality from colorectal cancer in various populations and the consumption of meat or lipids by these populations. These have directed considerable attention to possible relationships between diet and the occurrence of this neoplasm. We have carried out studies of the composition of bile from rats as influenced by diets of varying lipid content. Two cannulas were surgically implanted to form an externalized bile duct through which bile was drained from the common duct and returned to the duodenum. Small aliquots were analyzed for total bile acids by enzymatic assay and for individual bile acids by high-pressure liquid chromatography, gas chromatography and gas chromatography-mass spectrometry. Animals consuming diets highest in lipid content provided bile with the greatest amounts of bile acids. The primary bile acids, taurocholic, taurochenodeoxycholic, and tauro α- and β-muricholic acids made up more than 99% of the 3α-hydroxy bile acids and were found in approximate molar ratio of 2:1:1. Either complete drainage of bile without return to the duodenum, or biliary tract obstruction had pronounced influence on the rate of secretion of bile and its composition.

Cancer of the lower intestinal tract is a major health problem in the United States and many other countries. In this country during the present year, it is estimated that in excess of 100,000 new cases of cancer of the colon and rectum will be diagnosed (1). Finding a cause and a cure for a disease of this prevalence which accounts for 13% of all cancer-related deaths is among the highest priorities in health care.

As world-wide epidemiological studies of colorectal cancer have been undertaken, it has become increasingly apparent that mortality from this group of neoplasms has wide geographical variation. New Zealand, for example, has an annual mortality rate in excess of 25 per 100,000, whereas El Salvador has a rate ca. 1 per 100,000 per year (1). Among the many differences in world populations which could give rise to such a spectrum of mortalities are those of industrialization, farming practices, sanitation, health care, social customs, ecologic pollutants, gene pools, dietary habits, alcohol consumption and a whole host of others. The numbers of complexities of the variables have added greatly to the difficulty of arriving at a statistical identification of an etiologic agent for this cancer.

In 1968 Haenszel and colleagues published a comparison of the mortality rates of Japanese immigrants to the U.S. and relatives who remained in Japan (2). Within about two decades of their establishing residence in the U.S., immigrants had begun to assume the higher risk of mortality from colorectal cancer experienced by the native U.S. population, in contrast to the lower mortality rate of their country of origin. Children of the immigrants had a mortality rate that was undetectably different from that of the native U.S. population.

Dietary habits of immigrant Japanese living in Hawaii who had contracted colorectal cancer were the subject of a further study by Haenszel et al. (3). Significant associations were noted between the risk of this malignancy and the length of adaption to western culture, particularly western diet. Stratification of the data led these investigators to infer the strongest associations of risk among the cohorts to lie with the frequency in their use of beef, legumes, and certain sources of starch. Other demographic

TABLE I

Composition of Diets of Variable Lipid Content

Constituent	Range of Weight
	(g/kg diet)
Casein (20% of calories)	197 to 226
DL-Methionine (10% of wt of casein)	2.0 to 2.3
Salt mixture #446[a]	50
Water-soluble vitamins[b]	50
Nonnutritive fiber	20
Fat-soluble vitamins[c]	0.5
Lipid (corn oil or lard)	45 to 160
Powdered sucrose	635 to 491
Calories per kg	3941 to 4517

[a]1.5 mg $(NH_4)_2MoO_4$ added to each 50 g of salt mixture.

[b]Water-soluble vitamin mixture: each 50 g of mixture provides the following amounts. Folic acid, 0.5 mg; thiamine, 5 mg; pyridoxine, 5 mg; riboflavin, 10 mg; p-aminobenzoic acid, 10 mg; menadione, 5 mg; niacinamide, 25 mg; calcium pantothenate, 50 mg; inositol, 500 mg; choline chloride, 4 g; biotin, 0.5 mg; vitamin B_{12}, 0.1 mg.

[c]Fat-soluble vitamin mixture: the following ingredients were dissolved in sufficient light paraffin oil to provide 0.5 ml of the mixture. 16.3 mg (16,300 IU) vitamin A palmitate; 0.041 mg (1640 IU) vitamin D_3; and 19.2 mg (19.2 IU) DL-α-tocopheryl acetate.

studies have shown correlations between per capita consumption of meat (4) or lipids (5) and the incidence or mortality of colorectal cancer in various countries of the world. Since beef alone among several dietary meats, provided high relative risk, Haenszel et al. surmised that the quality of fat, in terms of fatty acid content or contaminating sterols, might be more important than quantity.

As a corollary to the finding that consumption of lipids may be a predisposing factor to the malignancy in question, we became interested in whether lipid content of the diet fed to rats may play a role in the kinds and amounts of bile acids secreted by the liver. The primary bile acids of the rat are cholic, chenodeoxycholic and α- and β-muricholic acids which are conjugated primarily with taurine prior to secretion.

These conjugates pass from the liver via the common (bile) duct to the duodenum where their surface-active properties play an important role in the digestion and absorption of dietary lipids. Having fulfilled this role, they continue their transit down the digestive tract where, in the presence of microorganisms of the lower small intestine, the conjugates become hydrolyzed and some become metabolically altered to provide secondary bile acids. In the region of the ileum, about 80% of the free bile acids are absorbed into the hepatic portal circulation to be returned to the liver via a process known as enterohepatic circulation.

The hypothesis which we undertook to investigate was that diet manifests an influence on composition of bile, particularly with respect to the kinds and amounts of bile acids. In most studies on bile composition that have been previously reported, bile has been totally collected from experimental animals via a cannula to the bile duct. It was our interest, however, to establish a model in which changes in bile composition could be observed in the animal with an uncompromised enterohepatic circulation. To achieve this, 250 g female Sprague-Dawley rats had two cannulas surgically implanted. One of the cannulas conducted bile from the common duct to the exterior of the animal, and the second was a conduit for return of bile from outside the animal to the upper portion of the duodenum, depositing the bile about 3 cm distal to the pylorus. Bile was collected over two time periods – 8:30 a.m. to 4:30 p.m. and 4:30 p.m. to 8:30 a.m. – while the animal was maintained in a restraining cage with free access to food and water. The bile was collected in sterile, ice-cold vessels and, after thorough mixing, a small sample (0.5 or 1.0 ml) was withdrawn for later analysis. The bulk of

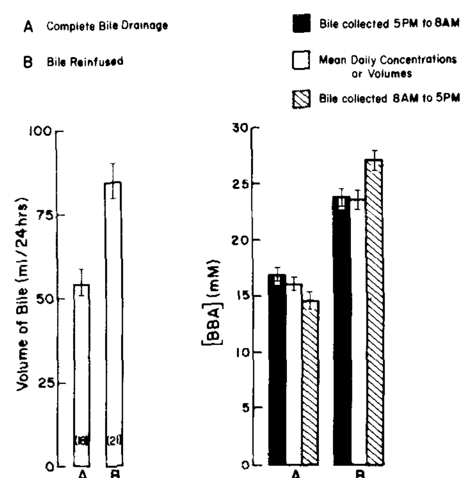

FIG. 1. The diurnal variations in volume of bile secreted and in the concentration of biliary bile acids ([BBA]) from a rat fed laboratory chow. None of the bile drained from the cannulated common duct was returned to the animal during the first 18 post operative days. Beginning on day 18, bile was returned via a cannula to the duodenum. The span of each vertical line measures the limits of one standard error above and below the mean, which is given by the total height of a bar.

the collected bile was placed in a syringe pump and returned via the second cannula to the small intestine at a selected rate. Each analytical sample was immediately mixed with 5 vol of absolute ethanol and stored at -20 C. For determination of bile acid content, an aliquot of the ethanolic sample was freed of protein by centrifugation and dried thoroughly at 60 C under a stream of nitrogen. The dried residue was dissolved in buffer and assayed for bile acids using 3α-hydroxysteroid dehydrogenase (3α-hydroxysteroid:NAD^+ oxidoreductase; E.C. 1.1.1.50) (6).

Animals were maintained on diets with a range of lipid compositions between 4.5% and 16% prepared with either corn oil or lard (Table I).

Experiments were initiated to ascertain whether preservation of the enterohepatic circulation provides information different from that obtained from an animal undergoing total biliary drainage. A rat prepared with two cannulas was maintained for 40 days on a diet of Purina Laboratory Chow. For the first 18 days, the bile was drained from the animal and not returned. On the 19th day of the experiment, bile infusion via the return cannula was begun, and the animal continued in this way for the remainder of the experiment. The data shown in the left hand set of bars in Figure 1 depict the nearly 50% greater daily mean volumes of

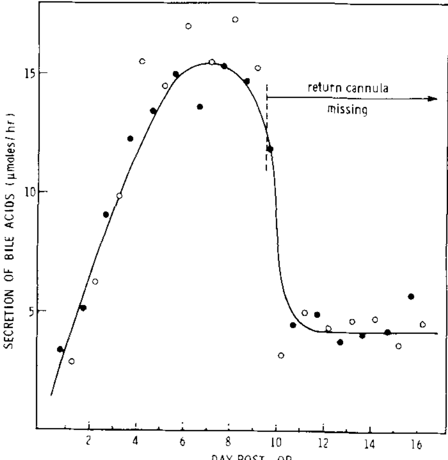

FIG. 2. A rat was maintained on 4.5% lard diet for 55 days before surgical emplacement of drainage and return cannulas. The rates at which bile acids were secreted by the liver were measured over the duration of each daytime (open circles) or nighttime collection period (black circles). During the evening collection of day 9-10 postoperatively, the rat chewed the return cannula where it entered the body, making return of the bile no longer possible.

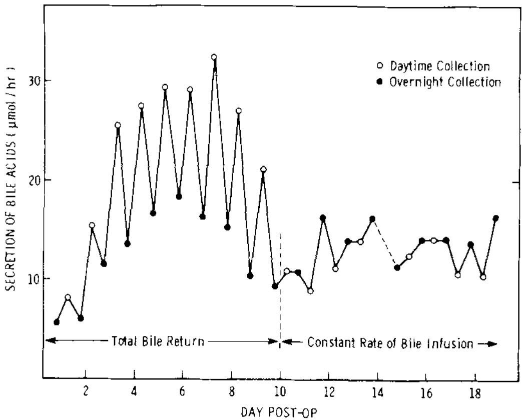

FIG. 3. A rat was maintained on 4.5% lard diet for 38 days prior to surgical emplacement of drainage and return cannulas. For the first 10 days postoperatively, the total volume of bile collected during the 8 hr day period was infused throughout the subsequent 16 hr overnight period, and the bile collected during the overnight period was returned to the duodenum throughout the following daytime period. Beginning day 11, the collected bile was divided and infused in such a way that the rat received bile at a nearly constant rate of return during both day and night.

bile secreted if bile was reinfused to the rat compared to those during complete drainage. The right hand set of bars indicates that the mean daily concentrations of biliary bile acids were also about 50% greater if bile infusion was practiced.

The diurnal fluctuation in concentrations of the bile acids was significant under both complete bile drainage and bile return but, interestingly, the changes were in opposite directions. The daytime collections provided lower concentrations than the overnight collections where bile was totally withdrawn; the reverse was true when bile was returned to the animal.

These results were verified in another experiment in which a rat, adapted to a diet containing 4.5% lard, was surgically fitted with cannulas. The postoperative course of bile acid excretion is shown in Figure 2. Over the first 5 days following surgery, there was a rapid increase in the amount of bile acids excreted, approaching a nearly constant value of ca. 15 µmoles/hour. On the 10th postoperative day, the animal destroyed the return cannula so that bile could not be returned to the small intestine. Immediately the amounts of bile acids secreted fell to a low, but relatively constant rate of about 5 µmoles/hour.

In consideration of the obvious effect of bile return on bile secretion, attention was turned to the diurnal fluctuation in the appearance of bile acids. In the initial experiments (e.g., Fig. 1), bile collected during an 8 hr daylight period, less the small sample removed, was infused during a 16 hr overnight period; bile collected overnight was infused during the shorter daytime period. In one experiment (Fig. 3), an animal maintained on 4.5% lard diet was infused for the first 10 days postoperatively in the manner just described. Beginning day 10, the infusion was changed so that approximately equal volumes of bile were returned each hour both day and night. Each overnight collection was divided and one-half of its volume was returned to the animal during the 8 hr daytime period. The remaining half was retained in the cold, mixed with the bile collected during the daytime period, and the mixture infused throughout the 16 hr overnight period. The results show a marked diminution in the variations between day and night samples upon institution of uniform infusion. This observation, with those gained from the experiments cited earlier, emphasize the great importance of enterohepatic return of bile acids as a mechanism for maintaining the volume of bile and the concentrations of bile acids being secreted by the liver.

In another experiment a rat was maintained on a rotation of diets during which time bile was sampled and returned to the small intestine at a uniform rate. Figure 4 shows the variation of mean daily concentration of bile acids from this animal. During a 6 to 8 day postoperative period, the concentration of bile acids rose

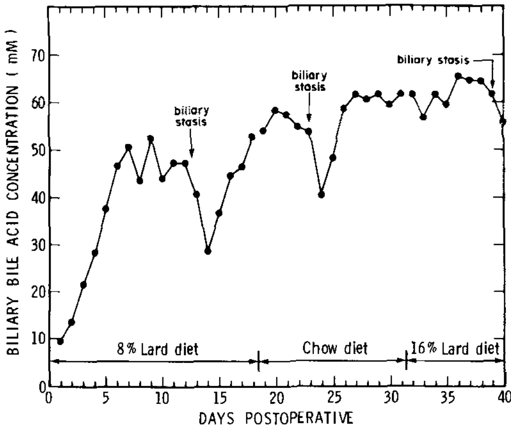

FIG. 4. The concentrations of bile acids were measured in the bile of a rat maintained for 18 days prior to surgery on 8% lard diet and for 18 days postoperatively before being rotated through the diets indicated. Each point is the concentration of bile acids that would have been found had the bile been collected as a single 24 hr sample.

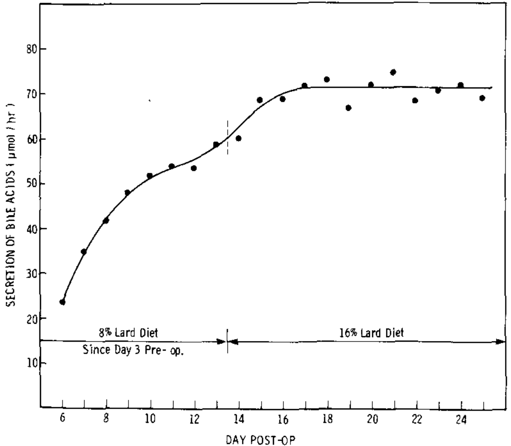

FIG. 5. A rat fed 8% lard diet for 3 days had cannulas surgically implanted in the common duct and the duodenum. For 13 days postoperatively, the animal was continued on 8% lard diet and then switched to 16% lard diet for the remainder of the experiment. The rates at which bile acids were secreted by the liver were averaged for each 24 hr period.

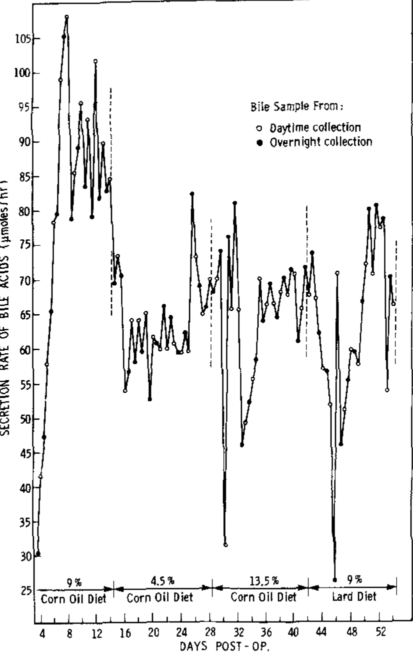

FIG. 6. Plot of secretion rate of bile acids with change in dietary regimen. After adaptation to a 9% corn oil diet (88 days), a rat had cannulas surgically implanted in the common duct and duodenum. The animal was continued on the 9% corn oil diet for 13 additional days and then rotated through the other diets shown. The plunges in secretion rate on days 30, 33, 46, 47 and 53 resulted from temporary bile stasis in the cannula draining the common duct.

rapidly. Beyond this time there were relatively slight alterations in concentration with changes in dietary regimen. On a diet containing 8% lard, the animal produced bile with a bile acid concentration of about 46 mM; the concentration rose to 60 mM as the diet was changed to laboratory chow and then to a diet containing 16% lard. Much more marked were the excursions in bile acid concentration occasioned by three seperate, temporary occlusions of the bile cannula by particulate matter in the bile. Each stasis of the bile flow lasted only a few hours until it was discovered and cleared, and led to only moderate decline (ca. 25%) in the volume of bile collected during that period. The recovery from each of the first two episodes (days 12 and 23) required about 48 hr for the bile acid concentration to return to its former value.

In another experiment (Fig. 5) the rates of secretion of bile acids varied with lipid content of the diet in a manner similar to that observed for concentrations of bile acids (Fig. 4). On an 8% lard diet, the rate of secretion began to plateau slightly above 50 μmoles/hr and increased 25% as the lard content of the diet was doubled. Biliary stasis was not observed in this experiment.

Results from corn oil diets (Fig. 6) were similar to those in which the lard content was varied. After an 8 day postoperative rise, the biliary secretion rate became constant at ca. 87 μmoles/hr on the 9% corn oil regimen. When the lipid content was diminished to 4.5%, the secretion rate fell to 62 μmoles/hr, but increased to about 69 μmoles/ hr when the corn oil

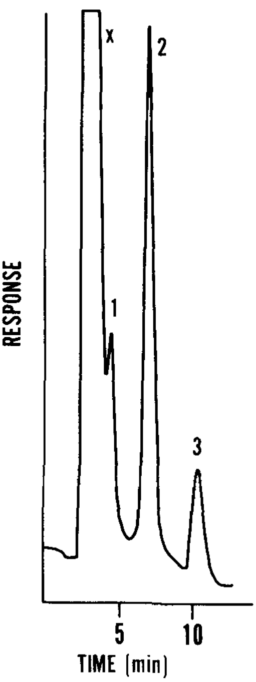

FIG. 7. High pressure liquid chromatogram of a sample of deproteinized rat bile. Peaks have been identified as: (a) a mixture of tauromuricholic acids; (b) taurocholic acid; (c) taurochenodeoxycholic acid. The peak designated X contained unknown materials.

level was raised to 13.5%. During the periods of consumption of the 13.5% corn oil and 9% lard diets, the animal experienced several episodes of biliary stasis. Although weight of diet consumed could not be accurately measured, it was felt that this animal did not have equivalent daily caloric intakes of the diet containing 13.5% corn oil and those of lower lipid content, especially of the 9% corn oil diet to which the animal had been accommodated for 3 months prior to surgery. In consideration of the frequent occlusions of the bile cannula in the final period, no useful information was obtained relative to changes in rate of bile secretion as the diet was changed from corn oil to lard.

The composition of rat bile with respect to the content of individual conjugates was studied using high pressure liquid chromatography (HPLC) with the solvent system and column conditions reported by Dr. Shaw in the preceding presentation (7). A chromatogram of rat bile is shown in Figure 7. For many samples of rat bile that have been analyzed by this technique, the bile acid pools appear to have relatively constant composition irrespective of the diet. Between 20 and 25% of the total bile acid content is a mixture of the muricholates (the 3α, 6β, 7β- and 3α, 6β, 7α-trihydroxycholanic acids), another 50 to 55% is taurocholate, and a relatively constant 25% is taurochenodeoxycholate. The isomeric tauromuricholates are not separated by this system of HPLC. Only traces of glycine conjugates were found.

For analysis of samples of bile acids by gas chromatography, bile samples were hydrolyzed with alkali, extracted, chromatographed on Amberlite XAD-2, and the bile acids were methylated with 2,2-dimethoxypropane and converted to their trimethylsilyl ethers. After chromatography on 3% OV-17 at 250 C, the trimethylsilyl ethers of methyl α-muricholate, cholate, chenodeoxycholate, and β-muricholate were found. These derivatives were accompanied by small amounts of substances with longer retention times, some of which were nonsteroidal. Identification of these biliary constituents was achieved by means of a gas chromatograph – mass spectrometer – data acquisition system (7). Fragment ion chromatograms, mass spectra, and relative retention times of these derivatives were compared with similar data from authentic compounds. In addition to the four primary bile acids, 3-oxo-7α, 12α-dihydroxy-5β-cholanate and 12-oxo-3α, 7α-dihydroxy-5β-cholanate, both bacterial metabolites of cholate, were detected. Small quantities of additional components have yet to be fully identified.

It is perhaps well to consider how the bile acids might initiate or influence carcinogenesis in the intestinal tract, and whether diet could play any role in the process. An often cited possibility is the conversion of bile acids to phenanthrene-like compounds under the anaerobic conditions of the lower intestine. Wieland and Dane in 1933 (8) apparently achieved such a conversion in the laboratory when they carried out a dry distillation of 12-ketocholanic acid. The resulting dehydronorcholene could be converted by treatment with selenium to the carcinogen, methylcholanthrene.

There has been speculation that such a process might be duplicated by intestinal flora; however, none of the many compounds resulting from action of microorganisms on bile acids is a hydrocarbon of the phenanthrene series (9). Knowledge as to whether some of these bacterial metabolites are themselves carcinogenic will require studies beyond those reported to date.

An alternative role for bile acids is that of co-carcinogen or potentiator of the carcinogenic activity of other compounds. Reddy and colleagues have provided evidence which is consistent with a potentiation of the carcinogenesis of N-methyl-N′-nitroso-N-nitroguanidine

(MNNG) by either cholic acid or chenodeoxycholic acid infused intrarectally (10). Whether the concentration of bile acids that the tissue is exposed to by this technique approximates those normally found in this region of the intestinal tract is a matter to be determined. Chomchai and colleagues have measured the incidence of generation of tumors by azoxymethane in colons of rats in which the common duct was surgically diverted to the mid-small intestine (11). They reported a "significant increase" in incidence (average number of tumors per rat) compared to that engendered by azoxymethane in animals not surgically modified. Lack of any measure of variance, however, makes it difficult to verify their conclusion. At this point there is no compelling evidence to suggest that either the conjugates or the free bile acids are involved in normal genesis of tumors in the intestinal tract. Diet, however, does play a role in the amounts of conjugates secreted by the liver. In our experiments, in which care was taken to maintain enterohepatic circulation, the lipid content of the diet had an influence on both the concentration and rate of secretion of conjugated bile acids. Of greater effect on this secretion, however, was the rate of return of bile to the duodenum. By using a uniform rate of return, it was possible to diminish greatly the diurnal variation in volume of bile and concentration of bile acids. Pathophysiologic factors such as temporary occlusion of the biliary duct can manifest large, transient changes in the secretion of bile acids as well as in the volume of bile from the liver. If chronic, such a condition could provide greatly altered amounts of bile acids to the colon and rectum.

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Influence of Dietary Fiber on Bile Acid Metabolism

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ABSTRACT

Fiber, when fed to animals or man, will generally cause increased excretion of bile acids. The level of bile acid excretion appears to be a function of the structure of the fiber. Fiber binds bile acids and bile salts in vitro. The extent of binding is characteristic for each type of fiber and each substrate. Bile acid binding may be one mechanism of the physiological action of fiber.

Fiber is a generic term that includes a number of substances, mostly carbohydrate in nature, that are not digested by man. Figure 1 presents a classification of these substances. As can be seen from Table I, different types of fiber have different structures and can be expected to exert different chemical and physiological effects.

Among the earliest investigations into the effects of fiber on bile acid metabolism were those of Portman (1,2). In one study he compared the effects of laboratory ration and a semipurified diet on bile acid metabolism in rats. When rats were maintained on commercial ration, their cholic acid half-life was 2.00 ± 0.23 days, and they excreted about 36 mg/kg of cholic acid and 75 mg/kg of sterol daily. When switched to a semipurified diet consisting of 20% casein, 8% corn oil and 67.6% starch, cholic acid half-time rose to 3.24 ± 0.53 days, and excretion of cholic acid and sterols fell to 10 and 48 mg/kg/day, respectively. Substitution of sucrose for starch resulted in another drop in steroid excretion. Replacement of about one-third of the sucrose with cellulose reduced cholic acid half-time to 1.44 ± 0.21 days, decreased steroid excretion to 30 mg/kg/day, and increased cholic acid excretion to 23 mg/kg/day. In a second experiment (2), Portman showed that extraction of the lipids from commercial ration did not affect its influence on cholic acid metabolism. He also found that addition of the lipid present in lab ration to a semipurified diet did not affect its inhibition of cholic acid metabolism to any appreciable extent (Table II).

Leveille and Sauberlich (3) fed pectin to rats maintained on 1% cholesterol and found a significant increase in bile acid excretion but none in sterol excretion.

We fed rats diets in which 50% of the calories were from either carbohydrate, protein or fat, and the remaining calories divided evenly between the other two sources of calories. The dietary fiber was either cellulose or alfalfa. Four days before the rats were killed, they were given a single dose of [4-^{14}C] cholesterol, and the radioactivity in serum, liver and feces determined. Rats fed alfalfa excreted more radioactivity, but the increase was mostly in the neutral steroid fraction (4). In another experiment, rats fed a semipurified diet with 1% alfalfa added excreted 37% more acidic steroid and 57% more neutral steroid than did controls

Class	Type				Digestibility
Monosaccharide		Glucose, fructose		Available carbohydrate	+
Oligosaccharide		Sucrose, lactose, maltose			+
Polysaccharide		Starch, dextrins			+
	Plant cell contents	Gums		Dietary fiber	-
		Mucilages			-
		Algae polysaccharides			-
		Pectins			-
		Hemicellulose			-
	cell wall	Cellulose	crude fiber		-
		Lignin*			-

*noncarbohydrate

FIG. 1. Dietary fiber.

TABLE I

Chemistry of Dietary Fiber

	Main chain	Side chains
Cellulose	glucose	– – –
Hemicellulose	xylose mannose galactose glucose	arabinose galactose glucuronic acid
Pectin	galacturonic acid	rhamnose arabinose xylose fucose
Lignin	sinapyl alcohol coniferyl alcohol coumaryl alcohol	

TABLE II

Influence of Components of Commercial Diet on Cholic Acid Metabolism in Rats[a]

	Cholic Acid		
Diet	t 1/2 (d)	Pool (mg/kg)	Excretion (mg/kg/day)
Chow (C)	2.0	100	35
Extracted chow[b]	2.2	97	31
Semipurified (SP)[c]	4.2	62	10
SP + C-lipid	2.8	50	12
SP + C-Non Sap.	3.7	64	12

[a]After Portman (2).
[b]Extracted with ethanol. Vitamins and corn oil added to level present in SP.
[c]67.6% sucrose; 20% casein; 8% corn oil; 4% salts.

TABLE III

Adsorption of Bile Acids to Fiber[a]

	Binding at pH 3.9		Binding at pH 8.0	
Fiber Source[b]	Cholic acid	Taurocholic acid	Cholic acid	Taurocholic acid
Barley husk	0.97	1.05	1.05	0.75
Oak husk	0.77	0.38	0.51	0.40
Corn meal	1.10	0.74	1.28	1.25
Apple	1.00	1.14	0.59	0.53
Brussels sprout	1.09	1.33	1.03	1.18
Carrot	0.92	0.81	0.51	0.00
Pear	1.00	0.90	0.59	0.58
Turnip	0.91	0.67	0.31	0.30

[a]After Eastwood and Hamilton (8).
[b]Bran mash = 1.00.

(5). The foregoing show that different types of fiber may have entirely different effects on bile acid excretion.

In a study in which baboons were fed semipurified diets containing different carbohydrates (the fiber was cellulose), we observed (6) that the ratio of biliary primary/secondary bile acids was lower in the test animals than in controls who were fed a diet of bread, fruit and vegetables. Specific activity of biliary cholesterol (after administration of [5-^{3}H]mevalonic acid) was similar in all baboons, but bile acid specific activity was 5-7.5 times higher in the control animals. These findings led us to postulate that in a fiber-free or fiber-poor diet, bile acids are not excreted at a rapid rate, and thus they accumulate, bile acid synthesis is shut off, and cholesterol accumulates in the serum. A reduction in synthesis of new bile acids concomitant with continuing bacterial degradation would reduce the ratio of primary/secondary bile acids (5,6). Kyd and Bouchier (7) reached a

TABLE IV

Relative Binding of Bile Acids and Bile Salts to Four Types of Fiber[a]

Substrate acid	% Binding			
	Alfalfa	Bran	Cellulose	Lignin
Cholic	19.9	10.2	3.0	43.7
Taurocholic	6.9	1.4	1.0	22.1
Glycocholic	11.5	3.8	1.2	22.5
Chenodeoxycholic	24.8	18.2	1.9	23.3
Taurochenodeoxycholic	15.1	9.8	0.0	25.4
Glycochenodeoxycholic	14.9	21.4	0.2	25.2
Deoxycholic	10.4	5.4	0.2	17.4
Taurodeoxycholic	11.4	3.4	0.7	30.9
Glycodeoxycholic	27.8	7.8	4.7	52.6

[a]After Story and Kritchevsky (13).

TABLE V

Effects of Bran on Bile Acid Excretion in Man

		Fecal steroids (% change)		
Dose gm/day	Duration (wks)	Acidic mg/day	Neutral mg/day	Ref.
16[a]	3	+32[a]	---	19
36	3	+40	+36	20
28	3	+40	+36	21
39	3	-2	N.C.	22

[a]130 mg/day increase during second control period.

TABLE VI

Bile Composition in Men Fed Bran[a]

	Control	Bran[b]
Cholate (%)	42.2	42.2
Chenodeoxycholate (%)	30.6	43.9
Deoxycholate (%)	27.1	13.8
P/S[c]	2.7	6.2

[a]After Pomare and Heaton (23).
[b]33 g/day; 6-10 weeks.
[c]Primary/secondary bile acids.

similar conclusion during experiments on cholelithiasis in rabbits.

Bile acids will bind to various types of fiber-rich substances, taurocholic acid to a variety of substances with the results shown in Table III. Birkner and Kern (8) tested binding of glycocholic and chenodeoxycholic acids to various foodstuffs and found differences in binding affinity. Balmer and Zilversmit (9) tested binding of taurocholate to stock diet (1.00) and to ground wheat (0.33), ground corn (0.80), ground oats (0.65) and soybean meal (0.69).

We (10) tested the binding of taurocholate to various foodstuffs and found that 100 mg of curry powder, cloves, parsley or oregano all bound significantly more of this bile salt than did 100 mg of alfalfa.

Other experiments with taurocholate showed that the two substances most frequently used as bulking agents in semipurified diets, cellulose and cellophane, bound very little of this material (11). In a later experiment (12), we tested the binding of cholic, chenodeoxycholic and deoxycholic acids and their taurine and glycine conjugates to alfalfa, bran, cellulose and lignin. Each bile acid or salt was bound to a different extent; i.e., each had a specific affinity for each binding substance (Table IV).

Human studies show that populations on a high fiber diet excrete a greater volume of feces and more bile acids than do populations on low fiber diets (13). Antonis and Bersohn (14) studied the effects of low and high fiber diets in groups whose diets also contained high or low levels of fat. On a high fat diet, level of fiber did not affect bile acid excretion; but when the dietary fat was low, fiber enhanced bile acid excretion. Thus, on the high fat (40 cal %)-high fiber (15 g/day) diet, neutral steroid excretion was increased by 50%, but acidic steroid excretion was unaffected. When the diet contained 15 cal % fat, neutral and acidic steroid excretion were increased by 90% and 36%, respectively.

Pectin has been found to enhance bile acid excretion in man. Kay and Truswell (15) fed nine subjects 15 g of pectin daily for 3 weeks. Their triglyceride levels were not affected, but cholesterol levels fell from 224 to 190 mg/dl. In the subjects fed pectin, excretion of neutral steroids rose by 16% and excretion of bile acids by 40%. Miettinen and Tarpila (16) obtained similar results by feeding subjects 50 g pectin per day for 2 weeks. They found a 57% increase in bile acid excretion and only a 10% increase in neutral steroid excretion.

Bran has been studied as a hypocholesteremic agent and is generally ineffective (17). When fed to human subjects, bran will cause a large increase in fecal bulk. The concentration of steroids in feces (mg/g) is lowered, but because of the increased stool weight, the total steroid excretion is enhanced. Table V summarizes four experiments in which bran (16-39 g) was fed to subjects for 3 weeks. An increase in bile acid excretion was observed. It is interesting that Eastwood et al. (18) observed a large increase in bile acid excretion after their subjects were returned to a control diet. When human subjects are fed bran for 6-10 weeks, the level of biliary cholic acid is unchanged, but chenodeoxycholic acid levels increased by 43% and those of deoxycholic acid decrease accordingly (Table VI). The ratio of primary to secondary bile acids is more than doubled (19).

Metamucil, a hydrophilic colloid, has been found to enhance bile acid excretion in man (20,21). The excretion of bile acids is about three times that seen when cellulose is fed and one-ninth that observed when a bile acid binding resin is administered. At a level of 15 g/day, cellulose gives a slight increase in bile acid excretion (20); when 100 g/day is fed to children who are also ingesting 4 g of cholesterol daily, bile acid excretion is increased by 45% (22). Bagasse (10.5 gm/day) did not affect serum lipid levels, but increased fecal bile acid excretion by 50% while reducing fecal neutral steroids by 10% (23).

In summary, most, but not all, types of fiber increase stool weight. In doing so, they may reduce concentration of fecal bile acids, but increase total excretion of bile acids. The effect on bile acid excretion varies with type of fiber. Fiber binds bile acids and bile salts in vitro. The extent of binding is characteristic for each type of fiber and each substrate. Bile acid binding may be one mechanism by which fiber reduces serum cholesterol levels and increases bile acid excretion.

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ERRATUM

In Lipids, Volume 13, No. 10, the following errors appeared on the title page 685. The symposium title should read: "Functions of Steroids and Other Isopentenoids." Chairman Erich Heftman should read Erich Heftmann, and he is from Berkeley, CA. R. Bittman's title should read: "Sterol-Polyene Antibiotic Complexation: Probe of Membrane Structure."

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